Biochemical Pharmacology, 1959, Vol. 3, pp. 1–9. Pergamon Press Ltd., Printed in Great Britain.

STUDIES ON THE NATURE OF RESISTANCE OF *STREPTOCOCCUS FAECALIS* TO FOLIC ACID ANTAGONISTS*

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(*Received* 4 *May* 1959)

Abstract—Mutants of *Streptococcus faecalis* were selected at different levels of amethopterin ("methotrexate") in a medium containing pteroylglutamic acid (PGA). An increased capacity to reduce pteroylglutamic acid to the tetrahydro-level was a consistent characteristic of each of the resistant strains. The amount of aminopterin bound by the sensitive and resistant variants did not differ significantly. One highly resistant strain, which had been maintained for 2 years as a stock culture, took up much greater amounts of PGA and aminopterin than did the sensitive parent strain or the newly-selected resistant strains. This example of the concomitant occurrence of increased uptake and decreased effectiveness of a drug demonstrates the need for methods to study specific and non-specific binding in relation to drug resistance. An eighty-fold higher concentration of amethopterin relative to 4-amino-4-deoxy-^{10}N-methylpteroic acid, was required to obtain comparable degrees of inhibition of the growth of this amethopterin-resistant strain. Similarly, an eighty-fold higher concentration of amethopterin was required to inhibit the conversion of PGA to folinic acid by the intact cells; however, the soluble enzyme in cell-free filtrates was equally sensitive to inhibition by the two antagonists. Additional studies are needed to explain the mechanisms by which the access of the inhibitors to the sensitive enzyme system is limited in the drug-resistant cells.

DETAILED examination of individual characteristics associated with drug resistance is desirable whenever practical methods can be applied. Resistance of *Streptococcus facealis* to amethopterin ("methotrexate"; 4-amino-4-deoxy-^{10}*N*-methylpteroylglutamic acid) has been used as one model for such studies.

Most instances of drug resistance probably result from the cumulative influence of several factors. In amethopterin-resistant strains of *S. faecalis*, changes observed include an increased capacity to metabolize pteroylglutamic acid,[1, 2] altered uptake of the drug,[3, 4] altered nutritional requirements[5, 6] and decreased access of the drug to a sensitive enzyme system.[7] When the stepwise selection of resistant strains progresses slowly or the cultures are transferred repeatedly for prolonged periods, the opportunity for multiple changes to occur may be increased. In the study of individual characteristics that are associated with resistance, it seems preferable to use resistant

*This investigation is included as part of a thesis submitted by Aaron H. Anton for the degree of Doctor of Philosophy.

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cells which are removed from the sensitive parent population by a minimum number of generations. Thus, in this investigation, newly selected variants of *S. faecalis* of differing levels of resistance to amethopterin were studied primarily with respect to their capacity to bind the drug and to utilize the corresponding metabolite, pteroylglutamic acid (folic acid), as indicated by the formation of 5-formyl-5:6:7:8-tetrahydropteroylglutamic acid (folinic acid).

MATERIALS AND METHODS

Selection of drug resistant cultures. Variants of *S. faecalis* (ATCC strain 8043) with differing degrees of resistance to amethopterin were developed by exposing a culture derived from a single colony of the sensitive strain to exponentially increasing concentrations of amethopterin in a folic acid-assay medium[8, 9] containing PGA (1 mμg/ml). Resistant cultures were selected at each tenfold increase in the concentration of the antagonist. The variants of low resistance were selected after a few daily transfers and were then maintained on solid medium, whereas growth at the highest concentration of the drug occurred only after some forty daily transfers. Cultures were selected which varied in their exposure to concentrations of the antagonist ranging from 1 mμg to 100,000 mμg/ml of medium. To determine whether the resistance of the selected strains was a stable characteristic, the cultures were transferred daily in the absence of amethopterin. After the fifth such transfer, each strain was still able to grow at that concentration of amethopterin which was used initially for its selection. Each resistant strain was maintained in media containing the same amount of amethopterin as that used for the initial selection. A resistant culture, designated *S. faecalis/A*, which grows well in the presence of 100 μg/ml of amethopterin, had been selected 2 years previously under similar conditions, and had been maintained in media containing this concentration of the drug.

