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# Genetic Studies of Sulfadiazine-resistant and Methionine-requiring *Neisseria* Isolated From Clinical Material

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Deoxyribonucleate (DNA) preparations were extracted from *Neisseria meningitidis* (four isolates from spinal fluid and blood) and *N. gonorrhoeae* strains, all of which were resistant to sulfadiazine upon primary isolation. These DNA preparations, together with others from in vitro mutants of *N. meningitidis* and *N. perflava*, were examined in transformation tests by using as recipient a drug-susceptible strain of *N. meningitidis* (Ne 15 Sul-s Met$^+$) which was able to grow in a methionine-free defined medium. The sulfadiazine resistance typical of each donor was introduced into the uniform constitution of this recipient. Production of *p*-aminobenzoic acid was not significantly altered thereby. Transformants elicited by DNA from the *N. meningitidis* clinical isolates were resistant to at least 200 $\mu$g of sulfadiazine/ml, and did not show a requirement for methionine (Sul-r Met$^+$). DNA from six strains of *N. gonorrhoeae*, which were isolated during the period of therapeutic use of sulfonamides, conveyed lower degrees of resistance and, invariably, a concurrent methionine requirement (Sul-r/Met$^-$). The requirement of these transformants, and that of in vitro mutants selected on sulfadiazine-agar, was satisfied by methionine, but not by vitamin $B_{12}$, homocysteine, cystathionine, homoserine, or cysteine. Sul-r Met$^+$ and Sul-r/Met$^-$ loci could coexist in the same genome, but were segregated during transformation. On the other hand, the dual Sul-r/Met$^-$ properties were not separated by recombination, but were eliminated together. DNA from various Sul-r/Met$^-$ clones tested against recipients having nonidentical Sul-r/Met$^-$ mutant sites yielded Sul-s Met$^+$ transformants. The *met* locus involved is genetically complex, and will be a valuable tool for studies of genetic fine structure of members of *Neisseria*, and of genetic homology between species.

Sulfonamides became available for clinical use in 1936-37. At that time, they were highly effective both in vivo and in vitro against most strains of *Neisseria gonorrhoeae* and *N. meningitidis*. Subsequently, they became decreasingly effective for treatment of gonorrhea, and 75 to 85% of *N. gonorrhoeae* strains isolated from 1944 to 1948 were sulfonamide-resistant (31, 37). However, *N. meningitidis* remained susceptible, and sulfonamides continued to be the drugs of choice for meningococcal meningitis until recently. In 1963, sulfonamide-resistant meningococcal strains were implicated in outbreaks of meningitis among military recruits (28), and subsequently have been isolated from civilian cases (12, 26).

As a basis for understanding the natural evolution of bacterial resistance to an antimicrobial agent, it is necessary to examine and compare strains isolated from clinical material. Yet sulfonamide resistance which arises by mutation in different strains is difficult to equate, inasmuch as the same mutation may not have the same phenotypic effect when it occurs in cells of different genetic constitution (29). The dilemma is resolved by introducing the mutant information from various clinical isolates into a selected recipient strain which has a uniform genetic and physiological constitution. The availability of a method to accomplish this genetic transfer between various species of *Neisseria* (2, 3), and of a completely defined culture medium for physiological studies (5), prompted this genetic study of sulfonamide-resistant clinical isolates of *N. meningitidis* and *N. gonorrhoeae*. The resistance of strains of both species could be introduced by transforming deoxyribonucleate (DNA) preparations into the sulfonamide-susceptible recipient, *N. meningitidis* strain Ne 15. Two classes of sulfonamide resistance could be distinguished by transformation. Resistance of one class was not associated with

significant nutritional change, whereas that of the other was invariably associated with a requirement for methionine.

Numerous studies have been made of the antibacterial action of sulfonamides and of the development of bacterial resistance (19). The discovery by Woods (40) that sulfonamide competitively inhibits bacterial utilization of *p*-aminobenzoic acid (PAB) was followed by the recognition that PAB is an essential component of folic acid. Folic acid coenzymes function as carriers of single carbon units in the biosynthesis of methionine and several other amino acids, and also of purines and thymine. Thus, the bacteriostatic action of sulfonamide is due to the ultimate shortage of the essential end products of folic acid metabolism (41). This explains the latent period that is observed before sulfonamide measurably reduces the rate of bacterial growth (23).

