Aminopterin Inhibition in *Aerobacter aerogenes*: Alanine and Valine Accumulation During the Inhibition and their Utilization on Recovery

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The inactivation of the folic acid antagonist aminopterin (4-amino-4-deoxypteroylglutamic acid) in cultures of *Aerobacter aerogenes* has been shown to occur by oxidative cleavage with the formation of derivatives of 6-carboxy- and 6-hydroxymethylpteridine (Webb, 1955). Thus during the period of inhibition of growth which follows the addition of bacteriostatic amounts of aminopterin to cultures of *A. aerogenes* in the early logarithmic phase of multiplication, there is a continual decrease in the concentration of the antagonist. Ultimately, this concentration falls to a non-inhibitory level and growth recommences. The growth rate of *A. aerogenes* in a simple chemically defined medium during this recovery phase frequently exceeds that of cultures grown in the absence of the antagonist, and it has been suggested that this is due to the utilization, on the resumption of growth, of metabolites which accumulate throughout the inhibition. Two of these intermediates have now been identified as alanine and valine, and this paper is concerned with the utilization of these amino acids during the recovery from inhibition by aminopterin. It is shown that both amino acids are utilized in the synthesis of leucine, the α - and carboxyl-carbons of which are derived from the accumulated alanine, and the remaining four carbon atoms from the valine. Evidence is presented which indicates that the carboxyl carbon of valine is eliminated, not as carbon dioxide, but as 'active' formate, which is utilized in the synthesis of purine nucleotides and the methyl group of thymine. It is suggested that the production of the C_2 and C_4 intermediates necessary for biosynthesis of leucine from pyruvic acid and 3-methyl-2-oxobutanoic acid is dependent upon folic acid and is inhibited by aminopterin.

MATERIALS AND METHODS

Source of organisms and preparation of cultures. *Aerobacter aerogenes* and *A. cloacae*, initially obtained from the National Collection of Type Cultures (strain numbers 418 and 408 respectively), were available from the Laboratory collection. *Escherichia coli* (American strain B) was provided by Dr W. J. Nickerson, Institute of Microbiology,

Rutgers University, New Brunswick, N.J., U.S.A. The strains were maintained on nutrient-agar slopes and transferred at approximately monthly intervals. Before being used in the following experimental work, the bacteria were serially subcultured in the simple defined medium of Monod (1942), which contained glucose as the sole organic component and was sterilized by filtration through sintered-glass funnels (UF porosity).

Cultures were grown at 37° in 18 ml. test tubes, 250 and 500 ml. Erlenmeyer flasks or 5 l. bolt-head flasks, containing 5, 50, 100 and 1000 ml. of medium respectively, according to the design of the experiment. When growth curves were plotted, measurements of turbidity were made every 15 min. with a Spekker absorptiometer (H. 508 filters), wooden mounts being used to hold the test tubes in the cell compartment of the instrument. To obtain uniform initial turbidities in these experiments a flask of medium (30 ml.) at 37° was first inoculated with 2 ml. of an 18 hr. culture, and portions (5 ml.) of this were transferred to sterile, matched test tubes towards the end of the lag phase. As the tubes were shaken before each determination the frequency of these measurements ensured the regular distribution of the cells and gave reproducible growth curves. In other experiments, in which the larger vessels were used, the cultures were either allowed to remain static or were agitated at intervals with a magnetic stirrer operated at low speed. Aeration was not employed since in media containing aminopterin this caused the rapid inactivation of the antagonist (Webb, 1955). Cell dry weights were obtained from the turbidity readings by means of a calibration curve prepared from cells harvested after growth for 18 hr. in the defined medium.

The final aminopterin concentration incorporated into the growth medium was usually 1:12 000 (≈ 0.19 mM). With this relatively high concentration the period of inhibition lasted for 15–18 hr., and thus it was possible to add the antagonist in the late afternoon and follow the subsequent recovery the next morning. The antagonist was added as a sterile (filtered) 1:2000 solution in Monod's medium to cultures in the early logarithmic phase of multiplication (turbidity about 0.15; ≈ 65 μ g. of dry cells/ml.).

In a series of experiments in which a number of compounds were examined for their ability to prevent inhibition by aminopterin when incorporated into the growth medium, the antagonist concentration was reduced to 1:25 000. At this concentration growth curves obtained over a period of 8 hr. were indistinguishable from those obtained at the higher level, although recovery usually occurred within 12 hr.

Paper chromatography. Paper chromatograms were developed by the descending method unless otherwise stated. The following solvent systems were used: (1) Amyl alcohol-methanol-0.2M-sodium citrate buffer, pH 5.0, on paper previously buffered with aq. sodium citrate sol. (J. A. Grunau, unpublished work); (2) butanol-acetic acid-water, 4:1:5 (Partridge, 1948); (3) methanol-aq. 1% (w/v) urea, 95:5 (ascending); (4) *tert.*-amyl alcohol saturated with water; (5) phenol-*m*-cresol-0.2M-borate buffer, pH 9.3, on buffered (borate, pH 9.3) paper (Levy & Chung, 1953); (6) butanol-5N-acetic acid, 7:3 (ascending) (Good & Johnson, 1949); (7) butanol-N-hydrochloric acid, 7:1 (Marshak & Vogel, 1951); (8) water-saturated butanol; (9) butanol-diethyleneglycol-water, 4:1:1 (Vischer & Chargaff, 1948); (10) butanol-2-methoxyethanol-0.5N-hydrochloric acid, 30.5:10.1:17.7 (Laland, Overend & Webb, 1952); (11) propanol-water, 4:1 (Greenberg, 1952); (12) propanol-water, 3:2 in an ammonia atmosphere (Greenberg, 1952); (13) butanol-formic acid, 95:5.

Of these solvent systems, nos. 1-5 were used for the separation of amino acids, no. 6 for pteridines, no. 7 for pyrimidines, nos. 7-10 for purines and pyrimidines, and nos. 11 and 12 for 5-amino-4-imidazolecarboxamide riboside. System 2 was also used for the separation of ribose and 5(4)-amino-4(5)-imidazolecarboxamide from the hydrolysis products of the riboside. In certain experiments, the keto acids isolated from the ether-soluble metabolic products in the medium at the end of the culture period were separated by direct chromatography in solvent system 13 (Wieland & Fischer, 1949; Magasanik & Umbarger, 1950); in others, separation was achieved by the method of Alfthan & Virtanen (1955), the amino acids obtained on reduction of the 2,4-dinitrophenylhydrazones being identified by chromatography in solvent system 2 in parallel with authentic amino acids as reference compounds. Solvent system 6 was used in conjunction with solvent system 2 for two-dimensional chromatography of protein hydrolysates according to the method of Levy & Chung (1953).

In general, the chromatograms were prepared on Whatman no. 54 paper. The use of other papers (Whatman no. 52 or no. 1) is specifically mentioned in the Results section. Amino acids were located on the developed chromatograms by spraying with 0.5% (w/v) ninhydrin in ethanol containing acetic acid (5%, v/v); purines, pyrimidines, pteridines and 5-amino-4-imidazolecarboxamide riboside by their absorption or fluorescence in ultraviolet light at 254 or 365-366 m μ . Ribose and 5(4)-amino-4(5)-imidazolecarboxamide were detected with aniline hydrogen phthalate (Partridge, 1949) and the Pauly reagents (Block, 1951) respectively.

Accumulation of amino acid in cultures treated with aminopterin. This was demonstrated by direct chromatography of culture filtrates in solvent systems 1 and 2. As relatively large amounts (120 μ l.) of the filtrates were necessary for the detection of the amino acids, the culture supernatants in certain experiments were concentrated *in vacuo*, then diluted with methanol (3 vol.), and filtered and again concentrated before the application of equivalent amounts to the paper. As observed by Dagley, Dawes & Morrison (1950), however, precipitation of salts in this way resulted in some loss of ninhydrin-positive material. For the estimation of amino acid concentration the culture supernatants were run in parallel with known amounts of

the appropriate reference compounds. After locating the amino acids with ninhydrin, areas containing the spots were cut from the paper and weighed on a microbalance before extraction of the colour with 60% ethanol in order to correct for the variable 'paper blank' (cf. Block, LeStrange & Zweig, 1952).

Chemical fractionation and analysis of *Aerobacter aerogenes*. The cells from each 100 ml. of medium were transferred to a 15 ml. Pyrex centrifuge tube, and washed three times with water and then extracted three times with 5% (w/v) trichloroacetic acid (5 ml.) at 0°. The cell residue was then washed with ethanol until the washings were free from trichloroacetic acid, and boiled for 1 hr. with 95% (v/v) methanol (5 ml.) to remove extractable fats. This extraction was repeated and material was then dried with ethanol and ether. In initial experiments nucleic acids were removed from the cell residue by extraction three times with 5% (w/v) perchloric acid at 90° (Ogur & Rosen, 1950). As observed by Bolton, Abelson & Aldous (1952), however, relatively large amounts of protein were removed with the nucleic acids under these conditions, and for most of the experiments described in this paper 5% (w/v) trichloroacetic acid (Schneider, 1945) was used in place of perchloric acid.

After the extraction of the nucleic acids the material was again dried with ethanol and ether. The centrifuge tube containing this residue was then drawn out near the open end before the addition of 6N-hydrochloric acid (0.5 ml.). The tube was flushed with nitrogen, and sealed and placed in a metal cylinder in an oven at 110° for 22 hr. to hydrolyse the protein. Hydrochloric acid was removed from the hydrolysate by the repeated addition and evaporation of water under reduced pressure, and then by concentration *in vacuo* at room temperature over phosphorus pentoxide and potassium hydroxide. The residue thus obtained was made to 5 ml. with water and portions were removed for the determination of total nitrogen. The remainder was again evaporated *in vacuo* and reserved for paper or ion-exchange chromatography.

