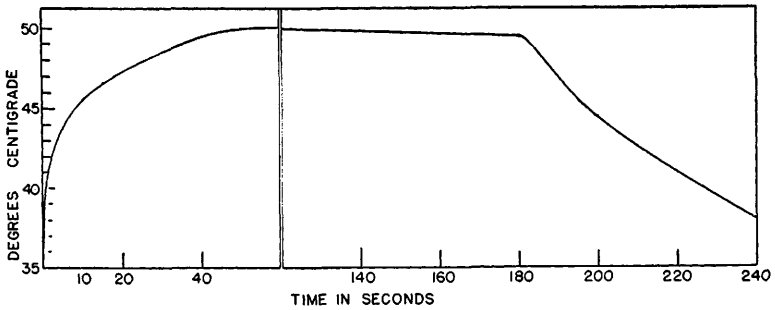

FIG. 3. Subcutaneous temperatures in a rat exposed to circulating water at 55°C. The rat was placed in water bath at T = 0 sec. and removed at T = 180 sec.

the skin before exposure, its position being verified by dissection following the thermal episode. A typical example of a subcutaneous temperature curve is shown in Fig. 3.

Summary. A simple and sensitive thermoelectric device for the measurement of temperature has been described which incorporates the thermistor as the temperature-sensing element. As a needle-type thermometer, this apparatus has been used extensively in our laboratory to obtain continuous recordings of abdominal subcutaneous temperatures attained during the production of hydrothermal burns in rats. On the basis of the many advantages inherent in this apparatus, its further utilization for temperature measurements in biological studies is advocated.

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Resistance to Folic Acid Analogues in a Strain of *Streptococcus faecalis.** (20332)

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The possibility that organisms which are resistant to certain antagonists of folic acid possess or develop the ability to convert such analogues to metabolically active compounds has formed an attractive hypothesis. Aminopterin (4-amino-pteroylglutamic acid) is readi-

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ly converted to pteroylglutamic acid (PGA) by alkaline hydrolysis(1). Kidder *et al.*(2) observed that Aminopterin and Methopterin (10-methyl PGA) promoted the growth of a species of *Tetrahymena* and suggested that this organism possesses enzymes capable of deaminating and demethylating these antagonists. Hutchison and Burchenal(3) also suggested that an antagonist-resistant strain of *Streptococcus faecalis*(4) utilizes in a similar manner these and related analogues for growth. In preliminary experiments designed to test this concept by measurement of precursors of citrovorum factor (CF) which might be derived from the antagonists, technics were developed for the separation of 4-amino and 4-hydroxy pteridines(5).

It has been found, employing paper chromatographic technics, that the folic acid-antagonists which are presently available contain sufficient PGA and pteroic acid to account for the apparent "utilization" of the analogues by *S. faecalis*. The remarkable increase in the resistance to certain folic acid antagonists which had been induced in the strain of *S. faecalis* studied(4) can be explained, in part, by its more effective utilization of PGA and pteroic acid, as shown in these studies. Similar observations were reported recently by Hutchison and Burchenal(6). That other phenomena are involved in the development of resistance will be discussed in detail in a subsequent communication.

Methods. Microbial assays—Streptococcus faecalis (ATCC 8043) was used for the microbial determination of folic acid-like activity, as described in the AOAC Methods of Analysis (7). An antagonist-resistant strain of *S. faecalis*§(4), designated as 8043AR, was used similarly for the assay of folic acid-like activity in the presence of the antagonists. In each case, a sample of pteroylglutamic acid was used as a reference standard. Citrovorum factor (CF) was determined using *Leuconostoc citrovorum* (ATCC 8081) grown in the medium described by Sauberlich(8). An antagonist-resistant strain of *Leuconostoc citrovorum*§(9), designated as 8081AR, was used in the same manner for the assay of CF in the presence of high concentrations of the antagonists. Leucovorin (synthetic CF) was used as a reference standard. The activity of the samples is expressed on the basis of the natural form of the free acid, since the naturally-occurring form of CF appears to be twice as active as leucovorin. The basis for this statement has been discussed elsewhere(10).