Measurement of degree of resistance. Each culture was grown for 24 hr in the specified medium. The organisms were harvested by centrifugation, washed twice with a solution of sodium chloride (0·9%) (subsequently referred to as "saline") and diluted with the same solution to give a reading of 10 units on a Klett–Summerson photoelectric colorimeter, using a red filter (no. 66). One drop of this dilute suspension of bacterial cells was then inoculated aseptically into sterile medium (10 ml) containing varying amounts of the inhibitor and a sufficient amount of pteroylglutamic acid for maximum growth (1·0 mμg/ml). After incubation for 20 hr at 37 °C, the turbidity of each culture was measured in a Klett–Summerson colorimeter.

Preparation of cells and extracts for incubation. The amethopterin-resistant strains of *S. faecalis* and the sensitive parent strain were grown for 16 hr in a medium[9] containing PGA (2 mμg/ml). The cells were harvested by centrifugation and washed once with cold saline. The dry weight of the cells in each preparation was determined. The cells were suspended in phosphate buffer (0·2 M; pH 6·4) and were added to the respective incubation vessels.

A saline suspension of the washed cells (5%) was subjected to sonication (Raytheon Model DF 101; 30 min) and then centrifuged at 5000 *g* to remove the cell debris. The protein content of the clear supernatant solution was determined by the differential spectrophotometric method of Kalckar.[10]

Incubation procedures. The incubations were performed at 37° under flowing nitrogen in 20 ml beakers which were shaken constantly. For the measurement of "folinic acid" formation, the incubation mixture contained cells (4 mg, dry weight), PGA (0·22 μmoles), glucose (40 μmoles), sodium formate (20 μmoles), sodium ascorbate (20 μmoles) and phosphate buffer (0·2 M, pH 6·4) in a total volume of 2·0 ml. Incubations with the cell-free extract (3 ml of an extract containing 3 mg protein per ml) required, in addition to the above ingredients, ATP (7·5 μmoles), DPN (9·5 μmoles) and $MgCl_2$ (25 μmoles) in a total volume of 5 ml.[7] After incubation for 1 hr, the samples were heated immediately at 120° for 20 min and were then centrifuged. The supernatant fluid was assayed with *Pediococcus cerevisiae* 8081 using the medium described by Sauberlich[11]. This procedure gives a satisfactory measure of the reduction of PGA even though several steps are involved in the formation of folinic acid. The enzymic reduction and formylation of PGA in this sytem yields a heat-labile ^{5}N : ^{10}N-methylene derivative of tetrahydro-PGA which can be converted upon heating in the presence of a reducing agent to a stable form, folinic acid or 5-formyl-tetrahydro-PGA.[12–15]

Measurement of drug uptake. Aminopterin was used in these experiments, since similar hydrolysis of amethopterin yields ^{10}N-methyl-PGA, which cannot easily be measured microbially. The analogue was purified by ion exchange chromatography, using Dowex-1[16] and contained 0·06 per cent of contaminating growth-promoting substances, as measured by a microbial assay.[2] Alkaline hydrolysis of aminopterin converts this antagonist quantitatively to pteroylglutamic acid.[17] This procedure, combined with the microbial determination of folic acid, provided a sensitive, specific determination of the amount of aminopterin present in the cells. Mixtures containing cells (25 mg, dry weight), PGA or aminopterin (0·11 μmoles), glucose (50 μmoles) and phosphate buffer (0·2 M; pH 6·4) in a total volume of 5 ml were incubated as described above. After the incubation, those samples in which the uptake of PGA or aminopterin was to be determined were washed three times with ice-cold saline, then hydrolysed in 0·5 N NaOH (120 °C for 30 min), neutralized and finally assayed for folic acid activity by using *S. faecalis* 8043.[9]