Phosphorus was determined by the method of Berenblum & Chain (1938) and nitrogen by the micro-Kjeldahl method, the apparatus described by Markham (1942) being used for the distillation of the ammonia. Deoxyribonucleic acid and ribonucleic acid contents of the hot perchloric acid or trichloroacetic acid extracts were measured by the Dische (1930) and Euler & Hahn (1946) methods respectively, purified samples of the corresponding nucleic acids of herring roe and yeast being used as standards. In the determination of extractable phospholipid phosphorus portions of the combined ethanol and ethanol-ether extracts were evaporated from water (2 ml.) in micro-digestion flasks before the addition of 60% (w/w) perchloric acid (1 ml.).

Separation of the amino acids from protein hydrolysates. The acidic and neutral amino acids of hydrolysates of *A. aerogenes* protein were separated on Dowex 50 according to the method of Moore & Stein (1951). The column used generally gave a satisfactory separation of all amino acids other than glycine and cystine, which were eluted together.

For analytical purposes a portion of the hydrolysate containing 150-200 μ g. of N was applied to the column; this was increased to 600-1000 μ g. when the column was used to separate the amino acids. Under the latter conditions,

alternate effluent fractions were analysed by the ninhydrin method (Yemm & Cocking, 1955) to locate the individual components. From the remaining fractions the contents of the appropriate tubes were combined and deionized on small columns (15 cm. $\times$ 0.8 cm.³) of Zeo-Karb 215 (H⁺) according to the method of Alfthan & Virtanen (1955). The amino acid eluates were evaporated to dryness on a steam bath, the residues dissolved in a little water and portions of these solutions checked for purity by paper chromatography in solvent system 2. For the separation of glycine from the small amount of cystine present in hydrolysates prepared by the foregoing method, the whole fraction containing both of these amino acids was applied as a streak across the width of a 25 mm. strip of Whatman no. 52 paper. After development for 48 hr. in solvent system 2, the glycine, located by cutting a lateral strip 3 mm. wide from the paper and treating this with ninhydrin, was eluted in water.

In experiments in which valine and leucine were the only amino acids to be isolated, practically quantitative recovery was obtained, since their location in the effluent fractions could be established from the analysis of the first 150 fractions from the column and reference to previous runs.

Hydrolysis of the nucleic acid fractions and isolation of the purine and pyrimidine bases. The extracts from the above fractionation, containing both ribo- and deoxyribonucleotides, were heated in boiling water until free from trichloroacetic acid (30–60 min.), then made *N* with respect to sulphuric acid and hydrolysed for 1 hr. at 100°. The purine bases and pyrimidine nucleotides thus liberated were separated as described by Kerr, Seraidarian & Wargon (1949). Adenine and guanine were isolated as the hydrochlorides from the purine fraction by chromatography on columns of Dowex 50 (H⁺) (Cohn, 1949), and recrystallized from 0.1 *N*-hydrochloric acid. The extracts containing the pyrimidine nucleotides were evaporated *in vacuo* over phosphorus pentoxide and potassium hydroxide, and the residual solids hydrolysed with 72% (w/v) perchloric acid (Marshak & Vogel, 1951) at 100°. The hydrolysates were diluted with water, and neutralized with potassium hydroxide and filtered to remove potassium perchlorate. The filtrates were evaporated under reduced pressure, and centrifuged and taken to dryness *in vacuo* over phosphorus pentoxide. The residues were dissolved in water and the solutions applied as streaks to sheets of Whatman no. 54 paper. The chromatograms were developed for 7 hr. in solvent system 7. Uracil (*R_F* 0.30), cytosine (*R_F* 0.05) and thymine (*R_F* 0.50) were located by examination through a willemite-coated quartz plate (Marsh, 1954) of the dry chromatograms mounted in front of a grid-type low-pressure mercury-vapour lamp (Thermal Syndicate Ltd., WallSEND, Northumberland, ref. T/M5/535) emitting high-intensity radiation at 254 m μ . Strips containing the bases were cut from the chromatograms and eluted with 0.1 *N*-hydrochloric acid. The eluates were concentrated *in vacuo* over phosphorus pentoxide and potassium hydroxide until crystallization occurred.

Radioactive amino acids. L-[¹⁴C]₅Alanine, L-[¹⁴C]₅valine and DL-[¹⁴C]valine were purchased from The Radiochemical Centre, Amersham, Bucks.

Location and measurement of radioactivity. Radioactive compounds on paper chromatograms were located by autoradiography on Crystalex and Industrex X-ray film

(Kodak Ltd.) with an exposure of 21–28 days. Solid samples were assayed for activity on flat aluminium or glass dishes (2 cm. diam.) with an assembly consisting of an end-window, Geiger-Müller tube (General Electric Co. Ltd., Magnet House, Kingsway, London, W.C. 2; type EHM 2^m) and Dynatron scaler (type 200, Dynatron Radio Ltd., Maidenhead, Berks). Carbon dioxide, either metabolic or from chemical degradations, was converted into barium carbonate, samples of which were counted to within 5% standard error and corrected for self-absorption. A correction for self-absorption was also applied when activity measurements were made on derivatives (e.g. 2:4-dinitrophenylhydrazones), whereas other compounds (e.g. amino acids, purines and pyrimidines) were plated at low concentrations (less than 100 μ g./cm.²) and were not so corrected.

Chemical degradation of labelled amino acids was done by established methods, references to which are given in the Results section. For the detection and degradation of formic acid, Pirie's (1946) method was used. The carbon dioxide thus obtained was trapped in *N*-sodium hydroxide and isolated as barium carbonate. To eliminate the possibility of the contamination of the latter with traces of volatile, radioactive fatty acids, the barium carbonate was decomposed with hydrochloric acid at 0° and the evolved carbon dioxide swept with a current of CO₂-free air through a trap cooled in ethanol-solid CO₂ into 0.2 *N*-barium hydroxide. The barium carbonate residues from these experiments were pooled and used as a source of ¹⁴CO₂ for the growth experiments summarized in Table 9.

Special chemicals. The aminopterin and leucovorin (calcium salt) used in the following experiments were gifts from Dr Nickerson, Rutgers University. The sample of synthetic 5(4)-amino-4(5)-imidazolecarboxamide, used as a reference compound, was kindly provided by Dr D. W. Woolley, of the Rockefeller Institute, New York. Coenzyme A (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) and pantothenic acid were purchased from V. A. Howe and Co., 46 Pembridge Road, London, W. 11, and Roche Products Ltd., Welwyn Garden City, respectively.

RESULTS

Identification of alanine and valine in cultures of Aerobacter aerogenes treated with aminopterin. Direct chromatography in solvent system 1 of supernatants from *A. aerogenes* cultures incubated for 12–16 hr. in the presence of aminopterin (1:12 000) revealed two amino acids of *R_F* values 0.145 and 0.29. These were not observed either in filtrates from control cultures incubated for the same period in the absence of the antagonist, or among the soluble components released when washed suspensions of cells from both experimental and control cultures were disintegrated at room temperature in a Mickle shaker (total shaking time 15 min., in five equal periods with 3 min. rest between each period). To isolate the amino acids from the experimental cultures the medium (100 ml.) was partially desalted with methanol as outlined under Materials and Methods and then chromatographed on four sheets of Whatman no. 54

paper in solvent system 2. The amino acids, located by spraying lateral strips from each chromatogram with ninhydrin, were eluted with water and the eluates evaporated *in vacuo*. These were rechromatographed by the ascending method on Whatman no. 1 paper in solvent system 6 to eliminate pteridines (detected by their fluorescence in ultraviolet light), which were derived from impurities and breakdown products of the aminopterin. The amino acids (R_f 0.38 and 0.60) thus isolated were identified as alanine and valine by co-chromatography with the authentic compounds in solvent systems 1-5. As the R_f values of valine in solvent systems 1, 2 and 5 were similar to those of methionine, differentiation between these amino acids was achieved by treatment with hydrogen peroxide (Dent, 1948).

For chemical characterization, the amino acids, separated from the culture medium (300 ml.) as above, were decomposed with chloramine-T (Ehrensward, Reio, Saluste & Stjernholm, 1951). The product from the degradation of the slow-moving component (R_f 0.38 in solvent system 6), distinguished from threonine by its greater resistance to periodic acid, was removed in a current of air and trapped in 2:4-dinitrophenylhydrazine (half-saturated solution in 2N-hydrochloric acid). The crystalline product which separated was recrystallized from aqueous ethanol to yield acetaldehyde 2:4-dinitrophenylhydrazone, m.p. 154° (uncorr.), alone and in admixture with the authentic compound. The volatile product, similarly separated from the decomposition of the second component (R_f 0.60 in solvent system 6) was trapped in 0.05N-potassium permanganate-0.05N-sodium bicarbonate. This solution was acidified and extracted with ether. Evaporation of the ether yielded an oil, from which an anilide was prepared in the usual way. The product, recrystallized first from water and then from aqueous ethanol, formed needles of m.p. 105° (uncorr.) alone and in admixture with the anilide of isobutyric acid similarly prepared from authentic DL-valine.

Accumulation of extracellular alanine and valine was also observed to follow the addition of aminopterin to cultures of *Escherichia coli* and *Aerobacter cloacae* in the early logarithmic phase of multiplication. In contrast with *A. aerogenes*, however, control cultures of these bacteria grown in the absence of the antagonist also contained detectable amounts of alanine and valine. The presence of these and other amino acids in filtrates from cultures of *E. coli* in a simple chemically defined medium has been described previously by Dagley & Johnson (1956).