Bioautographic technic. The distribution of the compounds on paper chromatograms was followed by means of a modification of previously described bioautographic technics (11,12). Solid medium (folic acid-assay medium containing 1% agar) was inoculated with a strain of *S. faecalis* immediately before the medium was poured into suitable trays. The paper strips were placed on the surface of the agar for 10 min; the trays then were placed in a 37° incubator. The zones of growth or inhibition were visible within 6 hours, but recordings were made after an 18-hour period. Growth-promoting compounds were readily located when PGA was omitted from the medium. When PGA was included in the medium at a level of 10 mμg/ml, the agar was clouded by growth except where inhibitory compounds caused clear zones. With a smaller amount of PGA in the medium, 1 mμg/ml, so that only limited growth resulted, both inhibitory and growth-promoting compounds could be readily located on a single bioautograph. By addition to the medium of 2,3,5-triphenyl tetrazolium chloride (200 μg per ml), which is reduced to an insoluble red compound by the growing cells, the visibility of areas of faint growth was improved and better contrast for photographic records was obtained. However, zones of growth caused by 0.5 mμg of PGA per strip were clearly visible without this aid. A convenient inoculum was prepared by lyophilization of the washed bacterial cells in a lactose suspending-medium(13). This preparation retained activity for a period of approximately 3 months when stored at room temperature in a desiccator (1 mg of the lyophilized cells was adequate for 100 ml of medium). Chromatograms, using strips or sheets of Whatman No. 1 paper, were developed by descending solvent (0.1 *M* phosphate buffer, pH 7.0) which moved 44 to 48 cm in 4 hours. When dry, the strips (1″ in width) were cut in half lengthwise. In order to locate both inhibitory and

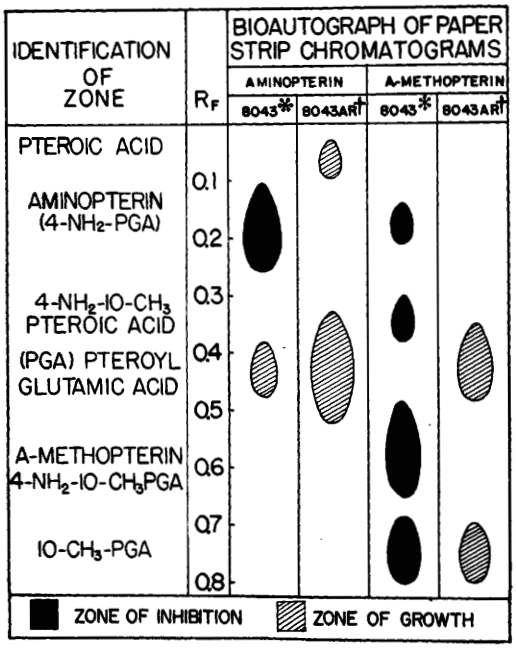

FIG. 1. Comparison of response of antagonist-sensitive (8043) and antagonist-resistant (8043-AR) strains of *S. faecalis* to Aminopterin and A-methopterin.
* Antagonist-sensitive strain of *S. faecalis*.
† " -resistant " " " " .

growth-promoting compounds, one half of each paper strip was placed on solid medium which contained PGA, the other half on medium without PGA. *Resting cell experiments.* Cells of strains 8043 and 8043AR were grown in a medium containing PGA (1 mμg/ml), harvested after 16 hours, washed twice in saline (0.9%), and the weight of the packed cells was recorded. The cell suspensions, in 0.1 *M* phosphate buffer of pH 6.5, were agitated constantly while incubating anaerobically at 37° with PGA or Aminopterin and the other additions indicated. At the end of the incubation period the samples were autoclaved at a pressure of 15 pounds for 30 min. The cells were spun down and the clear extract was appropriately diluted for microbial assay with *Leuconostoc citrovorum.*