EXPERIMENTAL AND RESULTS

Relationship between amethopterin resistance and growth in the presence of thymine, folic acid or folinic acid. S. faecalis 8043 responds equally well to either folic acid or folinic acid and the requirement for either of these forms of the vitamin can be obviated by the presence of thymine or thymidine in a medium which contains purines.[18, 19] Strains of *S. faecalis* were selected which could grow at concentrations of amethopterin ranging from 1 to 100,000 mμg/ml in media containing (1) PGA, 1·0 mμg/ml, or (2) 5-formyl-5:6:7:8-tetrahydro-PGA, 1·0 mμg/ml, or (3) thymine, 6·3 μg/ml. The level of resistance of these strains was then determined in the manner described, in each case the test medium containing PGA being used. Growth in the presence of even high levels of amethopterin was not accompanied by the selection of resistant strains when the medium contained thymine. Strains selected in the presence of folinic acid were somewhat less resistant than those selected in the presence of folic acid when tested under these conditions (Fig. 1). The level of resistance of strains selected in the medium containing folic acid was related to the final concentration of the drug used in each case during the selection.

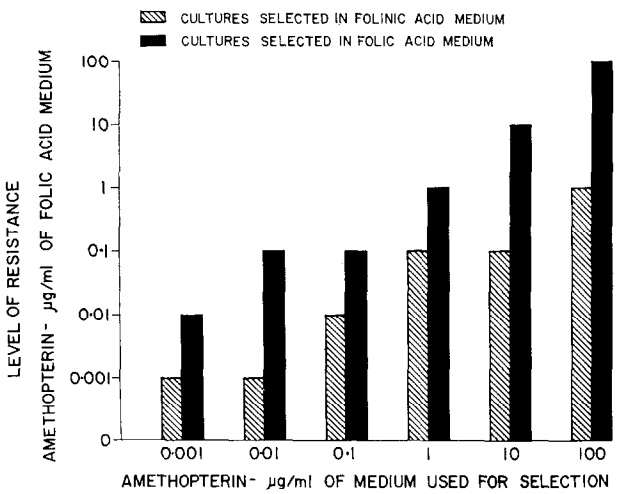

FIG. 1. Relationship of medium used for the selection of cultures to the level of resistance.

Relationship between degree of resistance and capacity to form a competing metabolite. The variants of *S. faecalis* with differing degrees of resistance to amethopterin (selected in the medium containing PGA) were compared with respect to their ability to form "folinic acid" from added PGA using intact cells and cell-free extracts of each strain (Table 1). The data indicate that the formation of folinic acid by the intact cells and the cell-free extracts was considerably greater in the resistant cells and was highest with the most resistant cultures. Also, the amount of antagonist needed to inhibit folinic acid formation by 50 per cent in the intact cells and cell-free extracts increased in a manner related to the degree of resistance (Table 1).

TABLE 1. SYNTHESIS OF FOLINIC ACID BY AMETHOPTERIN-RESISTANT STRAINS OF *Streptococcus faecalis*

Amethopterin tolerated by resistant strains during selection (μg/ml)	"Folinic acid" formed from PGA during incubation		Amethopterin conc. required for inhibition of "folinic acid" formation by 50 per cent	
	Intact cells (mμg/mg of cells, dry wt.)	Extract (mμg/mg of protein)	Intact cells (mμg/ml)	Extract (mμg/ml)
0	40	1	100	4
0·001	285	6	400	7
0·01	190	—	—	—
0·1	415	—	—	—
1	350	28	4000	10
10	880	—	—	—
100	910	120	20,000	25

Procedures were those described under Methods.

Uptake of aminopterin by different strains of S. faecalis. The uptake of the antagonist by the newly selected amethopterin-resistant strains of *S. faecalis* was not significantly different from that by the sensitive strain. However, it was found that *S. faecalis/A* consistently took up much greater amounts of aminopterin and of PGA than did the

sensitive strain (Table 2). The amounts of PGA and aminopterin taken up by the resistant organism were similar. It appears that in this instance the antagonist is bound in some manner that renders it innocuous.