In cultures of *A. aerogenes* traces of valine were present in the culture medium 1 hr. after the addition of aminopterin. At this stage of the

culture alanine was not detected. In samples removed after a further 90 min., however, both amino acids were present. As judged by the intensities of the colours formed on the developed chromatograms with ninhydrin, the concentrations of alanine and valine in the medium then increased progressively with time until 21 hr., after which recovery from the inhibition of growth began. Six hours after the onset of recovery, when the turbidity of the experimental culture had increased from 35 to 94 % of that of the fully-grown control, as well as during the subsequent increase in turbidity from 94 to 120 %, neither alanine nor valine was demonstrable by chromatography of portions of the culture medium. In three experiments in which the concentrations of the accumulated amino acids were determined at or near the estimated time of recovery, values of 2.45, 2.0 and 1.75 μ moles/ml. and 2.75, 2.5 and 1.76 μ moles/ml. were obtained for alanine and valine respectively.

Effect of aminopterin on the chemical composition of Aerobacter aerogenes. To determine if the accumulation of alanine and valine in the aminopterin-treated cultures was reflected in an alteration in either content or amino acid composition of the total cellular protein, cells from inhibited and control cultures were analysed as described under Materials and Methods. Results of these analyses are summarized in Tables 1 and 2.

As shown in Table 1, the amounts of the various cell components were measured relative to the deoxyribonucleic acid phosphorus (DNA-P) content, since this method generally provides the most suitable indication of the average cell composition and avoids difficulties inherent in the interpretation of per cent dry wt. values (e.g. Davidson & Leslie, 1950*a, b*). It is apparent, however, from the results expressed in this way, that growth inhibition by aminopterin in *A. aerogenes*, as in *E. coli* (Webb & Nickerson, 1956), *Lactobacillus casei* (Prusoff, Teply & King, 1948) and *Lactobacillus leichmanii* (Rege & Sreenivasan, 1954), causes a decrease in DNA-P content which is greater than that in any other cell component. Thus comparison of the ratios of DNA-P/ribonucleic acid phosphorus (RNA-P) in the two preparations from the inhibited cultures (E_1 and E_2) with the corresponding controls (C_1 or C_2 and C_3) reveals reductions in DNA-P content of 50 and 36 % respectively. These figures may represent minimum values, since the RNA-P content has been assumed to remain unaltered, whereas an analogous decrease (51 %) in DNA content of *L. leichmanii* under partial aminopterin bacteriostasis (0.25 μ g./ml.) is accompanied by a 23 % reduction in the amount of RNA (Rege & Sreenivasan, 1954).

Relative to RNA-P, a small decrease in protein content is induced in *A. aerogenes* by aminopterin

Table 1. *Effect of aminopterin on the chemical composition of Aerobacter aerogenes*

Analyses are recorded for *A. aerogenes* cells harvested during the growth inhibition which followed the addition of aminopterin (83 $\mu\text{g./ml.}$) to cultures in the early logarithmic phase (turbidity $0.20 \equiv 85 \mu\text{g. dry cell wt./ml.}$) (preparations E_1 and E_2), and after recovery from this inhibition (preparation E_R), together with the corresponding control preparations (C_1 , C_2 , C_3 and C_4) from cultures grown in the absence of the antagonist.

	E_1	C_1	C_2	E_2	C_3	E_R	C_4
Age of culture (hr.)	19	19	19	20	20	44	44
Time (hr.) of aminopterin addition	3	—	—	2.5	—	2.5	—
Cell density at time of harvesting ($\mu\text{g. dry cells/ml.}$)	200	665	660	260	658	612	628
Acid-soluble N/DNA-P	—	—	—	3.9	3.3	5.5	6.0
Acid-soluble P/DNA-P	12.1	6.23	6.48	6.5	5.5	4.0	3.7
RNA-P/DNA-P	9.6	4.7	5.1	7.1	4.5	4.0	3.1
Protein N/DNA-P	30.7	—	20.5	24.6	19.6	21.0	20.2
Acid-soluble N/RNA-P	—	—	—	0.6	0.7	1.4	1.9
Acid-soluble P/RNA-P	1.3	1.3	1.3	0.9	1.2	1.0	1.2
DNA-P/RNA-P	0.10	0.21	0.19	0.14	0.22	0.25	0.33
Protein N/RNA-P	3.2	—	4.0	3.4	4.0	5.5	6.6

Table 2. *Amino acid composition of Aerobacter aerogenes protein*

Contents of acidic and neutral amino acids in hydrolysates of the protein from aminopterin-inhibited *A. aerogenes* (preparation E_2) and the corresponding control cells (preparation C_3), determined according to the method of Moore & Stein (1951). Separation of alanine from glycine and cystine was incomplete.

Amino acid	Amino acid residues ($\mu\text{moles/100 } \mu\text{g. of protein N}$)	
	E_2	C_3
Aspartic acid	0.60	0.62
Threonine	0.32	0.32
Serine	0.28	0.28
Glutamic acid	0.62	0.68
Alanine	0.45	0.56
Glycine + cystine	0.74	0.58
Valine	0.42	0.44
Methionine	0.08	0.09
Isoleucine	0.30	0.29
Leucine	0.48	0.52

Table 3. *Incorporation of ^{14}C from generally-labelled L-alanine and L-valine by Aerobacter aerogenes during recovery from inhibition by aminopterin*

	Total activity (counts/min.)	
	Cultures supplemented before recovery with	
	$[^{14}\text{C}_3]\text{Alanine}$	$[^{14}\text{C}_2]\text{Valine}$
Initial activity added to culture medium	1.92×10^5	1.73×10^5
Residual activity in medium after recovery	420	3400
Cell fractions:		
Acid-soluble components	1620	690
Lipids	1950	2160
Nucleic acids	1.92×10^4	0.81×10^4
Protein	2.70×10^4	6.00×10^4

(Table 1). This is not associated, however, with any significant change in the amino acid composition of the total cell protein (Table 2).

With recovery from inhibition by aminopterin, the chemical composition of *A. aerogenes* becomes similar to that of normal cells grown in the absence of the antagonist (Table 1). As is to be expected from the limited viability of fully grown normal cultures of *A. aerogenes* in the simple chemically defined medium, cells harvested 44 hr. after inoculation (i.e. some 30–36 hr. after the end of logarithmic multiplication) differ significantly in chemical composition from cells isolated after 20 hr. (Table 1). The fact that the chemical composition of cells after recovery from the inhibitory effects of aminopterin appears to be intermediate between those of the 20 and 44 hr. control preparations, probably reflects nothing more than the absence of the appropriate control to the recovery culture. It is significant, however, that cells after recovery have essentially normal ratios of RNA-P/DNA-P and protein N/DNA-P.

Utilization of L-alanine and L-valine by Aerobacter aerogenes on recovery from inhibition by aminopterin. To investigate the metabolism of the accumulated alanine and valine during the recovery phase, aminopterin-treated cultures of *A. aerogenes* at the end of the period of growth inhibition were supplemented separately with each of these amino acids generally labelled with ^{14}C . After recovery, the cells were harvested and fractionated. The total ^{14}C -activities in the crude fractions thus separated are given in Table 3. The figures shown should be regarded as approximate rather than absolute values, as, for example, no allowance has been made for the contamination of the nucleic acid fractions with protein, whereas the activities of the protein components have been calculated from measurements made on samples of

the insoluble residues obtained on completion of the fractionation.

Under the conditions of these experiments considerable radioactivity from both [$^{14}\text{C}_3$]alanine and [$^{14}\text{C}_5$]valine was incorporated into the cell protein. The association of this uptake with recovery from the growth inhibition was shown by the absence of ^{14}C -activity from cells harvested during the inhibition phase of an aminopterin-treated culture to which DL-[^{14}C]valine (5 μ moles, 0.01 mc) had been added 2 hr. after the addition of the antagonist.

Table 4. *Specific activities of certain amino acids isolated from the proteins of Aerobacter aerogenes after recovery from aminopterin inhibition in the presence of L-alanine or L-valine generally labelled with ^{14}C*

Amino acid	Specific activity (counts/min./ μ mole) after recovery in the presence of	
	[$^{14}\text{C}_3$]Alanine	[$^{14}\text{C}_5$]Valine
Valine	0	1340
Alanine	1312	—
Leucine	186	1070
Aspartic acid	292	—
Threonine	306	—
Serine	314	—
Glutamic acid	470	—
Phenylalanine	178	—
Isoleucine	—	360

When recovery occurred in the presence of generally labelled L-alanine and L-valine, neither amino acid was detected in the medium at the end of the culture period. In each experiment the corresponding keto acids, pyruvic acid and 3-methyl-2-oxobutanoic acid (α -oxoisovaleric acid), were present, however, and accounted for much of the residual activity. The incorporation of ^{14}C -activity into the lipids and, at this stage in the investigation, into the nucleic acids, was not investigated further. The distribution of activity among the amino acid components of the cell protein was studied initially by autoradiography of two-dimensional chromatograms of acid hydrolysates of this fraction. The results obtained were as follows: when recovery occurred in the presence of generally labelled L-alanine, ^{14}C -activity was present to a high level in the alanine of the cell protein and to a lesser but still marked extent in glutamic acid, glycine, serine, threonine, aspartic acid, leucine, lysine, phenylalanine, cystine and histidine. When recovery occurred in the presence of generally labelled L-valine, however, significant activity was incorporated only into the valine and leucine components of the protein. This finding is in agreement with the work of Abelson & Vogel (1955), in which the addition of [^{14}C]valine to cultures of *Neurospora crassa* and *Torulopsis utilis* resulted in significant labelling of protein-bound