Various factors enhance the capacity of a given bacterial strain to multiply in the presence of a concentration of sulfonamide higher than the minimal inhibitory concentration established for that strain under standard conditions. These may be grouped into two categories depending on whether genetic change is or is not associated with the increased resistance: phenotypic resistance and genetic resistance.

In the absence of any genetic change (phenotypic resistance), the bacteriostatic action of sulfonamide is reduced by exogenous supplementation of the bacterial environment with PAB (40) or with one or more of the essential products of folate metabolism (41). Thus, methionine was shown to be an antagonist of sulfonamide for *Escherichia coli* (15); moreover, the methionine biosynthetic pathway was recognized as being the most sensitive to inhibition by sulfonamides (38).

Genetic changes resulting in increased endogenous supplies of PAB (24, 40), or in alteration of a PAB-utilizing enzyme with consequent decreased affinity for sulfonamides (7, 17), were proposed to account for increased resistance. Recent work with two cell-free systems has shown that enzymes which catalyze the formation of folate compounds from PAB indeed do differ structurally: those extracted from sulfonamide-resistant mutants combine with sulfonamide less readily than do the corresponding enzymes extracted from the original parent bacteria (30, 39). Furthermore, a genetic parallel to the end-product antagonism of sulfonamide bacteriostasis is found in certain mutational events that impose a nutritional requirement for methionine. It will be shown that *N. meningitidis* clones, which are unable to perform this folate-catalyzed biosynthetic reaction because of a genetic block introduced by spontaneous mutation or by transformation, are able to multiply in a defined medium containing methionine together with sulfadiazine at a concentration higher than that sufficient to inhibit growth of the parental strain in the same methionine-containing medium.

## MATERIALS AND METHODS

*Chemicals.* Inorganic chemicals and PAB were certified reagent grade obtained from Fisher Scientific Co., Pittsburgh, Pa. Crystalline vitamin $B_{12}$ was from Sigma Chemical Co. (St. Louis, Mo.). Calbiochem (Los Angeles, Calif.) supplied the *S*-adenosyl-L-methionine (B grade) and all amino acids (A grade, "chromatographically homogeneous") except DL-homocysteine free base (Nutritional Biochemicals Corp., Cleveland, Ohio). Homocysteine also was prepared immediately before use from L-homocysteine thiolactone hydrochloride (A grade, Calbiochem) by addition of alkali by two slightly different methods (11, 16). L-Homocystine ("chromatographically homogeneous"), found to be contaminated with methionine, was recrystallized from a solution in hot sodium acetate buffer (0.05 M, *p*H 6). Crystalline dihydrostreptomycin sulfate (USP; E. R. Squibb & Sons, New Brunswick, N.J.) and sodium sulfadiazine (solution, 0.25 g/ml; Lederle Laboratories, Pearl River, N.Y.) were the antibacterial agents used. Crystalline pancreatic deoxyribonuclease (sterile; Worthington Biochemical Corp., Freehold, N.J.) was used to destroy activity of transforming DNA.

*Media.* The complex medium (HIY-1) used routinely for growth and transformation tests of *N. meningitidis* Ne 15 has been described (2). It contains Difco Heart Infusion Broth, Yeast Extract, and Bacto-agar (1.4%, w/v, for hard agar and 0.7% for soft agar), together with calcium and other supplements.

*N. gonorrhoeae* was cultivated (35 C with 5 to 10% $CO_2$) on chocolate-agar prepared with Proteose No. 3 Agar (Difco) and 5% sheep blood.

The defined medium was prepared by methods detailed previously (5). The components of solutions A and B (mineral salts), and D (sodium lactate), were the same as before, but other components were changed. As the sodium glutamate employed earlier was found to be contaminated with methionine (a difficulty also encountered by others (9, 27), glutamic acid was used (final concentration, 8.0 mM). The sodium salt was prepared by slow addition of 2 N sodium hydroxide (to the 400 mM stock) to give *p*H 7.4. Serine was routinely added at a concentration of 0.2 mM; though not required, it promoted growth in defined fluid of some transformants (Sul-r $Met^+$). The remaining components of the routine medium were arginine (0.5 mM), glycine (2.0 mM), calcium chloride (0.2 mM), ferric sulfate (0.01 mM), and glycerine (0.5%, v/v). As emphasized before (5), the *p*H of the final medium must be rigidly controlled (7.4 ± 0.1) in order to obtain growth of *N. meningitidis* from small inocula. To avoid *p*H changes associated with autoclaving, the various stock solutions (except $CaCl_2$, glycerine, and agar) were sterilized separately by

filtration (HA membranes; Millipore Corp., Bedford, Mass.) and combined aseptically. The complete medium without agar is referred to as defined fluid. "Hard" and "soft" agar medium contained, respectively, 1.0 and 0.5% (w/v) Purified agar (Difco).