TABLE 2. UPTAKE OF PTEROYLGLUTAMIC ACID AND AMINOPTERIN BY SENSITIVE AND RESISTANT STRAINS OF *Streptococcus faecalis*

Compound	Incubation time (min)	Amount recovered from washed cells	
		Sensitive strain *S. faecalis* 8043 (μg/g cells, wet wt.)	Resistant strain *S. faecalis/A* (μg/g cells, wet wt.)
Pteroylglutamic acid (μg/ml)			
—	—	0·18	0·18
10	15	4·12	23·4
10	30	5·76	36·0
10	60	8·65	52·0
Aminopterin (μg/ml)			
—	—	0·29	0·18
10	15	0·54	32·4
10	30	0·77	40·8
10	60	2·16	56·0

Procedures were those described under Methods.

Resistant cells (*S. faecalis/A*) which had been incubated with aminopterin were fragmented in the Raytheon sonic oscillator. A compound was found in the cell extracts which inhibited the growth of the sensitive strain of *S. faecalis* and had the same R_f value (0·24 with descending phosphate buffer; 0·1 M; pH 6·0) on paper chromatograms as has a sample of highly purified aminopterin. Since any capacity to inactivate aminopterin could be related to the ability of the resistant cells to grow in the presence of this drug, the recovery of aminopterin was estimated in two ways. The concentration of aminopterin was measured in duplicate preparations, before and after incubation, by measurement of its ability to inhibit the growth of the sensitive strain in liquid medium. Aminopterin was readily recovered from the cells by autoclaving at 15 lb/in^2 pressure for 10 min. Also, alkaline hydrolysis of duplicate preparations before and after incubation was followed by assay of the folic acid activity of the solutions. No evidence was obtained by these procedures that aminopterin was altered or inactivated during the incubation with these resistant cells.

Relationship between molecular structure and inhibitory potency. The compound 4-amino-4-deoxy-^{10}N-methylpteroic acid duplicates the structure of amethopterin except that it lacks the glutamic acid portion of the latter compound (Fig. 2). This pteroic acid analogue was only one-twelfth as effective as amethopterin in inhibiting by 50 per cent the growth of *S. faecalis* 8043. Since complete cross resistance between amethopterin and aminopterin was observed in the study of *S. faecalis/A*, it might be expected that similar cross resistance would occur between amethopterin and the pteroic acid analogue. It was found, however, that the growth of *S. faecalis/A* was eighty times more sensitive to inhibition by 4-amino-4-deoxy-^{10}N-methylpteroic acid than by amethopterin; inhibition of the growth of *S. faecalis/A* by 50 per cent occurred

at a concentration of the former compound of $1{\cdot}0 \times 10^{-5}$ M, whereas a similar degree of inhibition by amethopterin required a concentration of 8×10^{-4} M.

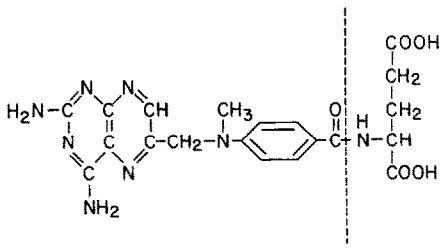

FIG. 2. Structural relationship between amethopterin and a pteroic acid analogue. 4-Amino-4-deoxy-^{10}N-methylpteroic acid duplicates the structure of amethopterin, with the exception of the glutamic acid-substituent.