Table 5. *Distribution of ^{14}C in certain amino acids isolated from the proteins of Aerobacter aerogenes after recovery from aminopterin inhibition in the presence of either L-alanine or L-valine generally labelled with ^{14}C*

Amino acid	Carbon atom or radical	Method of degradation	Activity (% of total activity) after recovery in the presence of	
			[$^{14}\text{C}_3$]Alanine	[$^{14}\text{C}_5$]Valine
Serine	C ₍₁₎	Sakami (1950)	50.1	—
	C ₍₂₎		45.6	—
	C ₍₃₎		2.2	—
Threonine	C ₍₁₎	Ehrensward, Reio, Saluste & Stjernholm (1951)	30.3	—
	C ₍₂₎		36.3	—
	C ₍₃₎ + C ₍₄₎		29.3	—
Aspartic acid	C ₍₁₎	Oxidation with chloramine-T (Ehrensward <i>et al.</i> 1951)	29.3	—
	C ₍₁₎ + C ₍₄₎	Ninhydrin decarboxylation (Van Slyke, MacFadyan & Hamilton, 1941)	42.6	—
Phenylalanine	C ₆ H ₅ ·CH ₂ -	Oxidation to benzoic acid with alkaline permanganate	0	—
Leucine	C ₍₁₎	Ninhydrin decarboxylation (Van Slyke <i>et al.</i> 1941)	46.0	0
	C ₍₂₎	Deamination with nitrous acid and oxidation with permanganate to isovaleric acid, which was then degraded by the Schmidt reaction (Phares, 1951)	50.6	0.26
	CHMe ₂ ·CH ₂ -	Total activity — (C ₍₁₎ + C ₍₂₎)	3.4	99.7
Valine	C ₍₁₎	Ninhydrin decarboxylation (Van Slyke <i>et al.</i> 1941)	—	22.7

valine and leucine only. Of the remaining amino acids in the hydrolysate of the *A. aerogenes* protein, only isoleucine, separated from leucine by chromatography for 96 hr. in solvent system 4, possessed activity sufficient to be detected by autoradiography.

The specific activities of certain acidic and neutral amino acids, separated from each protein hydrolysate by ion-exchange chromatography (Moore & Stein, 1951), are given in Table 4. After these determinations, a number of the isolated amino acids were supplemented with the corresponding unlabelled compounds and degraded by established methods, adapted where necessary to the micro scale, to locate the active carbon atoms. The results of these experiments are summarized in Table 5.

Effect of coenzymes and vitamins on inhibition by aminopterin. The results presented in Table 4 suggested the possibility that the accumulation of valine in the aminopterin-treated cultures was due to an inhibition of leucine synthesis, a process which appears to be either directly or indirectly dependent upon folic acid (Woods, 1953). Degradation of the isolated leucine, however, established that the carbon skeleton of this amino acid was not derived from that of valine by the addition of a C_1 unit, as might be expected if a folinic acid-dependent, aminopterin-sensitive reaction was involved in the synthesis. Thus the α -carbon of leucine contained insignificant ^{14}C -activity, whereas the carboxyl carbon of the isolated valine contained 22.7 % of the total activity of the molecule (Table 5). In addition, attempts to prevent the growth-inhibitory action of aminopterin on *A. aerogenes* by the addition to the culture medium of L-leucine in concentrations up to 5 μ moles/ml. were unsuccessful. As it thus seemed unlikely that aminopterin had a direct action on the formation of leucine, a number of experiments were done to investigate the possibility of an indirect effect of the antagonist on this synthesis through the inhibition of the production of an essential coenzyme.

It is known that 3-methyl-2-oxobutanoic acid, the precursor of valine, is also a precursor of the pantoic acid moiety of coenzyme A (CoA) in *E. coli*, *A. aerogenes* (Maas & Vogel, 1953) and *Brucella abortus* (Altenbern & Ginoza, 1954). Furthermore, the hydroxymethylation of 3-methyl-2-oxobutanoic acid to α -oxopantoic acid requires *p*-aminobenzoic acid (Purko, Nelson & Wood, 1953, 1954) or folic acid (e.g. Johnson, 1955). Recent work by T. Wainwright & D. D. Woods (personal communication) suggests that the biosynthesis of leucine may occur during the conversion of isobutyryl-CoA and acetyl-CoA. Thus the inhibition of CoA synthesis theoretically could result both directly and indirectly in the accumulation of 3-

methyl-2-oxobutanoic acid from which valine would be derived by transamination. Indeed, an accumulation of valine through an inhibition of CoA synthesis has been observed by Maas & Vogel (1953) in a pantoic acid-requiring mutant of *E. coli* when grown in the presence of limiting amounts of pantoic acid. In addition, if the acetyl-CoA required for leucine synthesis according to the above mechanism is derived from pyruvate (e.g. Chantrenne & Lipmann, 1950) a deficiency of the coenzyme could also result in the accumulation of alanine. The addition of pantothenic acid (3 μ g./ml.) or coenzyme A (0.3 μ g./ml.) to cultures of *A. aerogenes* either before or together with aminopterin, however, was without effect upon either the growth-inhibition or the accumulation of alanine and valine. Although *A. aerogenes* may be impermeable to coenzyme A, it seems unlikely that the lack of effect of pantothenic acid could be due to its inability to enter the cells, since this compound has been shown to prevent the inhibition of growth produced by sulphanilamide in cultures of *Bacillus linens* containing limiting amounts of *p*-aminobenzoic acid (Purko *et al.* 1953).

In further experiments, thiamine, riboflavin, nicotinic acid, biotin, cocarboxylase and pyridoxin at several concentrations (2, 5 and 10 μ g./ml.) were found also to be without effect upon either the inhibition of growth or accumulation of amino acid in the aminopterin-treated *A. aerogenes* cultures. The reasons underlying the choice of these substances are to be found in the work of Lascelles & Woods (1954), Lascelles, Cross & Woods (1954), Blakley (1955), Kitahara & Fukui (1955), Montfort (1955), Ekladius & King (1956), Guthrie, Hillman & Hyatt (1956), Trevelyan & Harrison (1956) and Wright & Stadtman (1956). Partial reversal of the inhibition was obtained, however, with leucovorin, even if this was added several hours after the antagonist, although high concentrations were required to produce a consistent effect. Thus when leucovorin and aminopterin in the ratio of 50:1 were added to a culture of *A. aerogenes* in the early logarithmic phase, the inhibition of growth after a further 6 hr. still amounted to about 60 % of that produced by aminopterin (50 μ g./ml.) alone. This partial reversal, however, was accompanied by a corresponding decrease (35–40 %) in the amounts of alanine and valine accumulated in the medium.

Prevention of inhibition by aminopterin. The inhibitory action of aminopterin (1:24 000) was almost entirely prevented (Fig. 1) by the addition to the simple growth medium of the following: L-serine, L-isoleucine, L-leucine, L-valine, glycine, L-histidine, L-tryptophan, L-glutamic acid, L-threonine, L-methionine, L-lysine, adenine and thymine at mM-concentrations. Furthermore, in

the presence of these supplements, the formation of filamentous cells normally observed in the aminopterin-treated cultures (Nickerson & Webb, 1956) was entirely prevented. The choice of these substances was determined in part by the knowledge that folinic acid is required for the synthesis of many of them, and in part from the distribution of ^{14}C -activity observed in the above experiments with the labelled alanine and valine. In addition, L-isoleucine and L-valine were included to obviate the possible growth-inhibitory effect of L-leucine alone (e.g. Gladstone, 1939; Rowley, 1953).

As a result of many growth experiments with media lacking one or more of the above components, it was possible to eliminate the following as being of secondary importance in the prevention of the inhibition: L-serine, L-isoleucine, L-leucine, L-valine, L-histidine, L-tryptophan, L-methionine, L-lysine and thymine. Thus in the presence of aminopterin the omission of these compounds singly from the medium produced little change in the form of the growth curve from that observed with the complete supplement, although when all were omitted together, their cumulative effects

upon the inhibition were apparent (Fig. 2, cf. Fig. 1). The effects of the remaining four (adenine, L-threonine, L-glutamic acid and glycine) were studied in a factorial experiment, the design of which together with the analysis of the data is given in the Appendix. The results obtained revealed adenine to be the active compound in the prevention of the inhibition. This finding has a parallel in the observation of Guthrie *et al.* (1956) that adenine blocks the inhibitory action of amethopterin (4-amino-4-deoxy-10-methylpteroyl-glutamic acid) on *Bacillus subtilis* in a minimal medium. The presence of the purine also reduced by 50–70% the amounts of alanine and valine chromatographically detectable in portions (120 μl .) of medium from aminopterin-treated cultures, although complete suppression of amino acid accumulation under the conditions of these experiments was not observed.

Hypoxanthine was as effective as adenine in preventing the inhibition due to aminopterin, whereas guanine at a concentration limited by solubility to 7 $\mu\text{g./ml.}$ was not only inactive but also appeared to inhibit the action of adenine. This observation contrasts with the known ability of normal *A. aerogenes* cells to utilize guanine, although less effectively than adenine (Hamilton, Brown & Stock, 1952; Balis, Brooke, Brown & Magasanik, 1956). It is possible, however, that

Fig. 1. Curves show the growth of cultures of *A. aerogenes* in the simple chemically defined medium with (x) and without (Δ) a supplement of L-serine, L-isoleucine, L-leucine, L-valine, glycine, L-histidine, L-tryptophan, L-glutamic acid, L-threonine, L-methionine, L-lysine, adenine and thymine (all mM). ●, ○, Effect of the addition of aminopterin (final concentration 1:24 000; about 0.1 mM) to cultures with and without supplement respectively at the times indicated by the arrows.