Experimental and results. Bioautographs of Aminopterin and A-methopterin (4-amino-10-methyl-pteroylglutamic acid), in which the response of strains 8043 and 8043AR were compared, are shown in Fig. 1. With the antagonist-sensitive strain, large clear zones indicated the location of Aminopterin and A-methopterin. No effect was visible in the corresponding areas of the other half of each strip which was placed on medium containing the antagonist-resistant organism. Aminopterin contained two growth-promoting compounds with R_f values which corresponded to those of PGA and pteroic acid, respectively. The PGA which was present on one half of the strip consistently gave larger zones with strain 8043AR and the amount of pteroic acid which was adequate for the growth of strain 8043AR was insufficient to cause visible growth of strain 8043. A-methopterin was contaminated with 3 inhibitory compounds with R_f values which corresponded to Aminopterin, 4-amino-10-methyl-pteroic acid and 10-methyl-pteroylglutamic acid, respectively. Also, a growth-promoting compound was present which apparently was PGA. Growth of the antagonist-resistant organism was consistently observed in the area corresponding to 10-methyl-PGA; however, 2-dimensional chromatography showed that this zone contained PGA.

Tentative *identification of the contaminants* found in Aminopterin and A-methopterin, based on their R_f values, was made by comparison of the bioautographs shown in Fig. 2. Growth promoting compounds were observed in pteroylaspartic acid and in 4-amino-pteroyl-aspartic acid. Aminopterin-2 was obtained by elution with distilled water of the zone (R_f-0.12 to 0.29) obtained when Aminopterin-1, the initial material, was chromatographed on paper sheets. The amount of Aminopterin-2 was determined by microbial and fluorometric assays(5). Although the zones of Aminopterin and PGA were distinctly separate, only partial purification of Aminopterin was obtained in this way. A-methopterin-2 was obtained by elution of the A-methopterin zone (R_f-0.43 to 0.62) from the chromatogram of the initial material. A-methopterin-3 (Fig. 2) was derived from A-methopterin-2 in the same manner. Although the materials were protected from light and heat during these operations, to minimize degradation of the compounds, the twice-chromatographed A-methopterin-3 still contained detectable amounts of two contaminants. The antagonist-resist-

ant strain of *S. faecalis* was used to study the growth-promoting activity of the analogues. The biological activity of the analogues (Fig. 2) cannot be expressed as a quantitative measure of the activity of a single compound, since most of the analogues studied contained pteroic acid as an active contaminant.

The requirement for CF was the same for each bacterial strain, but the resistant strain required much less PGA or pteroic acid than the sensitive strain (Table I). PGA and leucovorin were equally active for strain 8043, but PGA was more active than leucovorin for strain 8043AR (it should be noted that these findings take into account the circumstance that leucovorin appears to be composed of inert material to the extent of 50%) (10).

Cell suspensions of the antagonist-sensitive and antagonist-resistant strains of *S. faecalis* were incubated with PGA and the amount of CF formed was determined by microbial assay (Table II). Only an insignificant increase in the amount of CF formed by strain 8043 was obtained when the concentration of PGA in the suspending medium was greater than 0.5 μg/ml. However, with the resistant strain, the amount of CF formed increased as the concentration of PGA was increased; thus, at a PGA-level of 50 μg/ml this organism formed more than 200 times as much CF as the sensitive strain under the same conditions. In the same experiment, Aminopterin or A-methopterin in amounts of 0.1 μg per ml caused approximately 50% inhibition of the formation by strain 8043 of CF from PGA, while a similar degree of inhibition of the formation of CF from PGA by strain 8043AR required a 500-fold increase in the concentration of these antagonists.

The amounts of CF formed from the two samples of Aminopterin and from PGA are shown in Table III. The CF derived from Aminopterin-1 corresponded to approximately 10% of the amount added. The yield of CF from PGA was approximately 50%. Therefore, the precursors of CF in Aminopterin-1 can be estimated to be about 20% of the reagent material. The yield of CF from the