The effectiveness of these two compounds in inhibiting the formation of folinic acid from added PGA by cell suspensions of the resistant organism was compared (Fig. 3). The data indicate that the pteroic acid analogue is nearly one hundred times

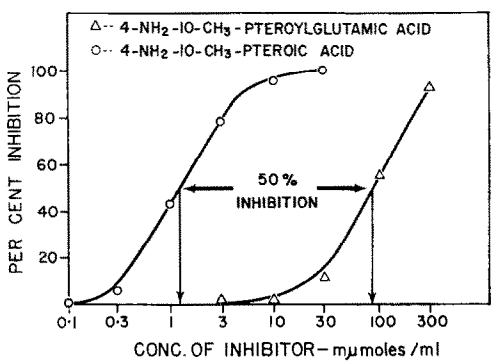

FIG. 3. Relative effectiveness of two similar analogues as inhibitors of folinic acid formation by cell suspensions of *S. faecalis/A*.

more effective than amethopterin in this respect. The parallelism of the two curves suggests that the inhibitory action of the two compounds was being exerted in a similar manner. When cell-free extracts of *S. faecalis/A* were used in a similar experiment, the formation of folinic acid was inhibited to the same extent by similar concentrations (2×10^{-7} M) of the two compounds.

DISCUSSION

A consistent characteristic of all of the newly selected resistant mutants was an increased capacity to metabolize PGA. Although there was not a close correlation between the magnitude of this change and the degree of resistance, the greatest amount of folinic acid was formed from PGA by the highly resistant cells. These findings are in agreement with earlier observations of an increased formation of folinic acid in amethopterin-resistant strains of *S. faecalis*.[1, 2] A similar alteration occurred in a sulphonamide-resistant strain of *Staphylococcus aureus* which formed more "folic acid"

than the sensitive parent strain when incubated with *p*-aminobenzoic acid.[20] Resistance to amethopterin does not appear to involve the development by *Streptococcus faecalis* of any amethopterin-insensitive pathway for the activation of PGA, as measured by the formation of reduced derivatives with folinic acid activity. Although somewhat higher concentrations of amethopterin were required to inhibit this reaction in extracts of the highly resistant cells, the insensitivity of the folinic acid-forming system in intact cells to high concentrations of amethopterin is in striking contrast to the sensitivity of this reaction in cell-free extracts to low concentrations of the drug. Two major characteristics associated with amethopterin-resistance in this organism, selected under these conditions, appear to concern the more efficient use of the metabolite, PGA, and the development of some means by which a sensitive enzyme system is protected from contact with the drug.

It is apparent that the composition of a medium can influence the level of resistance of cultures capable of growth in it. The degree of resistance of cultures selected in the presence of PGA was related to the concentration of the drug in the selecting medium. The level of resistance of cultures selected in the presence of 5-formyl-5:6:7:8-tetrahydro-PGA was considerably lower than the level of the drug in the selecting medium; however, a graded increase in resistance was observed. In the medium containing thymine, the organism grew readily at all concentrations of the inhibitor, and no selection of resistant cells occurred. The outgrowth of resistant variants would not be expected to occur unless the growth were limited by the concentration of thymine. Of particular interest in this regard are the observations that the presence of thymine in a *purine-free* medium containing folic acid reduced the lag-phase in the outgrowth of *S. faecalis* cells resistant to aminopterin[21, 22], and that thymine-requiring variants of *L. casei* were selected during the development of resistance to amethopterin[23] and pyrimethamine.[24] It would be expected, therefore, that in crude media or in a natural environment the availability either of metabolites produced by folic acid-dependent reactions or of folinic acid-like forms of the vitamin would condition both the ability of cells to grow in the presence of the drug and the emergence of a drug-resistant population.

The amount of aminopterin bound by different variants during incubation with cell suspensions was measured by a sensitive microbial determination of the PGA formed from aminopterin by alkaline hydrolysis. There was no consistent difference in the amount of aminopterin bound by the newly-selected resistant strains that could be related to the degree of resistance. Similar studies by Johnson and coworkers[4] based on the uptake of aminopterin-2-^{14}C indicated that other resistant strains of *S. faecalis* took up slightly more of the drug than did the sensitive strain. Only with the highly-resistant strain, *S. faecalis/A*, which had been selected similarly and maintained for more than 2 years in a similar medium containing amethopterin (100 μg/ml), was there a remarkable uptake of aminopterin, as compared to the sensitive "wild" strain of *S. faecalis*. This resistant organism also took up much greater amounts of PGA than did the sensitive parent. The occurrence of an increased uptake of aminopterin by cells which are not inhibited by it clearly indicates that the drug can be bound in an inactive form. This characteristic may be accentuated in *S. faecalis/A*. Similar binding of some fraction of the drug which penetrates the cell also may occur with other resistant variants. Even in the absence of any change in the total amount of the drug taken up, any decrease in the functionally effective proportion of

the antagonist taken up could be of real significance to the development of resistance.