Fig. 2. ○, Effect of the addition (at time indicated by arrow) of aminopterin to a concentration of 0.1 mM to a culture of *A. aerogenes* in Monod's medium supplemented with adenine, L-threonine, L-glutamic acid and glycine (all mM). x, Growth of the control culture in the same medium in the absence of the antagonist.

under conditions of purine deficiency, guanine may inhibit the utilization of adenine, as observed by Aaronson (1955) in cultures of the purine-requiring *Staphylococcus flavocyanus*.

Accumulation of 5-amino-4-imidazolecarboxamide riboside in aminopterin-treated cultures of *Aerobacter aerogenes*. The prevention by adenine both of the growth inhibition and the production of alanine and valine in the treated cultures, when considered together with the decreased deoxyribonucleic acid content of the cells (Table 1), suggested the possibility of a relationship between amino acid accumulation and inhibition of purine synthesis. In cultures of certain bacteria inhibition of purine synthesis by the folic acid antagonists, as well as by sulphonamides, is known to result in the accumulation of 5-amino-4-imidazolecarboxamide riboside (Woolley & Pringle, 1950; Edwards, Skipper & Johnson, 1952; Stetten & Fox 1945; Ravel *et al.* 1948; Greenberg, 1952). In previous work (Webb, 1955) attempts to demonstrate the presence of this imidazole derivative in cultures of *A. aerogenes* after recovery from inhibition by aminopterin were unsuccessful. In the present studies, however, when cultures in the inhibition phase were examined, high levels of 5-amino-4-imidazolecarboxamide riboside were detected. This compound was isolated with a yield of 1 mg./100 ml. of medium, by Greenberg's (1952) method, as this, in contrast with the most recent procedure (Greenberg & Spilman, 1956), gave an efficient and convenient elimination of pteridines derived from the aminopterin preparation. The product thus obtained contained equimolecular proportions of pentose and non-acetylatable diazotizable amine, the latter being determined by the method of Bratton & Marshall (1939) after treatment with acetic anhydride (Rosenthal & Bauer, 1939). The compound was characterized by its absorption spectra in 0.1N-hydrochloric acid ($\lambda_{\max} = 246$ and $268 \text{ m}\mu$; $\epsilon_{268 \text{ m}\mu} = 11\,560$) and 0.1N-sodium hydroxide ($\lambda_{\max} = 267 \text{ m}\mu$; $\epsilon_{267 \text{ m}\mu} = 14\,250$), in agreement with the data of Greenberg & Spilman (1956), and by its hydrolysis (0.5N-hydrochloric acid at 100° for 1 hr.) to 5(4)amino-4(5)-imidazolecarboxamide and ribose.

Incorporation of the carboxyl carbon of [1- ^{14}C]-valine into the nucleic acids of *Aerobacter aerogenes*. As glycine and aspartic acid, both of which can be derived from alanine (Table 4), are known precursors of purines and pyrimidines in bacteria as in mammalian tissues (see review by Carter, 1956), the utilization of labelled alanine by *A. aerogenes* would be expected to result in the incorporation of radioactivity into the nucleic acids by mechanisms other than those dependent upon folinic acid. For this reason subsequent experiments to investigate the relation between amino acid accumulation and

purine synthesis were done with DL-[1- ^{14}C]-valine. The labelled amino acid was added (a) in tracer amounts to a normal culture of *A. aerogenes* in the early logarithmic phase of multiplication, and (b) towards the end of the period of growth-inhibition in an aminopterin-treated culture as a supplement to the accumulated valine. This approach was used to determine if differences in utilization would result from the high level of exogenous valine in the inhibited culture (cf. Koch, 1955). A similar distribution of radioactivity was observed, however, in the fractions separated from cells grown under the conditions of (a) or (b). In consequence of this and of the limitation imposed on the scale of experiment (b) by the amount of aminopterin available, only the results obtained with *A. aerogenes* grown in the presence of tracer amounts of DL-[1- ^{14}C]-valine are summarized in Table 6.

Table 6. *Distribution of ^{14}C -activity in the fractions isolated from *Aerobacter aerogenes* after growth in Monod's medium containing DL-[1- ^{14}C]-valine*

The culture was grown in 1 l. of medium contained in a 5 l. bolt-head flask fitted with an inoculation port, an outlet tube and two inlet tubes, one of which terminated 1 in. above the liquid surface; the second tube reached to the bottom of the vessel. The two inlet tubes were joined externally through a two-way tap with a source of sterile, CO_2 -free air, which could thus either be passed through the medium, or used to replace the gas phase in the flask. An arrangement of two-way taps in the outlet tube enabled a trap containing N-sodium hydroxide to be inserted in the train. The medium was brought to 37° and inoculated with 10 ml. of a 16 hr. culture of *A. aerogenes* in the same medium. After 2 hr., CO_2 -free air was drawn rapidly through the medium for 15 min., and a solution of DL-[1- ^{14}C]-valine (0.04 mc) in Monod's medium (5 ml.) added. The sodium hydroxide trap was inserted, and a slow current of air drawn continuously through the gas phase of the vessel for a further 15 hr. The air stream was then directed through the medium for 20 min. to remove dissolved CO_2 , after which the culture was centrifuged and the cells were submitted to chemical fractionation. The carbonate formed in the sodium hydroxide trap was precipitated with barium, and washed and assayed for activity.

	Total ^{14}C -activity (counts/min.)
Initial activity in medium	4.6×10^6
Final activity in medium	
Ether-soluble	1.08×10^6
Ether-insoluble	21.2×10^6
Metabolic CO_2	2.64×10^{10} *
Lipids	0.03×10^6
Protein	4.7×10^6
Nucleic acids	3.6×10^6

* Specific activity = 10.2×10^3 counts/min./m-mole.

Table 7. *Specific activities of certain of the amino acids isolated from a hydrolysate of the cell proteins of Aerobacter aerogenes cultivated in the presence of DL-[1-¹⁴C]valine*

Amino acid	Specific activity (counts/min./μmole)
Aspartic acid	49.2
Serine	50.2
Glutamic acid	46.6
Alanine	12.2
Isoleucine	20.2
Leucine	22.2
Valine	970

Table 8. *Specific activities of the purine and pyrimidine bases isolated from the nucleic acids of Aerobacter aerogenes after growth in the presence of DL-[1-¹⁴C]valine*

Base	Specific activity (counts/min./μmole)
Adenine	420
Guanine	410
Uracil	0
Cytosine	0
Thymine	190

In confirmation of earlier conclusions, autoradiographs of paper chromatograms of hydrolysates of the cell proteins showed no activity in amino acids other than valine. Slight activity was detected, however, in certain of the amino acids separated from a protein hydrolysate by chromatography on Dowex 50 (Moore & Stein, 1951). These results (Table 7) indicate some re-cycling of the metabolic carbon dioxide during the course of the experiment.

Table 6 shows that almost 50% of the total activity initially present remained in the medium at the end of the growth period. As this probably reflects the inability of *A. aerogenes* to utilize the D-isomer of valine, the isolation of this activity was not attempted. The ether-soluble fatty acid components of the culture filtrate were separated by distillation in steam into volatile and non-volatile fractions, which required 0.3 and 1.05 m-mole of sodium hydroxide respectively for neutralization. The volatile fraction contained 0.05 m-mole of formic acid, which had a specific activity of 2.7×10^3 counts/min./m-mole.

From the nucleic acid fraction (mixed ribo- and deoxyribo-nucleotides) adenine and guanine were isolated as the crystalline hydrochlorides by the methods described earlier. Both bases possessed high radioactivity (Table 8), unaltered on chromatography in solvent systems 8–10. In contrast, the pyrimidine bases uracil and cytosine, separated from perchloric acid hydrolysates of the nucleotide fraction by paper chromatography in solvent system 7, were devoid of radioactivity. Thymine, however, had a specific activity approximately half

that of adenine or guanine (Table 8), which was unchanged on chromatography in solvent systems 8–10. The location of this activity solely in the methyl group of thymine follows from the known derivation of the latter from uracil, either as the free base as observed in *B. subtilis* (Rege & Sreenivasan, 1954), or more probably as the riboside (Hammarsten, Reichard & Saluste, 1950; Rose & Schweigert, 1953) or deoxyriboside (Friedkin & Roberts, 1956).

Degradation of the isolated guanine was carried out by the method of Brown, Roll, Plentl & Cavalieri (1948) after the addition of inactive guanine sulphate (15.2 mg.) as carrier. The yield of crude guanidine, isolated as the picrate (3.45 mg.), was insufficient for complete purification; nevertheless, after one recrystallization from water the specific activity of the product was 25% of that of the initial guanine. Thus at least 25% of the total activity of the guanine molecule was located in the C₂ atom.