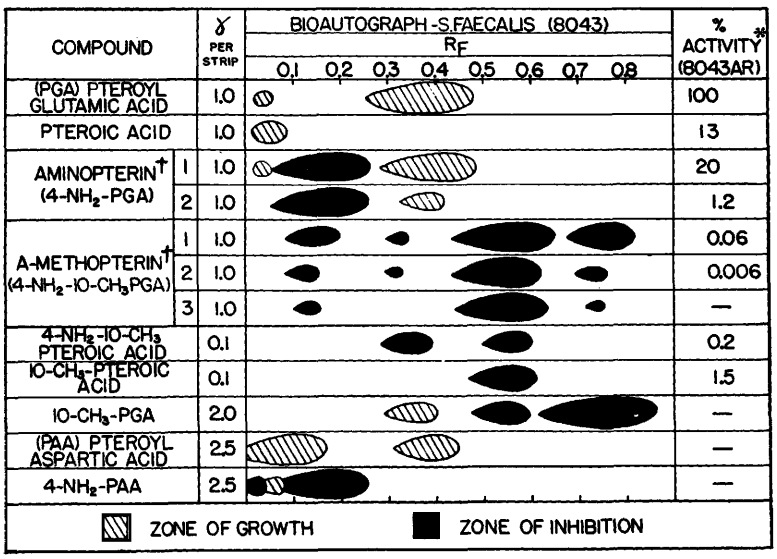

FIG. 2. Paper chromatograms and growth-promoting capacity of several analogues of pteroylglutamic acid.

* Comparison of growth-promoting capacity based upon microbial assay of analogues, using antagonist-resistant strain of *S. faecalis* (8043 AR). Large amounts of pteroic acid required to promote growth of antagonist-sensitive strain (8043) which was used for bioautographs shown here (see Fig. 1 and Table I).

† Aminopterin-2 obtained by elution of the Aminopterin zone of a chromatogram of Aminopterin-1, the commercial material. Similarly, A-methopterin-2 and -3 are, respectively, the once and twice chromatographed materials (see text for details).

partially purified Aminopterin-2 was exceedingly small.

Discussion. The presence of contaminants in various folic acid-antagonists has caused considerable confusion in the interpretation of the results of studies of resistance to these compounds. The antagonists which are presently available contain not only sufficient PGA and pteroic acid to account for the apparent utilization of the analogues by *S. faecalis* (3), but other inhibitory compounds may also be present, as found in A-methopterin. The growth-promoting capacity of Aminopterin (one-sixth that of PGA) reported for a species of *Tetrahymena* (2) actually corresponds closely to the amount of PGA found in these studies to be present in samples of the analogue. It will be of importance to determine the effect of the simultaneous presence of these growth-promoting compounds on the rate of development of resistance, since the resistant strain of *S. faecalis* utilized PGA and pteroic acid much more effectively than did the antagonist-sensitive strain.

Instead of deaminating the 4-amino analogues of PGA, it appears from these studies that in the resistant cell the ability to absorb the analogues may have been reduced greatly. Thus, the formation of CF from PGA by resistant cells of *S. faecalis* is inhibited only by high concentrations of Aminopterin or A-methopterin, while in extracts of these cells, the same reaction is inhibited by amounts of these antagonists which also inhibit the formation of CF by the antagonist-sensitive cells or

TABLE I. Requirement for PGA, Pteroic Acid, and CF of Antagonist-Sensitive (8043) and Antagonist-Resistant (8043AR) Strains of *S. faecalis.*

Compound	Requirement for half-maximal growth* I Strain 8043	II Strain 8043AR	Approx. ratio I/II
	mμg/ml of assay medium		
Pteroylglutamic acid	.18	.06	3
Pteroic acid	7.0	.6	12
Citrovorum factor†	.16	.17	1

* Turbidimetric reading of growth in 10 ml of assay medium.

† A standard solution of leucovorin was used; activity is expressed in terms of naturally-occurring CF.

TABLE II. Formation of CF from PGA by Resting Cells of Antagonist-Sensitive (8043) and Antagonist-Resistant (8043AR) Strains of *S. faecalis.*

Additions to suspending medium* PGA, μg/ml	Ratio	CF formed by cell suspensions of *S. faecalis* Strain 8043	Strain 8043AR
	Antagonist:PGA	μg CF/25 mg cells, wet wt	
0	—	0	0
.5	—	.16	.20
5	—	.18	3.21
50	—	.20	44.4
	Aminopterin:PGA		
50	1:1	—	17.0
"	1:10	—	39.9
"	1:100	.038	42.3
"	1:250	.084	—
"	1:500	.11	—
"	1:1000	.15	—
	A-methopterin:PGA		
50	1:1	—	23.1
"	1:10	—	37.5
"	1:100	.025	37.0
"	1:250	.070	—
"	1:500	.11	—
"	1:1000	.14	—

* Cells (25 mg/ml, wet wt) incubated at 37° for 4 hr under an atmosphere of nitrogen (95%) and carbon dioxide (5%) in 2 ml of a medium consisting of Na_2HPO-KH_2PO_4 buffer (0.1 *M*, pH 6.5), glucose (0.02 *M*), sodium formate (0.01 *M*), sodium ascorbate (0.005 *M*) and PGA, Aminopterin or A-methopterin, as indicated. Preparation of samples for assay described in text.