Insensitivity to a drug, as the result of the development of a *decreased* capacity to "bind" the agent or to convert it into a metabolically active form, can readily be related to the occurrence of resistance. This characteristic has been observed repeatedly in studies on the nature of resistance of trypanosomes to certain toxic agents and of microbial resistance to certain antibiotics.[25] A number of examples demonstrate that similar changes are associated with microbial resistance to certain antimetabolites, such as resistance of tubercle bacilli to isoniazid,[26] resistance of *L. casei* to 2 : 6-diaminopurine,[2] and resistance of *S. facealis* to 6-azauracil.[28] *Increased* binding of drugs by resistant organisms is more difficult to relate satisfactorily to the development of resistance. The occurrence of this characteristic, however, is not uncommon. More penicillin was taken up by some resistant strains of *Micrococcus pyogenes* and *S. faecalis* than by sensitive strains.[29, 30] Similarly, sulphanilamide was bound in larger amounts by strains of *Enterococci* and *E. coli* that were resistant to this drug.[31] Resistance of *Mycobacterium tuberculosis* to *p*-aminosalicylic acid was associated with the uptake of much larger amounts of the ^{14}C-labelled drug.[32] Larger amounts of unchanged aminopterin were extracted from an aminopterin-resistant strain of *Enterococci* than from the parent strain after incubation with this drug.[33]

The ability of *S. faecalis/A* to take up more aminopterin than the sensitive strain is of particular interest because of a difference in the inhibitory activity of closely related structures. Although some degree of cross resistance did occur, 4-amino-4-deoxy-^{10}N-methylpteroic acid was approximately eighty times more effective than amethopterin in inhibiting folinic acid formation by cells of this amethopterin-resistant strain. These observations suggest that the glutamic acid portion of the amethopterin molecule may be involved in the binding of this antagonist in an inactive form or with specific changes in transport mechanisms that prevent the drug from reaching a sensitive enzyme system. Two other observations of different responses to related glutamyl structures may be noted: *p*-aminobenzoylglutamate cannot supply the *p*-aminobenzoate requirement of *Leuconostoc mesenteroides* P-60 or of a nutritional mutant of *E. coli*;[13] pteroyltriglutamate (pteroylglutamyl-γ-glutamyl-γ-glutamate) cannot replace for the mono-glutamate as a vitamin for *S. faecalis*, even though enzymes capable of reducing this triglutamate to the corresponding derivative of folinic acid occur within these cells.[34, 35]

The concomitant occurrence of increased uptake and decreased effectiveness of a drug emphasizes the need for the development of methods of determining that fraction of the total amount of drug taken up by a cell which is accounted for by "specific-binding", that is, binding associated with inhibition of an enzyme. Comparison of the effectiveness of amethopterin and a related pteroic acid analogue in these studies implies that resistance in *S. faecalis/A* is associated with a structurally specific binding of amethopterin which prevents its access to a sensitive enzyme. The possible occurrence of similar drug-binding deserves examination in other instances of drug resistance.

Acknowledgements—This investigation was supported, in part, by a research grant from the National Cancer Institute, U.S. Public Health Service. Grateful appreciation is expressed to Professor A. D. Welch for his interest and advice, to Dr. S. F. Zakrzewski for his frequent help and to Mr. R. J. Leonard for his expert technical assistance. The analogues of folic acid and pteroic acid used in this investigation were generously supplied by the American Cyanamid Co., through the courtesy of Dr. J. M. Smith, Jr.

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