Genetic Basis of the Association of Sulphonamide Resistance with Methionine Auxotrophy in *Neisseria gonorrhoeae*

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Selection by sulphonamides was investigated in *Neisseria gonorrhoeae* because a sulphonamide-resistant (Sul^r), methionine-requiring (Met⁻) phenotype that was common in the era of sulphonamide therapy became rare in the penicillin era. Cultures of wild-type (Sul^s Met⁺) gonococci on a conventional medium containing sulphadiazine (2–10 µg ml⁻¹) yielded numerous, nonidentical mutations of two *met* genes. The requirement of MetI⁻ mutants was satisfied only by methionine, whereas MetII⁻ mutants utilized either homocysteine or methionine. My theory that increased resistance to sulphonamides is a pleiotropic effect of methionine auxotrophy was confirmed by the return of sulphonamide susceptibility in all Met⁺ spontaneous mutants. Furthermore, the Sul^r Met⁻ traits were introduced or eliminated together by DNA-mediated transformation. Sulphonamides are known to inhibit dihydropteroate synthase; consequently, they interrupt the entire sequence of reactions in the folate pathway including the methyl group transfer from N⁵-methyltetrahydrofolate to homocysteine to form methionine. The increased sulphonamide resistance of these Met⁻ mutants is discussed in terms of conservation of the pool of essential tetrahydrofolate derivatives. The ease with which spontaneous forward and reverse *met* mutations can be obtained is unique among gonococcal genes.

INTRODUCTION

The treatment of gonorrhoea with sulphanilamide commenced in 1937 (Dees & Colston, 1937). Thereafter, sulphadiazine and other improved sulphonamides were introduced and widely employed, but their effectiveness was curtailed increasingly by the development of resistance (Catlin, 1976). The advent of penicillin treatment was followed by a reduction of sulphonamide-resistant (Sul^r) *Neisseria gonorrhoeae* from a peak of 85% in 1949–1950 to 5% of strains examined ten years later (Reyn, 1961). Recent tests of the nutritional requirements of gonococcal strains isolated over a period of 40 years revealed that methionine auxotrophy was very common in the sulphonamide era, but had largely disappeared by 1970 (Catlin & Reyn, 1982; Knapp *et al.*, 1985).

The antibacterial effect of sulphonamides is due to inhibition of dihydropteroate synthase by binding to this target enzyme in competition with *p*-aminobenzoate, as foreseen in 1940 by Woods (1962). Dihydropteroate synthase performs an essential role in the biosynthesis of folates by condensing *p*-aminobenzoate with 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine pyrophosphate to yield dihydropteroate (Gale *et al.*, 1981) (Fig. 1). Furthermore, dihydropteroate synthase can accept sulphonamide as an alternative substrate to form a stable sulphonamide-containing dihydropteroate analogue (Brown, 1962). This product diffuses from *Escherichia coli* cells, thereby serving to deplete the pteridine pool (Roland *et al.*, 1979). By reducing dihydropteroate availability, the sulphonamide affects the entire sequence of enzyme-catalysed reactions in which the product of each reaction serves as the substrate for the next (Harvey, 1982). The final products of the folate pathway are essential for growth.

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Fig. 1. Relation between the folate and methionine biosynthetic pathways in *Escherichia coli*. The solid arrows represent enzyme-catalysed reactions; broken lines indicate the sites of direct action of inhibitors.

Some mutations of *N. gonorrhoeae* (Ho *et al.*, 1974), *E. coli* (Pato & Brown, 1963) and *Streptococcus pneumoniae* (Lopez *et al.*, 1987) result in structural alterations of dihydropteroate synthase. A reduction in the relative affinity of the *N. gonorrhoeae* dihydropteroate synthase for sulphonamide compared to *p*-aminobenzoate causes increased resistance. An additional mechanism of sulphonamide resistance in *E. coli* was postulated to involve methionine auxotrophy (Kohn & Harris, 1942). Evidence for such a mechanism in *Neisseria* came from interspecific transformation experiments with DNAs from Sul^r *N. gonorrhoeae* strains that had been isolated from patients in the 1940s (Catlin, 1967). *N. meningitidis* cells that were Sul^s and did not require methionine (Met⁺) were transformed to Sul^rMet⁻ in a single step. This phenotype could be reversed to Sul^sMet⁺ by parental DNA. Thus, the two traits were not separated by recombination. Confirmation that the Sul^r DNA donor strains were Met⁻ was obtained when a defined medium for growth of *N. gonorrhoeae* later became available (Catlin, 1973).

The relation between sulphonamide action and methionine biosynthesis is indicated in Fig. 1 and will be discussed hereafter. This communication examines the selection exerted by sulphonamides on *N. gonorrhoeae*. I describe the isolation and characteristics of certain *met* mutations, their profiles of recombination, and the return of susceptibility to sulphonamides that accompanies reversion to Met⁺.

