

Relationship Between Sulfadiazine Resistance and the Failure to Ferment Maltose in *Neisseria meningitidis*

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The genetic marker for sulfadiazine resistance was transferred by means of purified deoxyribonucleic acid from sulfadiazine-resistant *Neisseria meningitidis* and *N. perflava* to a sulfadiazine-sensitive strain of *N. meningitidis*. Over 80% of the isolates from these experiments, selected on the basis of sulfadiazine resistance, failed to produce acid from maltose. The same proportion of naturally occurring isolates that are sulfadiazine-resistant failed to ferment maltose. The enzymatic block in 17 isolates tested was the loss of maltose permease activity; in two cases, maltose phosphorylase activity was lost also. The permease was present in these cells, however, and could be activated by the addition of sulfadiazine. The results obtained support the hypothesis that these organisms, in becoming resistant to sulfadiazine, have undergone a single mutation.

The recent rise in the number of cases of meningococcal meningitis and the concomitant rise of sulfadiazine-resistant meningococci (10) has focused our attention on these drug-resistant microorganisms. To understand adequately the epidemiological situation, it is imperative that drug-resistant organisms be isolated and properly identified.

The members of the genus *Neisseria* are separated primarily on the basis of the fermentation of four sugars: glucose, maltose, sucrose, and fructose. *N. meningitidis* ferments only glucose and maltose; *N. gonorrhoeae* ferments glucose only. Strains of *N. meningitidis* which ferment lactose have been recovered (11); however, no reports have appeared describing the loss of the ability to utilize a sugar, or changes in sugar fermentations associated with acquisition of drug resistance.

The enzymology of maltose utilization by *N. meningitidis* has been carefully studied (4-6). After maltose has entered the cell, presumably by enzymatic action, it is split by the enzyme maltose phosphorylase. The resulting products, glucose-1-phosphate and free glucose, are further degraded in the same manner as added glucose. This report deals with the isolation and enzymatic characterization of maltose-negative strains of *N. meningitidis*, occurring naturally or obtained in the laboratory and which are also sulfadiazine-resistant.

MATERIALS AND METHODS

N. meningitidis strains were recent isolates of both case and carrier origin. The identity of the strains and the procedures for identification and maintenance in the laboratory have been described earlier (7). The *N. perflava* strains, isolated from healthy carriers, were identified and treated in the same manner.

The enzymes used in this study and where they were obtained are as follows: ribonuclease and deoxyribonuclease, Worthington Biochemical Corp., Freehold, N.J.; and Pronase, Calbiochem, Rockville, Md. The ribonuclease was heated to 80 C for 1 hr to remove any deoxyribonuclease activity. The maltose-¹⁴C was purchased from Calbiochem.

The microorganisms were grown in Trypticase Soy (TS) Broth (BBL), Franz modified broth (12), or on Mueller Hinton agar (Difco). Carbohydrate fermentation reactions were done in Cystine Trypticase Agar (BBL) containing 0.75% of the carbohydrate to be tested. Broth cultures were placed in a reciprocal shaker at 37 C, and the agar slants and plates were incubated in a moist incubator (37 C) with an atmosphere of 10% CO₂ in air. The fermentation tubes were incubated in air at 37 C. Sulfadiazine resistance was determined by plating the microorganisms on Mueller Hinton agar containing the concentration of sulfadiazine to be tested.

Deoxyribonucleic acid (DNA) was extracted from cells grown in broth by a modification of the procedure of Marmur (9). The bacteria were lysed with 0.5% sodium deoxycholate and Pronase. The lysate was extracted with phenol, and, after removal of the aqueous phase, 2 volumes of cold ethyl alcohol were

added and the DNA "spooled" onto a glass rod. After two extractions, the DNA was treated with ribonuclease (50 $\mu\text{g}/\text{ml}$ at 37 C for 2 hr) to remove contaminating RNA. This step was followed by two additional phenol extractions. Before use, the DNA was dialyzed overnight against a solution of 0.15 M NaCl and 0.015 M sodium citrate at pH 7.3 and was checked for sterility. Only bacteria-free preparations were used.