TABLE III. Formation of Citrovorum Factor from Precursors in Commercial and Partially Purified Samples of Aminopterin.

Conc. of PGA or Aminopterin in suspending medium,* μg/ml	CF formed by cell suspensions of *S. faecalis* (8043AR) PGA	Aminopterin–1†	Aminopterin–2†
	μg CF/25 mg cells, wet wt		
0	0	0	0
1.5	4.76	.56	—
3	7.26	1.16	.05
6	12.2	2.53	.04
12	18.2	5.36	.03

* Cells (6.25 mg/ml, wet wt) incubated at 37° for 4 hr under an atmosphere of nitrogen (95%) and carbon dioxide (5%) in 4 ml of a medium consisting of Na_2HPO_4-KH_2PO_4 buffer (0.1 M, pH 6.5), glucose (0.02 *M*), sodium formate (0.01 *M*), sodium ascorbate (0.005 *M*) and PGA or Aminopterin as indicated. Preparation of samples for assay described in text.

† Aminopterin–1 refers to the commercial material, while Aminopterin–2 is that which had been purified by paper chromatography, described in text.

their extract(14). No detectable effect of these antagonists was observed on bioautographs obtained with the *intact* resistant organism: neither growth, indicating utilization of these compounds, nor inhibition, which might be anticipated if the compounds were able to exert their expected effect within the cells. Hutchison and Burchenal(9) reported that the requirement for CF of the antagonist-resistant strain of *Leuconostoc citrovorum* was the same as that of the parent strain and also, this organism, like the parent strain, was unable to utilize PGA effectively. Conceivably, the primary mechanism of resistance in *Leuconostoc citrovorum* involves an altered permeability to the antagonists, since a more efficient utilization of the factor required for growth does not appear to be implicated.

Paper chromatography is an aid to the identification and purification of these compounds but does not ensure either. In the system used, A-methopterin has the same R_f as 10-methyl-pteroic acid. The inhibitor at R_f 0.55, which was present in the sample of 10-methyl-PGA, was found, by the use of a different solvent, to migrate in the same manner as 10-methyl-pteroic acid. "Trailing" of compounds is frequently observed on paper chromatograms. Consequently, the presence of some PGA at the zone of Aminopterin was not surprising. However, twice chromatographed A-methopterin "carried" a slower moving inhibitor. Thus, it appears that these similar compounds have considerable ability to "carry" each other. A two-dimensional chromatogram of 10-methyl-PGA showed that the discreet zone of R_f 0.75 separated into two components, in the second dimension; one of these apparently was PGA. Although 10-methyl-PGA and 10-methyl-pteroic acid were apparently "utilized" by the resistant strain, as indicated by distinct zones of growth on the bioautographs, clarification of this possibility must await additional purification of the compounds.

Summary. Bioautographs of several 4-amino analogues of folic acid indicated the presence of contaminants which corresponded to the demethylated, deaminated, or pteroic acid analogues. All the antagonists studied contained sufficient pteroylglutamic acid or pteroic acid to account for their apparent utilization for growth by an antagonist-resistant strain of *Streptococcus faecalis*. This strain had a lower requirement for PGA and pteroic acid and a much greater capacity to convert PGA to CF than did the parent antagonist-sensitive strain. Significant inhibition of the formation of CF from PGA by the resistant cells was obtained only with very high concentrations of Aminopterin or A-methopterin.

We are indebted to the Lederle Laboratories Division and the Calco Chemical Division of the American Cyanamid Co. for supplies of leucovorin, and for pteroylglutamic acid, and its various analogues.

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