Incorporation of carbon dioxide into the proteins and nucleic acids of Aerobacter aerogenes. In the foregoing experiment a significant percentage of the activity initially supplied to the culture was eliminated as carbon dioxide (Table 6) and it was necessary therefore to determine whether this formed an intermediary for the incorporation of the valine carboxyl carbon into the purine bases and thymine. Although the activities of the purine bases (approx. 4.1×10^5 counts/min./m-mole, Table 8) were greater than that of the metabolic carbon dioxide (10.2×10^3 counts/min./m-mole, Table 6) evolution of carbon dioxide of considerably higher activity may have occurred during the early stages of logarithmic growth when the rates of synthetic processes were maximal. This would be shown by a maximum in the curve expressing the variation in specific activity of the metabolic carbon dioxide with time, as observed by Andersson-Kottö, Ehrensärd, Högström, Reio & Saluste (1954) in cultures of *Neurospora crassa* containing ¹³CH₃¹⁴CO₂H. Later, when the culture entered or approached the stationary phase, the formation of unlabelled carbon dioxide from other sources would result in the dilution of the activity of the total carbon dioxide without a corresponding decrease in the activity previously incorporated into cell components.

To investigate the utilization of carbon dioxide by *A. aerogenes* for the synthesis of protein and more particularly of nucleic acids, a culture was grown for 15 hr. in a medium containing NaH¹⁴CO₃ and in an atmosphere enriched with ¹⁴CO₂. Details of this experiment are summarized in Table 9.

Most of the amino acids separated from a hydrolysate of the cell protein exhibited slight radioactivity, although the total counts recorded for the

Table 9. *Specific activities of certain amino acids and of the nucleic acid bases isolated from Aerobacter aerogenes after growth in the presence of [^{14}C]carbon dioxide*

A. aerogenes was inoculated into 900 ml. of Monod's medium at 37° contained in the 5 l. bolt-head flask used in the previous experiment (Table 6). After 2 hr. the air inlet to the culture flask was closed, and the flask evacuated by suction from a water pump attached to the outlet tube. Sterile, CO_2 -free air was then admitted and the flask again evacuated. A solution of $\text{Na}_2^{14}\text{CO}_3$ [prepared by the absorption of $^{14}\text{CO}_2$ from $\text{Ba}^{14}\text{CO}_3$ (2 g., 6.6×10^4 counts/min./g.) in NaOH, followed by precipitation of the carbonate with ethanol] in Monod's medium (25 ml.) was titrated with $\text{m-KH}_2\text{PO}_4$ to pH 7.5 and immediately drawn into the evacuated culture vessel through the longer of the two inlet tubes. $^{14}\text{CO}_2$ (generated from 3 g. of $\text{Ba}^{14}\text{CO}_3$ and collected in a gas burette over $\text{N-H}_2\text{SO}_4$) was introduced through the second inlet, and the internal pressure restored to atmospheric by the admission of CO_2 -free air. The inlet and outlet leads were closed and the culture was incubated for a further 15 hr. At the end of this time a trap containing $\text{N-sodium hydroxide}$ was inserted between the outlet tube and the water pump, and suction applied and the outlet tap opened. After a few seconds the inlet taps were opened, 5 ml. of 5N- H_2SO_4 was drawn into the vessel and CO_2 -free air drawn alternately through the medium and the gas phase for 20 min. A total of 4.2×10^4 counts/min. was recovered as barium carbonate from the contents of the sodium hydroxide trap. The ether-soluble components of the culture medium at the end of growth were separated by distillation in steam into the volatile and non-volatile components; these had total activities of 322 and 913 counts/min. respectively. The volatile fraction contained 0.26 m-mole of formic acid of specific activity 920 counts/min./m-mole.

Amino acid	Specific activity (counts/min./m-mole)	Purine/pyrimidine base	Specific activity (counts/min./m-mole)
Aspartic acid	4780	Adenine	9380
Threonine	4600	Guanine	9060
Glutamic acid	3000	Uracil	9510
Isoleucine	4600	Cytosine	8750
Leucine	2440	Thymine	7530

samples (approx. 50 $\mu\text{g.}$) of valine, alanine and glycine + cystine were only 12–15% greater than background. The incorporation of ^{14}C into the amino acids listed in Table 9 is in agreement with previous work by Ehrensvald (1948) and Abelson, Bolton & Aldous (1952) with *Torulopsis utilis* and *E. coli*, and the approximately equal activities of aspartic acid, threonine and isoleucine, and the greater activity of aspartic acid over glutamic acid (Table 9) are similar to the findings of Abelson & Vogel (1955) for both *T. utilis* and *N. crassa*.

As observed by Bolton *et al.* (1952) with cultures of *E. coli*, the purine and pyrimidine bases isolated from the nucleic acids of *A. aerogenes* (Table 9) possessed similar specific activities. Although this finding is possibly not immediately reconcilable with the known biosynthetic mechanisms, the significant fact which emerges from the data of Table 9 is that carbon from $^{14}\text{CO}_2$ is incorporated into all pyrimidine bases. Thus the fixation of carbon dioxide into formic acid in the anaerobic cultures of *A. aerogenes* (Table 9) is accompanied by a concomitant fixation into the pyrimidines. As the uracil and cytosine of *A. aerogenes* grown in the presence of [^{14}C]valine are unlabelled (Table 8), it follows that fixation of metabolic carbon dioxide does not occur to any significant extent under the conditions of the latter experiments.

DISCUSSION

Several examples are available of the accumulation of valine, or its immediate precursor 3-methyl-2-

oxobutanoic acid, as a result of specific deficiencies of unrelated amino acids, such as phenylalanine, lysine, proline, histidine and arginine and of vitamins (e.g. biotin) in various microbial mutants which have requirements for these substances (e.g. Mitchell, 1953; Vogel & Bonner, 1954; Davis, 1955; Katsuki, 1955). In addition, an excretion of alanine and an accumulation of pyruvic acid have been observed in wild strains of *E. coli* and *A. aerogenes*, cultivated in simple chemically defined media under various conditions (Dagley *et al.* 1950). Thus the accumulation of alanine and valine in aminopterin-treated cultures of *A. aerogenes* cannot be assumed as evidence that folinic acid-dependent reactions are involved in the metabolism of these amino acids.

It is evident from the results in Table 4 that much of the alanine which accumulates in aminopterin-treated cultures of *A. aerogenes* during the inhibition phase must undergo deamination at the onset of recovery. Thus in cultures supplemented with alanine generally labelled with ^{14}C , the pyruvate formed from the exogenous amino acid is pooled with pyruvate from other sources and provides an intermediate for the synthesis of a number of amino acids. Utilization of pyruvate via the Krebs cycle, which is known to form an important mechanism for synthesis of amino acid in other micro-organisms (e.g. Roberts & Abelson, 1953; Wang, Christensen & Cheldelin, 1953; Abelson & Vogel, 1955; Vavra & Johnson, 1956) would explain the incorporation of activity into aspartic and glutamic acid and the amino acids derived from them.

The distribution of ^{14}C -activity in serine (Table 5) encountered in these experiments, and in particular the low activity of the β -carbon as compared with the α - and carboxyl-carbons, either indicates an extremely high turnover of the hydroxymethyl group, or suggests that this amino acid is not formed directly from pyruvate, but from a C_2 fragment which may be derived from it. Such a mechanism has been postulated in yeast, where serine appears to be synthesized from glycine (Wang *et al.* 1953), although in *E. coli* (Meinhart & Simmonds, 1955), as in mammalian cells (Arnstein & Keglević, 1956), serine forms a precursor of glycine.

Although pyruvate has been shown to be the sole source of valine in *A. aerogenes* (Rafelson, 1955), as in yeast (Strassman, Thomas & Weinhouse, 1953, 1955), no activity is incorporated into this amino acid when recovery from aminopterin inhibition occurs in the presence of generally labelled alanine (Table 4). Thus the valine which accumulates together with alanine in the medium during the inhibition phase must reach a concentration sufficient to prevent its further synthesis during the recovery period. This finding, which provides a further example of the inhibition by an exogenous amino acid of its synthesis (e.g. Ehrens-värd, 1955), has a close parallel in the observations of Adelberg & Umbarger, 1953) that the presence of valine in cultures of *E. coli* inhibits the synthesis not only of the amino acid but also of its precursors.

The synthesis of the carbon chain of valine is of interest for arguments developed later, as it must include the elimination of the carboxyl group from one of the two molecules of pyruvic acid. According to the hypothesis put forward by McManus (1954) and Strassman *et al.* (1953) as a result of experiments with *Torula* yeast, and the work of Umbarger, Brown & Eyring (1957) with a valine-requiring mutant of *E. coli*, acetolactate is an early intermediate in this synthesis. This compound, which is thought to undergo a pinacol-type rearrangement to 3-methyl-2-oxobutanoic acid, may be formed by a ketol condensation between pyruvate and acetaldehyde, the latter being derived by the action of carboxylase on a second molecule of pyruvate (but cf. Wang *et al.* 1953; Vavra & Johnson, 1956).

During the recovery of *A. aerogenes* from inhibition by aminopterin the accumulated valine is used extensively for the synthesis of leucine (Table 4), and provides the *isobutyl* moiety of the carbon skeleton of this amino acid. The remaining atoms, the α - and carboxyl-carbon atoms, are derived from the accumulated alanine via pyruvic acid (Table 5). Leucine biosynthesis in *A. aerogenes* thus appears to be identical with that in *E. coli* (Abelson, 1954), *T. utilis* (Strassman, Locke, Thomas & Weinhouse,

1955; Abelson & Vogel, 1955) and *N. crassa* (Andersson-Kottö *et al.* 1954; Abelson & Vogel, 1955). In these organisms it is known that the leucine carbon chain is formed from the acetyl and *isobutyryl* radicals, although the mechanism of the reaction has yet to be established experimentally. Abelson & Vogel (1955) assume a direct combination, whereas Strassman *et al.* (1955) propose a more elaborate sequence of reactions which includes the condensation of the methyl carbon of acetyl-CoA with α -oxoisovaleric acid to yield α -hydroxy- α -isopropylsuccinic acid as an intermediary. More recently, T. Wainwright & D. D. Woods (personal communication) have suggested that a condensation occurs between *isobutyryl*-CoA and acetyl-CoA in a manner analogous to the formation of straight-chain fatty acids.