Defined agar was supplemented with amino acids and other substances for special studies. When solubility of the compound permitted, a stock solution (100 to 1,000 times the required concentration) was prepared, filter-sterilized, and added aseptically. Difficultly soluble (cystine, homocystine) or expensive (adenosylmethionine) compounds were weighed aseptically and added directly to the prepared sterile medium; contaminant colonies were rarely observed.

*Abbreviations.* Until the precise mechanism relating the methionine requirement and sulfonamide resistance of *Neisseria* is established, it is convenient to indicate the various genetic changes which affect the test strain *N. meningitidis* Ne 15 in terms of their recognized phenotypic effects. In concordance with recent recommendations (10), the following phenotypic abbreviations will be used. Sul-r20 $Met^+$ will refer to mutant strains or transformants which are able to produce colonies on HIY-1 agar containing sulfadiazine at a concentration of 20 $\mu$g/ml, but not at 100 $\mu$g/ml, and which do not show a nutritional requirement for methionine in defined medium. Sul-r $Met^+$ indicates strains that produce fast-growing colonies on 100 $\mu$g of sulfadiazine per ml, and do not require methionine. Sul-r/$Met^-$ will refer to strains which reproduce on sulfadiazine-containing agar more slowly and require methionine for growth in defined medium; the two characteristics are acquired concurrently during transformation. Str-r will refer to strains which are resistant to dihydrostreptomycin (DHS) incorporated in HIY-1 agar at a concentration of 500 $\mu$g/ml.

*DNA donor strains.* Strains of *N. meningitidis* and *N. gonorrhoeae* which were resistant to sulfadiazine when isolated from clinical material were received from other laboratories (Table 1). The four meningococcal strains were all serological group B, as were the streptomycin-resistant mutants derived from them. Methods used at Statens Seruminstitut to isolate and identify gonococci, and results of susceptibility studies, have been published (31, 32). To simplify the presentation and discussion of transformation results, roman numerals will be used to designate DNA preparations from these clinical isolates.

DNA preparations I–VI were obtained from Str-r strains. The Str-r mutants of *N. meningitidis* were derived by the method previously described (4). *N. gonorrhoeae* 1197/61 was resistant to DHS as well as to sulfadiazine when isolated. *N. gonorrhoeae* 7764/45 Str-r was obtained by selecting an isolated mutant colony which grew on heavily inoculated chocolate-agar containing 500 $\mu$g of DHS/ml. The *str* gene provided a valuable reference marker for work with these six transforming DNA preparations. DNA preparations VII–XI, on the other hand, did not bear this marker. These gonococcal strains were cultivated on drug-free chocolate-agar exclusively. After revival from the lyophilized state, they were subcultured only the several times required to establish their purity and to obtain physiologically active cells for extraction of DNA. This precaution was taken to avoid the possibility of selecting nutritionally deficient strains.

DNA preparations, which were used for reference purposes in many tests, were obtained from two different sulfadiazine-resistant strains derived in vitro from the wild-type *N. meningitidis* Ne 15. Ne 15 (Sul-r I) $Met^-$ Str-r had a mixed origin. A Sul-r transformant colony, obtained by treating Ne 15 with DNA I, was picked from HIY-1 agar containing 50 $\mu$g of