METHODS

N. gonorrhoeae strains. Strain G10, isolated in 1937 from an eye-infection, is Met⁺Arg⁺Pro⁻ (Catlin & Reyn, 1982). Strain 89 was designated SS41835 when isolated in 1943 from a Danish patient; it is Met⁺Arg⁺Pro⁺ and was used in genetic experiments by Koelbl & Catlin (1986). Strains G10 and 89 are highly susceptible to sulphonamides (resistant to 0.5 µg sulphadiazine ml⁻¹) and are streptomycin-susceptible. Strain 251 was isolated in 1972 from a cervical specimen from a patient in Milwaukee, Wisconsin; it is Met⁺Arg⁻Pro⁺Sul^s (resistant to 1.0 µg sulphadiazine ml⁻¹) and Str^r (resistant to streptomycin at a concentration ≥ 200 µg ml⁻¹). Strain CDC, designated 78/002802 by the Centers for Disease Control, is Met⁺Arg⁺Pro⁻, resistant to 75 µg sulphadiazine ml⁻¹, and Str^r. The identity of the bacteria as *N. gonorrhoeae* was confirmed by conventional methods (Catlin, 1978). Additional differences between strains 89 and 251 include the serological type of outer-membrane protein I (Catlin & Shinnors, 1985) and a five- to tenfold higher resistance of strain 251 than strain 89 to benzylpenicillin, oxacillin, erythromycin, tetracycline and chloramphenicol.

Media. GCMBS agar is GC medium base (Difco) with 1% (v/v) White-Kellogg supplement added aseptically to the sterile molten medium (Catlin, 1978). GCMBS broth has the same composition as GCMBS agar, except for the omission of agar. 'Soft' agar is a mixture of equal volumes of GCMBS agar and GCMBS broth. NEDA (*Neisseria*

defined agar) and the auxotyping media lacking various compounds possibly required for growth have been described (Catlin, 1973, 1978). NEDA-minus-Met is NEDA medium lacking added methionine. DEFT is a defined fluid for transformation that lacks methionine and various other compounds (Catlin, 1974; Koelbl & Catlin, 1986).

Incubation and preservation of cultures. Inoculated media for genetic tests, auxotyping and other phenotype determinations were incubated at 36 °C in an atmosphere of humidified air plus 8% (v/v) CO₂. The strains were preserved at -60 °C and revived, as described previously (Catlin, 1978).

Colony purification and multitip inoculation. The independent origin of mutants and transformants was assured by picking isolated colonies. Colonies were chosen that displayed the morphology indicative of piliation needed for competence in transformation (Catlin, 1974). Sul^r gonococci were picked after incubation of sulphonamide-containing agar for 1 d or 2 d. They were transferred to a medium containing the sulphonamide at a concentration above that needed to inhibit the background parent cells, but below the concentration used for primary selection in order to avoid selecting second-step resistant mutants. Colonies from NEDA media were subcultured on the corresponding medium. GCMBS agar was used for a second subculture of isolated colonies from all media. In preparation for inoculating media with the Steers multitip replicating device (Catlin, 1978), growth from 16 h cultures was suspended homogeneously in DEFT to a density of $1-2 \times 10^7$ c.f.u. ml⁻¹. A tenfold further dilution was made before filling the seed plate cups.

Sulphonamide resistance. The test compounds were sodium sulphadiazine (a gift from Lederle Laboratories, Pearl River, NY, USA, and also purchased from Sigma); sulphamethoxazole (a gift from Hoffmann-La Roche, Nutley, NJ, USA); and sodium sulphathiazole (from Sigma). Spontaneous mutation to resistance was determined quantitatively by inoculating a suspension of gonococci into liquefied soft agar containing the sulphonamide and immediately pipetting 4.0 ml volumes on top of solidified GCMBS medium containing the corresponding sulphonamide concentration. The suspension was assayed for total c.f.u.. ml⁻¹ in drug-free soft agar overlays. The apparent frequency of mutation was expressed as the ratio [no. of Sul^r colonies (after incubation for 3 d)]/[$10^{-6} \times$ no. of Sul^s c.f.u. inoculated]. It should be noted that the cellular divisions that occur before sulphonamide exerts its antibacterial action affect both the calculated mutation frequency and the assay of sulphonamide resistance.

To determine the level of gonococcal resistance, GCMBS agar containing a wide range of sulphonamide concentrations was inoculated with the aid of the multitip device. Each test site received $5-10 \times 10^3$ c.f.u. of the inoculum (prepared as described above). Resistance is given in terms of the highest concentration of the agent that permitted growth of part of the population (10 or more colonies) after incubation for 48 h. A haze of growth at the inoculation site was disregarded.