Irrespective of the nature of this condensation reaction, however, it is apparent that at some stage in the biosynthesis of leucine the valine carboxyl carbon is eliminated. The studies with $[1-^{14}\text{C}]$ valine (Tables 6–8), considered in relation to those with $^{14}\text{CO}_2$ (Table 9), strongly suggest that this carboxyl group is eliminated, not as carbon dioxide, but as some other C_1 unit which is utilized in the synthesis of nucleic acid purines and thymine. Evidence from other systems shows that the source of the thymine methyl carbon atom is either formate, or the β -carbon of serine (Totter, 1954; Elwyn & Sprinson, 1954). It is apparent from Tables 7 and 8, however, that under the conditions of the present experiments the activity incorporated into the entire serine molecule is less than that incorporated into thymine. On this evidence therefore formate appears to be the more likely intermediate in the transfer of the valine carboxyl carbon to thymine.

The fact that the metabolic formate present at the end of logarithmic growth of cultures supplemented with $[1-^{14}\text{C}]$ valine contains ^{14}C also suggests that the valine carboxyl carbon forms one source of formate in *A. aerogenes*. The presence of this radioactive formic acid cannot be attributed to the fixation of metabolic carbon dioxide, since it is obvious from the results of Table 9 that, under conditions where formic acid is derived from $^{14}\text{CO}_2$, activity is also incorporated into uracil and cytosine. Indeed, the absence of ^{14}C from the pyrimidine bases when $[1-^{14}\text{C}]$ valine forms the source of isotopic carbon may be considered to support the thesis that the valine carboxyl carbon forms a direct source of formate, since in both mammalian and microbial cells formate is not incorporated into uracil (Heinrich & Wilson, 1950; Edmunds, Delluva & Wilson, 1952).

Formic acid is known to participate in two stages in purine synthesis, providing a source from which $\text{C}_{(2)}$ and $\text{C}_{(8)}$ of the complete molecule are derived (e.g. Carter, 1956). Thus if the incorporation of ^{14}C

from [1- ^{14}C]valine into adenine and guanine of normal cultures of *A. aerogenes* proceeds through the intermediary of formic acid it would be expected that the major part of the total activity of the purine molecule would be shared between $\text{C}_{(2)}$ and $\text{C}_{(8)}$. The finding that at least a quarter of the total activity of the guanine molecule is located at $\text{C}_{(2)}$ is therefore further evidence for the direct formation of metabolically active formic acid from the valine carboxyl group. In aminopterin-treated cultures of *A. aerogenes*, however, the accumulation of 5-amino-4-imidazolecarboxamide riboside as well as valine suggests that in the presence of the folic acid antagonist, $\text{C}_{(2)}$ and $\text{C}_{(8)}$ of the purine nucleus are derived from different sources.

Although it is uncertain whether the synthesis of the purine nucleotide from 5-amino-4-imidazolecarboxamide ribosyl phosphate occurs via inosinic acid or adenylosuccinic acid or both, the biosynthetic mechanisms leading to the formation of the imidazole derivative from glycine in certain cells seem well established (see, for example, Carter, 1956). It is particularly interesting that 5-amino-4-imidazolecarboxamide riboside (or ribotide) accumulates in the presence of aminopterin, since in the sequence of reactions leading to the formation of this compound the formylation of the intermediary glycinamide ribosyl phosphate is also dependent upon a folinic acid coenzyme (Goldthwait, Peabody & Greenberg, 1956*a, b, c*). It is possible, however, that the mechanism of synthesis of the imidazole intermediate in bacteria differs from that of avian and mammalian cells. Thus although Edmunds *et al.* (1951, 1952) and Koch (1955) found [2- ^{14}C]glycine to give rise to [5- ^{14}C]guanine in yeast and *E. coli* respectively, Gots & Gollub (1956), in a study of 5-amino-4-imidazolecarboxamide ribotide synthesis by cell-free extracts of an *E. coli* mutant (B-96), were unable to demonstrate any requirement for glycine, formate, bicarbonate or aspartic acid.

The accumulation of the imidazole derivative by *A. aerogenes* is in accordance with previous findings in *E. coli* (Woolley & Pringle, 1950; Edwards *et al.* 1952), and the known inhibition by the folic acid antagonists of the incorporation of formate into nucleic acid purines (Skipper, Mitchell & Bennett, 1950; Goldthwait & Bendich, 1952). In the present system, however, there is an inhibition not only of the incorporation, but also of the production of formate. In this respect there seems to be some analogy between the accumulation of amino acids in the aminopterin-treated *A. aerogenes* cultures, and the depression of the synthesis of glycine from serine (a reaction which contributes to the formate 'pool') by folic acid deficiency in animal tissues (Elwyn & Sprinson, 1956).

The incorporation of formate into the methyl group of thymine, which in chick and rabbit bone-marrow cells is known to be inhibited by aminopterin (Totter & Best, 1955; Friedkin & Roberts, 1956), must involve a reductive step. Elwyn, Weissbach, Henry & Sprinson (1955) consider that two different types of folic acid coenzyme, in which the active intermediates are hydroxymethyl and formyl derivatives, are required for the introduction of the methyl group into thymine (and also into choline and methionine) and in the synthesis of purines respectively. Although these two forms of the coenzyme are interconvertible by a reversible di- (or tri-) phosphopyridine nucleotide enzyme, at least in the serine hydroxymethylase system (Kisluk & Sakami, 1955), studies in which the β -carbon atom of [$^{14}\text{CD}_2\cdot\text{OH}$]serine has been shown to be transferred to the methyl group of thymine (Elwyn & Sprinson, 1950) and choline (Elwyn *et al.* 1955) without the loss of a deuterium atom, suggest that the rate of this interconversion is small compared with the rates of incorporation of the formyl and hydroxymethyl units. From these considerations the incorporation of ^{14}C from [1- ^{14}C]valine into thymine observed in the present work (Table 8) seems particularly high, although the value of approximately two for the ratio of the specific activity of adenine or guanine to that of thymine (Table 8) agrees with the figure given by Goldthwait & Bendich (1952) to express the ratio of formate incorporated into adenine and thymine by rat tissues.

The mechanism of the production of formate from the valine carboxyl group remains to be established experimentally. On the assumption that the accumulation of both alanine and valine is the result of the inhibition of the metabolism of the corresponding keto acids, it is possible that there is a common folinic acid-dependent step in the metabolism of both 3-methyl-2-oxobutanoic acid and pyruvic acid. It has been suggested by Kidder (1953), although without experimental justification, that a formyl derivative of folinic acid (CoF) may be a primary product of the dissimilation of pyruvate to acetyl-CoA and formic acid (e.g. Chantrenne & Lipmann, 1950). If this hypothesis is correct, as suggested by the recent observations of Chin, Krampitz & Novelli (1957) on the stimulation by tetrahydrofolic acid of the incorporation of formate into pyruvate by cell-free extracts of *E. coli*, a deficiency of CoF would be expected to lead to an inhibition of one pathway of pyruvate utilization. This would offer an explanation for both the reduced ability of folinic acid-deficient *Leuconostoc citrovorum* cells to decompose pyruvic and lactic acids (Chang, Silverman & Keresztesy, 1951), and the accumulation of alanine by *A. aerogenes* in the presence of aminopterin. The

mechanism of leucine biosynthesis suggested by T. Wainwright & D. D. Woods (personal communication) includes the formation of isobutyryl-CoA from 3-methyl-2-oxobutanoic acid, a reaction which is known to occur in the degradation of valine by extracts of rat heart and liver (Coon, 1955; Robinson, 1956; Robinson, Nagle, Bachhawat, Kupiecki & Coon, 1957). The cleavage of 3-methyl-2-oxobutanoic acid to isobutyryl-CoA and formic acid thus appears analogous to the cleavage of pyruvate to acetyl-CoA and formic acid, and it is possible that both of these reactions are dependent upon folinic acid.

SUMMARY

1. Throughout the period of inhibition of growth which follows the addition of the folic acid antagonist, aminopterin, to cultures of *Aerobacter aerogenes* in a simple chemically defined medium, alanine, valine and 5-amino-4-imidazolecarboxamide riboside accumulate in the medium.

2. Inhibition of growth of *A. aerogenes* by aminopterin is accompanied by a decrease in deoxyribonucleic acid content, which markedly exceeds that of any other cell component.

3. With the resumption of growth, which occurs after 15–18 hr. through the inactivation of the aminopterin, the accumulated intermediates are reutilized rapidly, and the chemical composition of the cells becomes similar to that of normal cells grown in the absence of the antagonist.

4. In the recovery cultures the accumulated alanine is utilized in the synthesis of a number of amino acids including leucine, for which it provides the α - and carboxyl-carbon atoms. The accumulated valine, however, is incorporated to a significant extent only into valine and leucine of the protein of *A. aerogenes*.

5. The synthesis of leucine from valine involves the elimination of the carboxyl group of the latter amino acid. In normal cultures of *A. aerogenes* this occurs with the formation of a C_1 unit other than carbon dioxide, which is utilized in the synthesis of the purine nucleus and the methyl group of thymine, and is probably formic acid.

6. An analogy is drawn between the cleavage of 3-methyl-2-oxobutanoic acid with the production of formic acid, and the dissimilation of pyruvate to acetyl-coenzyme A and formic acid, and it is suggested that both of these reactions may be dependent upon folinic acid and susceptible to inhibition by aminopterin.