The transformation procedures of Catlin (2) were followed. Growth on 18-hr slants was suspended with 10 ml of TS broth, and 0.5 ml of suspension was placed in 9.5 ml of TS containing 0.01 M CaCl_2 (8). This mixture was incubated for 1 hr, and the DNA was added to a final concentration of 10 $\mu\text{g}/\text{ml}$. The cells were exposed to the DNA for 30 min; then deoxyribonuclease was added to stop the transformation process. The total number and the number of bacteria resistant to sulfadiazine were determined at this time, and transformation frequencies computed after subtraction of the number of resistant bacteria found on identical control plates. The controls in this study were both cells exposed to deoxyribonuclease-treated DNA and cells not exposed to DNA.

Maltose phosphorylase activity was determined by measuring the production of free glucose from maltose by cell-free extracts (4). Broth cultures were washed and resuspended in phosphate-buffered saline, and an equal volume of size 130 glass beads (Minnesota Mining and Manufacturing Co., Minneapolis) was added. The mixture was then placed in a Sorvall Omnimixer and stirred for 5 min at 4 C. The beads were removed by filtration, and the resulting extract was clarified by centrifugation at $20,000 \times g$ for 10 min. The extracts were incubated at 37 C for 1 hr in a reaction mixture consisting of 0.5 ml of crude extract and 0.5 ml of a solution containing 0.10 M maltose and 0.1 M sodium phosphate buffer (pH 7.2). Glucose concentrations were determined with the Calbiochem Calsul for glucose.

Permeability of the cell to maltose was determined by exposing bacteria suspended in dilute (1:5) TS broth, to which was added 0.02 M maltose- $I^{14}\text{C}$ (1 $\mu\text{c}/\text{mmole}$). At various times, samples were removed and the cells were washed by filtration onto membrane filters. The radioactivity on the filters was determined in a liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.).

The metabolic experiments were carried out with 0.02 M maltose- $I^{14}\text{C}$ (1 $\mu\text{c}/\text{mmole}$) in dilute TS broth by the procedure of Weiss (14). The reactions, in a final volume of 1.5 ml, were carried out in a 25-ml Erlenmeyer flask. The CO_2 was trapped by the use of Hyamine (Packard Instrument Co., LaGrange, Ill.). The disintegrations per minute were computed by dividing the counts per minute by the efficiency of counting.

The intracellular level of sulfadiazine was determined by the procedure of Bratton and Marshall (1). Cell suspensions in TS broth containing 50 μg of sulfadiazine per ml were incubated for 2 hr at 37 C and centrifuged. The cells were washed three times and resuspended in cold distilled water. Cell-free

extracts were prepared as previously described, and the sulfadiazine content was determined.

RESULTS

In the course of characterization of fresh isolates of *N. meningitidis* from cases of meningococcal disease or from asymptomatic carriers, several strains were found to be maltose-negative. All these strains were resistant to greater than 10 μg of sulfadiazine per ml. Similar results were obtained by Rust and Hunter (Annual Report Walter Reed Army Institute of Research, 1965), who found among 1,637 isolates, a 95% correlation between sulfadiazine resistance and the inability to ferment maltose.

The first step in the study of the relationship of maltose utilization and resistance to sulfadiazine was to determine whether the two properties were transferred together in genetic transformation. We had noted that naturally occurring sulfadiazine-resistant strains of *N. perflava*, in contrast to *N. meningitidis*, did not lose the ability to ferment maltose.

Since interspecific transformation has been demonstrated in the genus *Neisseria* (3), two types of transformations were possible. In the first experiments, the transforming DNA was obtained from strain 4-4 of *N. meningitidis* which is sulfadiazine-resistant and maltose phosphorylase-positive and maltose "permease"-negative. The second type of DNA was derived from a sulfadiazine-resistant and maltose-positive strain of *N. perflava*. The recipient strain was strain 174 of *N. meningitidis*, sensitive to 0.5 μg of sulfadiazine per ml, and maltose-positive.

The results of these experiments are shown in Table 1. The transformants were selected on the basis of resistance to sulfadiazine (50 $\mu\text{g}/\text{ml}$), not the ability to utilize maltose. The percentages of sulfadiazine-resistant strains that were also maltose-negative, resulting from the homologous and heterologous crosses, were almost identical. It is clear that, even though the *N. perflava* DNA did not carry the maltose-negative marker, almost all the resulting transformants were maltose-negative. Spontaneous mutants of the recipient strain (174) showed a similar frequency of maltose-negative cells.