Methionine pathway. L-Homoserine, O-succinyl-L-homoserine and L-cystathionine were used at final concentrations of 0.5 mM and 0.05 mM. L-Homocysteine thiolactone hydrochloride was used at 0.8 or 0.5 mM. The final concentrations of crystalline vitamin B12 ranged between 0.01 mM and 0.00005 mM. Solutions of these compounds were made up in sterile glass-distilled water on the day they were added to NEDA-minus-Met medium, which was freshly prepared. The complete NEDA medium contains 0.1 mM-L-methionine. *N. gonorrhoeae* has an absolute requirement for cysteine (Catlin, 1973) that is of interest since in *E. coli* the pathways for the biosynthesis of cysteine and O-succinylhomoserine converge in the formation of cystathionine (Smith, 1971). Multitip inoculation was used routinely, but all reported results were confirmed by streaking cellular suspensions on quadrants of methionine pathway media. Thus, the growth of isolated colonies could be inspected during incubation for 5 d. The phenotypic designations MetI⁻ and MetII⁻ are given to the mutants pending characterization of the gene loci and their products. The test compounds were purchased from Calbiochem and Sigma.

Other traits. The phenotype of mutants was examined in order to assess the specificity of the forward and reverse *met* mutations. Auxotyping, which tests the capacity for growth on NEDA media lacking various compounds, has been described in detail (Catlin, 1978). Mutations of loci that lead to requirements for arginine (*argA*, *argE*, *argG*), or uracil (*pyrB*, *pyrE*), or simultaneous requirements for arginine and uracil (*carA*, *carB*) have been documented (Shinners & Catlin, 1982). Other mutations in *N. gonorrhoeae* can impose requirements for hypoxanthine, proline, histidine, glutamine, lysine, threonine or thiamin pyrophosphate (Catlin, 1973, 1976; Catlin & Reyn, 1982). In addition to tests for the biosynthetic functions encoded by these 14 genes, the responses to six antibacterial agents were examined routinely. These were gain (by strain 89 derivatives) or loss (by strain 251 derivatives) of resistance to streptomycin ($\geq 200 \mu\text{g ml}^{-1}$); increased resistance to rifampin ($\geq 5 \mu\text{g ml}^{-1}$), spectinomycin ($\geq 25 \mu\text{g ml}^{-1}$) and nalidixic acid ($\geq 5 \mu\text{g ml}^{-1}$); and loss of typical resistance to vancomycin ($3 \mu\text{g ml}^{-1}$) and colistin ($5 \mu\text{g ml}^{-1}$) (Koelbl & Catlin, 1986).

Transformation. The methods for preparing DNA and performing transformation tests in DEFT fluid were as described by Koelbl & Catlin (1986). Transformation results, such as those illustrated in Fig. 2, were obtained by use of a slightly modified semiquantitative plate method described earlier (Catlin, 1974). One or more sections of the inoculated NEDA-minus-Met medium were left without added DNA to detect the possible occurrence of

Fig. 2. Met⁺ transformation obtained in crosses between MetI⁻ mutants. All MetI⁻ mutants were derived from *N. gonorrhoeae* strain 251, except 89-108, which was from strain 89. An inoculum of 3.0×10^8 c.f.u. of mutant 2 (in *a*) or 1.5×10^8 c.f.u. of mutant 35 (in *b*) was uniformly spread to dryness on the surface of NEDA-minus-Met medium; solutions of donor DNA (0.05 ml containing 5 to 10 µg) were added and spread out. DNA donor strains for both (*a*) and (*b*) were: upper left, mutant 34; upper right, mutant 35; middle left, mutant 1; centre, mutant 2; middle right, mutant 56; lower left, mutant 89-108; lower right, Met⁺ control DNA⁺ from parent strain 251.

Met⁺ mutations. DNA from the Met⁺ parent strain provided a positive control of recipient competence. Expression of the transformant phenotype occurs during the limited number of cellular divisions produced by Met⁻ gonococci in the absence of methionine.

RESULTS

Spontaneous mutations to sulphonamide resistance

A limited number of bacterial divisions can occur in the presence of a concentration of sulphonamide that ultimately will be inhibitory (Gale *et al.*, 1981; Roland *et al.*, 1979; Woods, 1962). During this period of residual growth, which is due to the activities of preformed tetrahydrofolates, bacteria undergo mutations. The apparent frequency of mutation to increased resistance was determined for three *N. gonorrhoeae* strains (Table 1). Higher frequencies were found for strain 251 than for strains 89 or G10 on medium containing sulphadiazine at concentrations of 5 or 10 µg ml⁻¹, in part because the slightly higher innate resistance of strain 251 permitted a longer period of residual growth. The mutation frequencies were inversely related to the concentration of sulphonamide.