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ADDENDUM

A Factorial Experiment to Study Factors which Reverse Aminopterin Inhibition

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The experiments reported in the preceding paper (Webb, 1958) showed that the inhibition of growth produced by aminopterin in cultures of *Aerobacter aerogenes* was partially prevented by the addition of glycine, adenine, L-threonine and L-glutamic acid to the culture medium. However, when attempts were made to investigate the significance of the individual components of this mixture, by omitting them one at a time from the culture medium, equivocal results were obtained. This appeared to be due to the marked stimulatory effect of L-glutamic acid on the growth of *A. aerogenes* in the simple medium, and to interactions between the effects of the remaining components. The effects of these substances were investigated therefore by means of a factorial experiment, such an experiment being ideally suited for the exploration of complex situations of this type.

METHODS

The culture conditions and details of the turbidity measurements are given by Webb (1958). Each of the four factors were employed at two levels, absent or present, giving the sixteen treatment combinations which are listed in Table 1. These mixtures were prepared in duplicate in 4 ml. of culture medium, and then inoculated with either an aminopterin-treated or untreated culture of *A. aerogenes*. This procedure was adopted in favour of that used in the growth experiments described in the main paper in order to overcome the variations in growth induced by the presence or absence of L-glutamic acid.

The tubes were incubated at 37° and the turbidity of each was measured at 0, 2.5, 5 and 21 hr. A replicate of the above procedure was carried out with a different overnight culture of *A. aerogenes* and different samples of aminopterin.

Mixtures. Mixtures were prepared in test tubes. When a factor was present in the treatment combination an amount sufficient to give a final concentration of mM was added in 1 ml. of the culture medium. All tubes were made up to a final volume of 4 ml. with the medium. Two sets of sixteen tubes, each corresponding to a separate treatment combination, were prepared in each replicate; numbers A1-A16 were inoculated from an aminopterin-treated culture, and numbers C1-C16 were inoculated from a control culture.

Aminopterin-treated culture. A volume (75 ml.) of medium was inoculated with an overnight culture of *A. aerogenes* and incubated at 37° until the turbidity reached

an optical density of 0.40-0.45 ($\approx$ 190-220 μ g. of dry cells/ml.); 35 ml. of this culture was then transferred to a flask containing 5 ml. of a 1:1000 solution of aminopterin at 37° and 2 ml. of this final mixture was added to each of the tubes A1-A16, giving a final aminopterin concentration of 1:24 000 ($\approx$ 0.1 mM).

Control culture. A volume (35 ml.) of the culture was similarly treated with 5 ml. of medium and 2 ml. samples from this mixture were added to tubes C1-C16.

EXPERIMENTAL DESIGN

An examination of the method by which the organisms from the overnight culture were allotted to the treatments shows that two stages were involved. In the first stage aminopterin-treated and control cultures were prepared; then, in the second stage, these cultures were each used to inoculate the appropriate set of sixteen tubes containing the mixtures under study. Thus the experiment is of the split-plot type; the aminopterin-treated and control cultures may be considered whole-plots, and each contains 16 split-plots arranged in a 2⁴ factorial scheme. A discussion of this design and its statistical analysis is given in many textbooks on the design of experiments (e.g. Kempthorne, 1952).

RESULTS

Results obtained after incubation for 5 hr. are shown in Table 1, and the analysis of variance is given in Table 2. The analysis for the split-plot design is in two parts with separate error terms: the whole-plot analysis, which compares the overall difference between the control and aminopterin-treated cultures (error *a*), and the split-plot analysis, which compares the effects of the factorial treatments within each whole-plot (error *b*). A substantial difference was obtained between the overall effects of the control and aminopterin-treated cultures. The experimental design used, however, does not allow an effective estimate of the error of this comparison to be made since it is based on only 1 degree of freedom. Thus no useful test of significance of the whole-plot effect is possible. Nevertheless this does not matter since inhibition of growth by aminopterin is well established.

In the split-plot analysis there are 15 degrees of freedom, which allow an examination to be made of the main effects and all the interactions of the sixteen factorially arranged treatments. Moreover, a further 15 degrees of freedom allow an examination to be made of the interaction of the effects of the whole-plot and split-plot treatments, i.e. an examination can be made of whether or not the effects of the sixteen factorial treatments are identical in the aminopterin-treated and control cultures. All of these thirty components are investigated with equal precision and their mean squares are tested for significance against error *b*. In the full analysis all of these individual mean squares were computed, but, for brevity, only the main effects and significant interactions are shown in Table 2.

It can be seen that the effects of adenine and L-glutamic acid are highly significant, and the effects

of glycine and L-threonine are not significant. Further, the adenine $\times$ aminopterin (BE), L-glutamic acid $\times$ aminopterin (DE) and adenine $\times$ L-glutamic acid $\times$ aminopterin (BDE) interactions are all highly significant, whereas all other interactions are not significant at the $P=0.05$ level. If the experiment is analysed in two independent parts corresponding to the control and aminopterin-treated whole-plots it is found that in both the adenine $\times$ L-glutamic acid interactions are significant. Since these interactions are in opposite directions, however, the same interaction in the overall analysis is not significant.

The above analysis indicates that glycine and L-threonine have no demonstrable influence on the system and that the effects of adenine and L-glutamic acid depend on whether or not aminopterin is also present in the culture medium. Fig. 1 demonstrates graphically the BDE three-way

Table 1. *Growth of Aerobacter aerogenes in aminopterin-treated and control media with and without glycine, adenine, L-threonine and L-glutamic acid*

The turbidity readings (optical density) of cultures after incubation for 5 hr. at 37°, after addition of various combinations of glycine, adenine, L-threonine and L-glutamic acid in aminopterin-treated and control media. +, The component was added to give a final concentration of mM; -, the component was omitted. The experiment was carried out in two replicates, with different samples of aminopterin.

Tube no.	Glycine	Adenine	L-Threonine	L-Glutamic acid	Turbidity (<i>E</i>)	
					I	II
Aminopterin-treated series						
A1	-	-	-	-	0.209	0.150
2	+	-	-	-	0.221	0.166
3	-	+	-	-	0.366	0.184
4	+	+	-	-	0.351	0.198
5	-	-	+	-	0.244	0.188
6	+	-	+	-	0.212	0.173
7	-	+	+	-	0.376	0.219
8	+	+	+	-	0.402	0.228
9	-	-	-	+	0.188	0.176
10	+	-	-	+	0.221	0.191
11	-	+	-	+	0.460	0.287
12	+	+	-	+	0.544	0.313
13	-	-	+	+	0.241	0.200
14	+	-	+	+	0.254	0.186
15	-	+	+	+	0.533	0.352
16	+	+	+	+	0.535	0.374
Control series						
C1	-	-	-	-	0.630	0.718
2	+	-	-	-	0.653	0.752
3	-	+	-	-	0.641	0.758
4	+	+	-	-	0.622	0.730
5	-	-	+	-	0.648	0.732
6	+	-	+	-	0.678	0.744
7	-	+	+	-	0.631	0.738
8	+	+	+	-	0.631	0.722
9	-	-	-	+	0.896	0.892
10	+	-	-	+	0.918	0.886
11	-	+	-	+	0.828	0.832
12	+	+	-	+	0.823	0.842
13	-	-	+	+	0.899	0.877
14	+	-	+	+	0.884	0.897
15	-	+	+	+	0.822	0.818
16	+	+	+	+	0.816	0.837

Table 2. *Analysis of variance of the data shown in Table 1*

Levels of significance are given in parentheses in the last column.

Source of variation	Degrees of freedom	Mean square
<i>A. Whole-plot analysis</i>		
Between replicates	1	0.0162
Control v. aminopterin-treated cultures (E)	1	3.9268
Error (a)	1	0.0998
<i>B. Split-plot analysis</i>		
Factorial treatments:	(15)	
Glycine (A)	1	0.0011
Adenine (B)	1	0.0558 ($P < 0.001$)
L-Threonine (C)	1	0.0031
L-Glutamic acid (D)	1	0.2385 ($P < 0.001$)
Interactions	11	0.0006
Whole-plot $\times$ split-plot treatments, interactions:	(15)	
BE	1	0.1516 ($P < 0.001$)
DE	1	0.0386 ($P < 0.001$)
BDE	1	0.0319 ($P < 0.001$)
Remainder	12	0.0008
Error (b)	30	0.00194

Fig. 1. Effect of adenine, L-glutamic acid and aminopterin on the growth of *A. aerogenes* after incubation for 5 hr. Values are the means of the BDE three-way table. (a) Without aminopterin; (b) with aminopterin.

table. It can be seen that aminopterin depresses growth under all conditions. Also the addition of L-glutamic acid causes greater growth, but the magnitude of this effect depends considerably on the absence or presence of the other two substances. The effect of adenine, however, is opposite in the aminopterin-treated and control cultures; in

the control cultures the adenine depresses the stimulating effect of L-glutamic acid, whereas in the aminopterin-treated cultures the addition of adenine enhances growth even when glutamic acid is not present in the medium. If L-glutamic acid is present, however, the enhancement of growth is greater still.

Similar results were obtained after incubation for 2.5 and 21 hr. and are not presented in this paper since they add little extra information to the above results.

SUMMARY

1. Glycine and L-threonine have no effect on the growth of *Aerobacter aerogenes* when they are added to aminopterin-treated or control media.
2. L-Glutamic acid stimulates growth in both aminopterin-treated and control media.
3. Adenine stimulates growth in aminopterin-treated media and in control media inhibits the effect of L-glutamic acid.

REFERENCES

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