A number of naturally occurring maltose-negative strains as well as a number of transformants were selected for enzymatic characterization (Table 2). All of the maltose-negative bacteria studied were unable to bind maltose ("permease negative"). In addition to the permeability barrier, two strains showed no maltose phosphorylase activity. These two phosphorylase-negative strains were selected, because they failed to ferment maltose when sulfadiazine was added.

TABLE 1. Transformation of *Neisseria meningitidis* strain 174 to sulfadiazine resistance

Source of donor DNA	Transformation frequency ^a	No. tested	Maltose negative	
			No.	Per cent
<i>N. perflava</i> strain CL65-66	0.1×10^{-5} – 2.5×10^{-5}	160	150	93.8
<i>N. meningitidis</i> , strain L1	2×10^{-5} – 100×10^{-5}	199	185	93.0

^a Transformants per milliliter divided by the number of exposed cells per milliliter.

TABLE 2. Enzymatic characteristics of sulfadiazine-resistant meningococci

No. and type of strains	Maltose enzymes		Reaction in the presence of	
	Permease	Phosphorylase	Maltose	Maltose + sulfadiazine (25 µg/ml)
Transformants				
10	–	+	Basic	Acid
2	–	–	Basic	Basic
Isolates				
5	–	+	Basic	Acid
Control	+	+	Acid	No growth

TABLE 3. Uptake of sulfadiazine by *Neisseria meningitidis*

Strain	Type	Amt (µg) of sulfadiazine bound/ml of crude lysate
SD6	Sensitive	4.5
174	Sensitive	5.0
4-4	Resistant	6.0
L1	Resistant	5.0
TS-10	Resistant	5.4

The frequency of phosphorylase-negative cells is probably very low.

Since permeability to maltose was clearly a factor in the inability of these strains to use it, the question of the permeability to sulfadiazine was also investigated, although there have not been any reports of sulfadiazine resistance being due to impermeability of the drug. Sulfadiazine uptake experiments were performed with the resistant strains used in this study, and the intracellular levels of drug were compared to those obtained in sensitive controls. In all cases, the resistant organisms took up the same amounts as the controls (Table 3).

In the course of the sulfadiazine uptake experiments, the enzymatically characterized strains of maltose-negative *N. meningitidis* were placed in maltose fermentation tubes with added sulfadiazine. The results obtained are summarized in Table 2. Clearly, when sulfadiazine was added, these organisms were able to ferment maltose.

The data of Fitting and Scherp (5) suggest that the maltose enzymes are constitutive in *N. meningitidis*, although the authors indicated that their data did not conclusively prove this point. To elucidate more closely the mechanism of the correlation between sulfadiazine resistance and activity of the maltose enzymes, we needed to know: first, whether the maltose enzymes were constitutive or inducible; second, whether the expression or synthesis of these enzymes had been repressed in

sulfadiazine-resistant strains; and, third, which of the effects is derepressed by the addition of sulfadiazine. A series of experiments was designed to answer these questions.

Figure 1 shows representative results of experiments designed to determine whether the maltose system is inducible in the "wild-type" maltose-

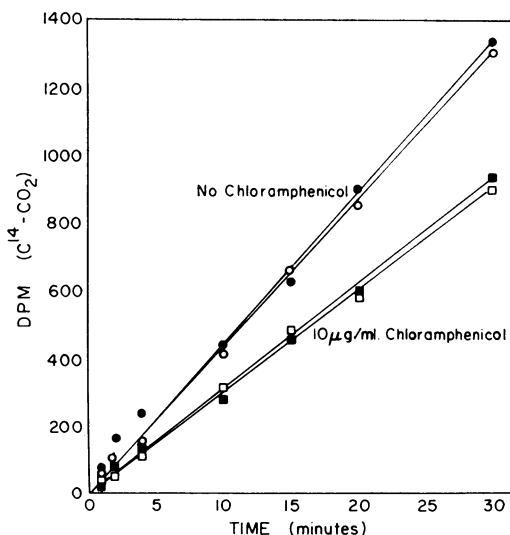


FIG. 1. Production of CO_2 from maltose by *Neisseria meningitidis*. The solid and open symbols represent cells obtained by growth in the presence and absence of 1% maltose, respectively. The cells were washed and resuspended in dilute (1:5) TS broth supplemented with 0.02 M maltose-1- ^{14}C (specific activity 1 µc/mmole).

positive cell. It seems clear that, as predicted by Fitting and Scherp (5), the maltose enzymes are constitutive.