Sul^r mutants obtained in these tests were purified by repeated subculture of isolated colonies and examined for resistance and other characteristics. The levels of resistance differed for mutants selected on a given concentration of either sulphonamide. For example, resistance to sulphadiazine of strain 89 mutants ranged between 10 µg ml⁻¹ (nine mutants) and 35 µg ml⁻¹ (two mutants) for 31 colonies selected on 2 µg ml⁻¹, and resistance to 35 µg ml⁻¹ was found in 3 of 22 mutants and 6 of 42 mutants selected on 5 and 10 µg ml⁻¹, respectively.

The large number of methionine-requiring mutants was the striking feature of these results (Table 1). Practically all of the strain 89 Sul^r mutants were Met⁻, as were large fractions of the strain 251 and G10 mutants. Methionine auxotrophs were also recovered in corresponding tests of two other Sul^r *N. gonorrhoeae* strains. In contrast, methionine auxotrophs were not found in exploratory tests of the well-known *N. gonorrhoeae* strain F62 inoculated on GCMBS medium

Table 1. Apparent frequency of spontaneous mutation to sulphonamide resistance, and methionine requirement of mutants

Gonococcal strain	Agent* ($\mu\text{g ml}^{-1}$)	Apparent frequency†	Sul ^r colonies	
			No. examined	No. requiring methionine‡
251	SDZ, 2	ND	38	17
	SDZ, 5	12.23	42	8
	SDZ, 10	1.28	16	4
	SMX, 5	ND	15	11
	SMX, 10	ND	13	7
	SMX, 20	1.21	5	5
89	SDZ, 2	ND	31	31
	SDZ, 5	0.69	22	22
	SDZ, 10	0.17	42	42
	SMX, 2	3.98	18	17
	SMX, 5	1.46	18	18
G10	SDZ, 5	1.26	17	10
	SDZ, 10	0.44	6	4

* Sulphadiazine (SDZ) or sulphamethoxazole (SMX) was incorporated at the indicated concentration in conventional GCMBS (bottom layer) agar and in molten GCMBS soft agar held at 46 °C; the latter was inoculated with the Sul^rMet⁺ population and distributed in 4.0 ml volumes on top of the solidified bottom layer in five or more Petri dishes.

† Apparent frequency expressed as the ratio [no. of Sul^r colonies (after incubation for 3 d)]/[10⁻⁶ × no. of Sul^s c.f.u. inoculated]. ND, Not determined.

‡ Determined on NEDA-minus-Met medium inoculated with purified Sul^r mutants (described in Methods under Colony purification).

containing sulphadiazine at various concentrations. The Met⁻ mutants, if any, presumably were not resistant to a concentration of sulphadiazine high enough to suppress multiplication of the parent strain, which is resistant to 50 $\mu\text{g ml}^{-1}$.

Characteristics of methionine auxotrophs

A detailed study was made of 25 Met⁻ mutants found among the first 80 Sul^r colonies picked from cultures of strain 251 after incubation for 1 d or 2 d on GCMBS medium containing sulphadiazine at 2 and 5 $\mu\text{g ml}^{-1}$ (Table 1). Increased resistance to sulphonamides and methionine auxotrophy were the only phenotypic traits that differentiated the mutants of strain 251 from the Met⁺Arg⁻Str^r parent strain. Since these tests could detect possible mutations affecting 20 genes (cited in Methods), the results refuted the idea that sulphonamides were exerting a mutagenic action.

Nutritional requirements. Intermediate compounds in the classical methionine biosynthetic pathway (Fig. 1) were added separately to NEDA-minus-Met medium. Methionine was the sole utilizable compound for 11 of the strain 251 mutants and for mutant 89-108. These mutants, designated MetI⁻, are listed in Table 2. The other 14 mutants (MetII⁻) could use homocysteine for growth in place of methionine, but were unable to substitute vitamin B12, L-cystathionine, O-succinyl-L-homoserine or L-homoserine. MetII⁻ mutants required homocysteine concentrations of 0.5 to 0.8 mM and incubation for up to 4 d for colonial diameters to equal the diameters of 2 d colonies on NEDA medium, which contains 0.1 mM-methionine. Also, the growth of MetII⁻ mutants was slower than that of MetI⁻ mutants on NEDA, and cellular lysis set in sooner.

Spontaneous reversion to met⁺. In the reversion tests, suspensions of Met⁻ mutants containing $<4 \times 10^8$ c.f.u. were inoculated on numerous plates of NEDA-minus-Met medium to avoid overcrowding of the inoculum which could possibly inhibit colony-formation by revertants. All 14 metII mutations were subject to reversion. The apparent frequencies ranged between 0.08×10^{-8} and 4.32×10^{-8} for 12 MetII⁻ mutants (Table 2). Mutants 22 and 31 displayed very

Table 2. *Spontaneous reversion to Met⁺ by N. gonorrhoeae MetI⁻ and MetII⁻ mutants*

NEDA-minus-Met medium in multiple Petri dishes was inoculated by spreading the surface uniformly with 0.1 ml of each Met⁻ cellular suspension made up in DEFT fluid; cultures were incubated at 36 °C for 3 d in air plus 8% CO₂. All mutants were derived from strain 251, except 89-108, which was from strain 89.

MetI ⁻ mutant designated:	10 ⁻⁸ × No. of Met ⁻ c.f.u. (A)	No. of Met ⁺ revertants (B)	Ratio (B)/(A)	MetII ⁻ mutant designated:	10 ⁻⁸ × No. of Met ⁻ c.f.u. (A)	No. of Met ⁺ revertants (B)	Ratio (B)/(A)
89-108	11.0	2	0.18	3	8.2	4	0.49
33	10.3	184	17.86	4	1.2	2	1.67
34	3.8	12	3.16	5	4.9	2	0.41
35	11.0	49	4.45	6	12.5	1	0.08
79	13.1	82	6.26	8	4.8	1	0.21
				21	20.4	2	0.10
1	11.5	0	0	22	1.0	349	349.0
2	72.5	0	0	23	7.7	3	0.39
43	12.6	0	0	24	20.9	3	0.14
56	30.9	0	0	25	2.1	5	2.38
61	23.5	0	0	29	23.4	2	0.09
68	18.0	0	0	31	11.9	151	12.69
69	49.5	0	0	32	6.9	6	0.87
				38	4.4	19	4.32

high frequencies and excessive residual growth, suggesting that methionine synthesis persisted at low rates. Reversion to Met⁺ occurred in four MetI⁻ mutants of strain 251 and in strain 89-108. Revertants were not detected, however, in cultures of seven mutant strains listed in Table 2, or in MetI⁻ mutants 90, 151, and 153 (Table 3).

Met⁻ × Met⁻ transformation. Recombination between the sites of *met* mutation was investigated as a means of characterizing the mutations and possibly differentiating various mutants. The 26 Met⁻ mutants listed in Table 2 were examined in reciprocal transformation tests. As expected of mutations affecting two genes, each of the MetI⁻ recipients was transformed to Met⁺ by DNA preparations from all of the 14 MetII⁻ strains, and vice versa. Intragenic crosses, however, revealed genetic diversity. Tests of strain 89-108 and 28 MetI⁻ mutants of strain 251 identified 14 distinct profiles of transformation (Table 3). Mutants 33 and 79 were indistinguishable in all tests, suggesting that their sites of mutation might be either identical or proximate. Additionally, 47 MetI⁻ spontaneous mutants isolated from strain 89 were examined in crosses with the 11 DNAs from strain 251 MetI⁻ mutants. Twelve transformation profiles (not shown) were differentiated, five of which resembled profiles illustrated in Table 3. Thus, 21 nonidentical *metII* mutations were identified in these 76 Sul^r isolates.

Crosses between the MetII⁻ strains 5, 23, 24, 25, 29 and 32 and each of the six corresponding DNAs did not yield Met⁺ transformants. Transformation was obtained, however, in crosses between these six recipients and each of the other DNAs. Mutants 3, 4, 6, 8, 21, 22, 31 and 38 were transformed by all heterologous DNAs (results not shown). The findings with these 14 revertible strains suggest that they carry single-site mutations of at least nine *metII* sites.

Selection of Sul^r Met⁻ mutants. It was of interest to compare the mutants isolated by selection with sulphonamides that have different structures. The selection medium for eight of the strain 251 MetI⁻ mutants listed in Table 3 contained sulphadiazine at 5 µg ml⁻¹ and mutants 151 and 153 came from sulphamethoxazole at 10 µg ml⁻¹. Mutant 153 and two strain 89 mutants obtained with sulphadiazine (5 and 10 µg ml⁻¹) displayed similar profiles. The profiles displayed by strain 251 mutants 33 and 79 resembled those of 11 mutants of strain 89; three of the 11 were isolated on medium containing 2 µg sulphathiazole ml⁻¹ and the remainder were from sulphadiazine at concentrations of 2, 5 and 10 µg ml⁻¹ (two, four and two mutants, respectively). In spite of their differing mutations and differences between the particular sulphonamide and the concentration used for selection, all of the MetI⁻ mutants examined were resistant to levels of sulphadiazine ≥ 10 µg ml⁻¹.

Table 3. *Recombination between metI mutational sites*

NEDA-minus-Met medium in multiple Petri dishes was inoculated with each MetI⁻ recipient strain followed by addition of DNA (0.05 ml containing 5 to 10 µg) from each MetI⁻ donor; typical results found after incubation are illustrated in Fig. 2. Met⁺ transformants: +, numerous; ±, <25; ●, none found in test with homologous DNA; 0, none found in test with heterologous DNA.