Figure 2 shows the results of similar experiments with maltose-negative cells, with sulfadiazine added at a specified time. Permease activity began immediately upon the addition of the sulfadiazine, as indicated by the maltose being available for metabolic activity. Figures 1 and 2 also show the same experiments done in the presence of a level of chloramphenicol adequate to prohibit protein synthesis in these organisms. No protein synthesis was required for permease activity in either case.

DISCUSSION

The emergence of sulfadiazine-resistant meningococci has been very dramatic in the past few years. In many areas, especially in military recruit training centers, the proportion of the total isolates that are sulfadiazine-resistant exceeds 50% (J. H. Rust, *personal communication*). The existence of naturally occurring maltose-negative strains of meningococci is of importance to epidemiologists, because they may be overlooked. The correlation between sulfonamide resistance and the inability to ferment maltose makes identification especially important. It is possible that, in some cases, reported rates of sulfadiazine resistance are low only because many strains are not identified as meningococci because they do not ferment maltose.

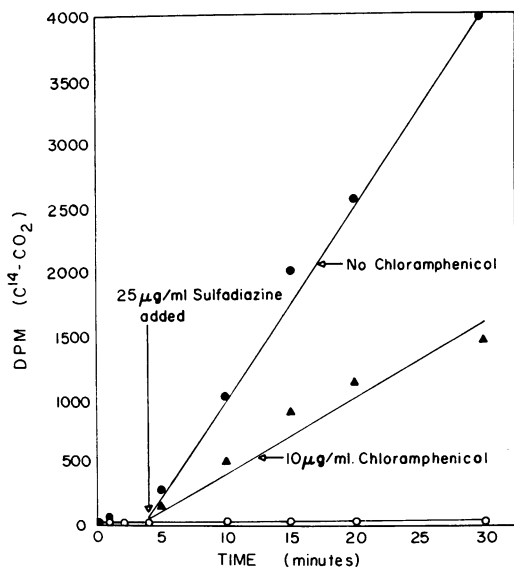


FIG. 2. Production of CO_2 from maltose by sulfadiazine-resistant *Neisseria meningitidis*. The cells were grown in the absence of maltose.

The genetic evidence obtained in this study on the relationship between maltose fermentation and sulfadiazine resistance indicates that only one mutation or the transfer of one gene was involved. This evidence is provided by: (i) the very high frequency of both markers being transferred together, and (ii) the existence of the maltose enzymes in the sulfadiazine-resistant cells, although one was inactive.

The mechanism of inactivation of the maltose permease is not clear from these studies. The means of expression of the sulfadiazine resistance may be the important factor. The exact mechanism of sulfadiazine action in the meningococcus is not known, but it is reasonable to assume that it is similar to that in other organisms. The best explanation for sulfadiazine resistance is that a single enzyme is changed and that this change results in a difference in the ability to bind the sulfadiazine molecule.

The evidence presented here clearly demonstrates that in these mutants and transformants we are dealing with a sulfadiazine-dependent enzymatic step. Two possible mechanisms are immediately apparent: the permease may need to bind sulfadiazine to make the active site functional, or the sulfadiazine may be removing an inhibitor from the enzyme, thus freeing it to act.

The first alternative would be favored by the results of Spotts (12), who studied streptomycin dependence in *Escherichia coli*. These studies clearly indicate that there are a number of active sites in the macromolecular fraction of the cells that need to be saturated for balanced growth. In the present study, however, this alternative would be difficult to reconcile with a single genetic change. The second possibility would assume that a factor within the cell is removed from the enzyme by the addition of sulfadiazine, for which this factor has an affinity. This factor would also be responsible for the resistance to the action of the drug. To date, we have not been able to obtain evidence to support or to refute either of these postulates.

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