Recipient mutant designated*:	Transformation with DNA from mutant designated:										
	34	35	69	43	56	1	2	61	68	33, 79	89-108
34	●	+	+	+	+	0	+	+	+	+	+
35	+	●	+	+	+	0	+	+	+	+	+
69	+	+	●	±	0	0	±	+	+	+	+
43	+	+	±	●	0	0	±	+	+	+	+
56	+	+	0	0	●	0	±	+	+	+	+
1	0	0	0	0	0	●	0	±	±	+	+
90†	+	0	0	0	0	0	±	+	+	+	+
151†	0	0	0	0	0	0	0	0	0	+	+
153†	0	0	0	0	0	0	0	0	0	0	+
2	+	±	±	±	±	0	●	0	0	+	+
61	+	+	+	+	+	+	0	●	0	0	+
68	+	+	+	+	+	+	0	0	●	0	0
33, 79	+	+	+	+	+	+	+	0	0	●	+
89-108	+	+	+	+	+	+	+	+	0	+	●

* Mutant 89-108 was isolated from strain 89; the 14 other mutants were from strain 251.

† DNA was not prepared from this mutant strain.

In contrast, the 14 MetII⁻ mutants were isolated on medium containing 2 µg sulphadiazine ml⁻¹. Tests of these mutants in pure culture found that their resistance was limited to concentrations ≤8 µg ml⁻¹. The MetII⁻ phenotype apparently did not adapt the cells for competition with the parental strain undergoing residual growth on medium containing 5 µg sulphadiazine ml⁻¹.

Return of sulphonamide susceptibility

The theory that increased resistance to sulphonamides is a pleiotropic effect of certain *met* mutations requires that a return to sulphonamide susceptibility must accompany the restoration of methionine prototrophy. This was demonstrated unequivocally by the response to sulphadiazine found in Met⁺ derivatives obtained by mutation and transformation.

Met⁺ revertants that arose by spontaneous mutation displayed the parental level of resistance. For example, tests of 14 Met⁺ revertants derived from strain 251 MetI⁻ mutants 34 and 35 showed that resistance had declined from 10 µg ml⁻¹ to 1.0 µg ml⁻¹. Also, the resistance of a spontaneous Met⁺ mutant isolated from 89-108 was equal to the parental level of resistance, 0.5 µg ml⁻¹. Additional tests of these revertants showed that possible mutations had not occurred in 20 other genes.

Furthermore, a level of resistance similar to that of the Met⁺ parent strain 251 was found in Met⁺ transformants derived by treatment of Met⁻ recipients with DNA from any one of five MetI⁻ mutants or the MetII⁻ mutants 5 and 24 (Table 4). Although the Met⁺ Arg⁺ strain CDC is naturally resistant to sulphonamides, its DNA elicited Met⁺ transformants that were Sul^s. Unaccountably, however, their resistance was not reduced to the parental level. For control purposes, Arg⁺ transformants were isolated from the reaction mixtures containing CDC DNA. These transformants retained the Met⁻ Sul^r phenotype typical of the particular recipient.

A second round of sulphadiazine selection

How will revertants respond to a second challenge with sulphadiazine? The question was explored with a spontaneous mutant isolated from the MetII⁻ mutant 38. This Met⁺ Sul^s revertant was inoculated on GCMBS medium containing 2.0 µg of sulphadiazine ml⁻¹, similar to the first-round selection medium. Sul^r colonies were picked and tests identified six MetII⁻

Table 4. *Levels of sulphadiazine resistance of Met⁺ and Arg⁺ transformants*

Cellular suspensions of MetII⁻ mutant 5 and four MetI⁻ strains were treated in DEFT fluid with each indicated DNA (1.0 µg ml⁻¹); the uptake of DNA was terminated after 20 min by deoxyribonuclease and samples were plated on NEDA-minus-Met or NEDA-minus-Arg medium. Transformant colonies were purified and inoculated on various media for phenotype characterization. Parent strain 251, the source of the mutants, is resistant to 1.0 µg sulphadiazine ml⁻¹. The levels of sulphadiazine resistance of the Met⁻ recipient strains, as determined in multiple tests, were: mutant 5, 4.0 µg ml⁻¹; mutant 2, 35 µg ml⁻¹; mutant 43, 50 µg ml⁻¹; mutant 56, 25 µg ml⁻¹; and mutant 69, 30 µg ml⁻¹.

Recipient strain	DNA donor strain designated:	Transformants		Nutritional phenotype	Resistance to sulphadiazine µg ml ⁻¹	
		Selection	No. examined		Mean	(range)
Mutant 5	2, 43, 56, 61, 69	Met ⁺	40	Met ⁺ Arg ⁻	1.0	(1.0)
5	CDC	Met ⁺	24	Met ⁺ Arg ⁻	1.2	(1.0-3.0)
5	CDC	Arg ⁺	8	Met ⁻ Arg ⁺	4.1	(4.0-5.0)
Mutant 2	24	Met ⁺	16	Met ⁺ Arg ⁻	1.0	(1.0)
2	CDC	Met ⁺	26	Met ⁺ Arg ⁻	2.4	(1.0-6.0)
2	CDC	Arg ⁺	9	Met ⁻ Arg ⁺	32.2	(30.0-35.0)
Mutant 43	24, 61	Met ⁺	31	Met ⁺ Arg ⁻	1.0	(1.0)
43	CDC	Met ⁺	25	Met ⁺ Arg ⁻	2.2	(1.0-5.0)
43	CDC	Arg ⁺	8	Met ⁻ Arg ⁺	44.4	(35.0-50.0)
Mutant 56	5, 24, 61	Met ⁺	32	Met ⁺ Arg ⁻	1.0	(1.0)
56	CDC	Met ⁺	8	Met ⁺ Arg ⁻	2.5	(1.0-4.0)
56	CDC	Arg ⁺	8	Met ⁻ Arg ⁺	24.4	(20.0-25.0)
Mutant 69	5, 24, 61	Met ⁺	23	Met ⁺ Arg ⁻	1.0	(1.0)
69	CDC	Met ⁺	11	Met ⁺ Arg ⁻	3.2	(2.0-5.0)
69	CDC	Arg ⁺	9	Met ⁻ Arg ⁺	33.9	(30.0-35.0)

mutants that could be transformed to Met⁺Sul^s by each of the 25 DNAs from strain 251 Met⁻ mutants. Seven MetI⁻ mutants included three with recombination profiles unlike any in Table 3: one was transformed by all DNAs except those of mutants 61 and 68, and two mutants with revertible sites were transformed by all except DNA 1. The other profiles resembled those of mutants 43, 56, 61 and 153.

In addition, Met⁺Sul^s transformants obtained from MetII⁻ mutant 5 by treatment with DNAs from MetI⁻ mutants 2, 43 and 56 were exposed in the second round to 5 µg sulphadiazine ml⁻¹. Fifteen MetI⁻ mutants and no MetII⁻ mutants were recovered. Their recombination profiles were among those illustrated in Table 3. Interestingly, none duplicated the profiles of mutants 2, 43 or 56. Thus, the second round *met* mutations were not constrained by the ancestral *met* genotypes. In agreement with previous results, MetII⁻ mutants were isolated in the presence of sulphadiazine at a concentration of 2 µg ml⁻¹, but not 5 µg ml⁻¹.

DISCUSSION

The relation between sulphonamide resistance and methionine auxotrophy in isogenic gonococci was investigated because of findings with diverse isolates from patients over a period of 40 years. A group of 70 *N. gonorrhoeae* strains isolated before 1950 displayed resistance to sulphadiazine that ranged between 0.2 µg ml⁻¹ and ≥100 µg ml⁻¹, and 24 (34%) were Met⁻ (Catlin & Reyn, 1982). The resistance of some Met⁺ gonococci presumably was due to structural alterations of dihydropteroate synthase, as described by Ho *et al.* (1974). Resistance to ≥35 µg sulphadiazine ml⁻¹ was found in eight strains, seven of which were MetI⁻. Lower levels of resistance characterized other strains, including eight MetII⁻ isolates from disseminated gonococcal infections.

The results given in Tables 1 and 2 support the theory that the high incidence of Met⁻Sul^r gonococci in the 1940s was due to selection of mutants by sulphonamides widely used for therapy

of various susceptible bacterial diseases. When penicillin replaced sulphonamides in the clinic, the selective advantage of sulphonamide resistance was lost, with the result that the Met⁻Sul^r phenotype virtually disappeared within ten years (Catlin, 1976; Knapp *et al.*, 1985).

N. gonorrhoeae strains 89 and 251 used here came from patients on different continents and separated by 30 years. The evolutionary opportunities afforded strain 251 in the post-sulphonamide era led to substantial changes (cited in Methods). Nevertheless, numerous *metI* mutations were easily recovered from both strains by selection with sulphadiazine, sulphamethoxazole or sulphathiazole, and some profiles of *metI* transformation were similar.

The increased resistance enabling the mutants to grow on sulphonamide-containing medium appears to be a pleiotropic effect of methionine auxotrophy. This theory that the two phenotypic changes are due to one mutation is substantiated by the evidence that Met⁻ revertants derived by spontaneous mutation reacquired sulphonamide susceptibility equal to that of the Met⁺Sul^s parent. Further support for the theory is provided by transformation in which the Met⁻Sul^r traits were introduced or removed together. The selective action of sulphonamides appears to be specific for these *met* mutations since mutations were not found in 14 genes that encode other biosynthetic functions or six genes that affect responses to other antibacterial agents.

The role of *met* mutations in sulphonamide resistance can be understood by analogy with the resistance to trimethoprim afforded by *thy* mutations responsible for a thymine requirement. Sulphonamides and trimethoprim each interrupt a step in the folate pathway, sulphonamides by binding to dihydropteroate synthase and trimethoprim by binding to dihydrofolate reductase (Gale *et al.*, 1981; Harvey, 1982). Inhibition of growth ensues in either case because of the critical role of folates. Tetrahydrofolate is a versatile carrier of activated one-carbon units. The unit can exist in three oxidation states which are interconvertible and are utilized in the synthesis of various cellular components, including methionine, thymidine, purines and formylmethionyl-tRNA_f (Stryer, 1981). *N*⁵,*N*¹⁰-Methylenetetrahydrofolate is the source of the methyl group used by thymidylate synthase to form deoxythymidylate. The absence of thymidylate synthase activity in mutants that require an exogenous source of thymidine reduces depletion of the pool of tetrahydrofolates. Thus, *E. coli* Thy⁻ mutants gain increased resistance to trimethoprim because of the greater availability of tetrahydrofolate derivatives (Amyes & Smith, 1974; Harvey, 1982). This resistance provides a way to isolate *thy* mutations (Stacey & Simson, 1965).

The enzymes affected by the *metI* and *metII* mutations have not been identified. Clues are provided, however, by knowledge of the folate and methionine pathways in *E. coli* (Fig. 1). The *metF* gene of *E. coli* encodes *N*⁵,*N*¹⁰-methylenetetrahydrofolate reductase, which reduces *N*⁵,*N*¹⁰-methylenetetrahydrofolate to *N*⁵-methyltetrahydrofolate (Rowbury, 1983). The formation of methionine by transfer of this *N*⁵-methyl group to homocysteine is catalysed in *E. coli* by either of two homocysteine methyltransferases, encoded by *metE* and *metH* (Smith, 1971). On the other hand, most bacteria produce only one homocysteine methyltransferase (Flavin, 1975).

Homocysteine does not satisfy the requirement imposed by mutations of *metF*, *metE* or *metH*, but it can replace methionine for MetC⁻ mutants, which fail to convert cystathionine to homocysteine due to the absence of cystathionine β -lyase activity (Rowbury, 1983). Similarly, the MetII⁻ gonococcal mutants utilize homocysteine, but not cystathionine. This suggests that they may lack cystathionine β -lyase or, alternatively, are unable to take up cystathionine. The MetI⁻ mutants which utilize only methionine most likely correspond to *E. coli* MetF⁻. The choice of *N*⁵,*N*¹⁰-methylenetetrahydrofolate reductase over homocysteine methyltransferase as the missing enzyme activity is based on the similarity of the effects of sulphonamides and trimethoprim on the folate pathway as described above. The absence of *N*⁵,*N*¹⁰-methylenetetrahydrofolate reductase activity in MetI⁻ mutants would eliminate one function that depletes the pool of essential tetrahydrofolate derivatives.

The importance of conserving the pool, according to this model, accounts for the greater resistance conferred by mutations of *metI* than *metII*. Whereas MetI⁻ mutants display resistance to $\geq 10 \mu\text{g}$ sulphadiazine ml⁻¹, the resistance of MetII⁻ mutants is limited to $\leq 8 \mu\text{g}$ ml⁻¹. The sulphadiazine-containing GCMB medium provides all mutants with methionine. Nevertheless, the *N*⁵,*N*¹⁰-methylenetetrahydrofolate reductase activity continuing in the presence of

sulphadiazine would further impair the capacity of MetII⁻ mutants to perform activities catalysed by tetrahydrofolate derivatives. The return to the parental level of resistance in Met⁺ revertants verifies that the metabolic defects imposed by the *metI* and *metII* mutations were solely responsible for the differing resistances.

The 76 MetI⁻ mutants of strains 251 and 89 examined in tests with DNAs from strain 251 MetI⁻ donors displayed 21 distinct transformation profiles. Thus, sulphonamide selection yields numerous distinguishable mutations. The revertible mutations of mutants 34, 35 and 89-108 may represent different base-pair changes. On the other hand, the 11 nonreverting *metI* mutations that fail to recombine with contiguous mutational sites appear to be deletions of various nucleotide sequences (Table 3). The deletion of mutant 135 spans a great length of the *metI* locus. The findings suggest that the genetic fine structure of *metI* could be analysed by deletion mapping (Smith & Childs, 1966).

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