TABLE 1. *Sources of the Neisseria strains, resistant to sulfadiazine upon primary isolation, from which transforming deoxyribonucleate preparations were obtained*

| Species | Strain | Source | Year isolated | Str-r | Designation for DNA |
|---|---|---|---|---|---|
| *N. meningitidis* | NM-131[a] | Spinal fluid | 1963 | M[e] | I |
| | 64-84[a] | Spinal fluid | 1964 | M | II |
| | 64-129[a] | Blood | 1964 | M | III |
| | 28988[b] | Blood | 1965 | M | IV |
| *N. gonorrhoeae* | 1197/61[b] | UD[c] | 1961 | PI[f] | V |
| | 7764/45[b] | UD | 1945 | M | VI |
| | W 18[b] | UD | 1946 | No | VII |
| | W 58[b] | UD | 1946 | No | VIII |
| | 18000[b] | UD, SP[d] | 1940 | No | IX |
| | 18273[b] | UD, SP | 1940 | No | X |
| | 31956[b] | UD | 1941 | No | XI |

[a] Strain (18) received from D. Ivler.
[b] Strain received from A. Reyn, Statens Seruminstitut, Copenhagen, Denmark.
[c] Discharge from male urethra.
[d] Separate isolations from the same patient.
[e] Mutant obtained in vitro; resistant to 500 $\mu$g of dihydrostreptomycin per ml.
[f] Primary isolate was resistant to 2 mg of dihydrostreptomycin per ml.

sulfadiazine/ml; it was resistant to 200 μg as revealed by equal growth on agar containing 100, 150, and 200 μg of sulfadiazine per ml. A mutant colony obtained by growth at 300 μg of sulfadiazine per ml was subcultured on higher concentrations. A single colony capable of growth on 500 μg of sulfadiazine per ml was isolated, and a Str-r clone was obtained from it. The DNA preparation was obtained from cells which had been subjected repeatedly to single-colony isolation, thereby minimizing the possibility that a mixed culture was used. Nevertheless, subsequent tests of its transforming activity (e.g., test 10, Table 2) revealed two kinds of sulfadiazine-resistant transformants, Sul-r $Met^+$ and Sul-r/$Met^-$. Standard practice involves freezing (at −60 C) a sample of the inoculum used for all DNA cultures. Therefore, it was possible (a year later) to examine the culture on defined agar. The total population was found to be composed of methionine-requiring cells.

Ne 15 Sul-r/$Met^-$ Str-r 64 was obtained by sequential selections of isolated mutant colonies from cultures on HIY-1 agar containing increasing concentrations of sulfadiazine, each culture incubated for 2 days at 35 C in humidified air atmosphere. The first mutation in the sequence conferred resistance to 100 μg of sulfadiazine per ml. A number of subsequent mutations occurred during growth on 150, 250, 400, and finally 500 μg of sulfadiazine per ml. Lastly, a Str-r clone was selected.

In the same manner, a multistep sulfadiazine-resistant strain was obtained from *N. perflava* Ne 16. The final mutant colony was selected by use of 250 μg of sulfadiazine per ml. It proved to be Sul-r/$Met^-$.

*Transforming DNA. N. meningitidis* strains were cultivated in HIY-1 broth containing half the concentration of sulfadiazine and DHS to which the strain was resistant. After 16 to 20 hr of incubation (with shaking in air at 35 C), cells were harvested by centrifugation, suspended in a solution containing NaCl (0.15 M) and sodium citrate (0.015 M), and frozen. *N. gonorrhoeae* strains were cultivated on drug-free chocolate-agar, petri dishes being incubated at 35 C in candle jars, or in a $CO_2$ incubator with 10% $CO_2$ when large dishes were used to obtain more cells. Growth was removed from agar with NaCl-citrate solution, washed once by centrifugation, and frozen. Rapid lysis of the thawed cell suspensions was obtained with 0.5 to 1.0% sodium dodecyl sulfate. DNA was extracted and partially purified (3).

*Transformation.* For streptomycin resistance, the method described previously (2, 4) for quantitative transformation of *N. meningitidis* Ne 15 was used, except that soft agar inoculated with a sample of the reaction mixture was overlaid in 4-ml volumes on five plates of antibiotic-free agar. These plates were incubated at 35 C until removed for addition of a 6-ml layer of HIY-1 soft agar containing DHS sufficient to give a final concentration of 500 μg/ml after diffusing throughout the agar. This challenge dose of antibiotic was added 5 hr after the time of mixing recipient bacteria with DNA, the intervening period having been found optimal for phenotypic expression of resistance to DHS.

For sulfadiazine resistance, only slight modification of the test method was required. Phenotypic expression of resistance to sulfadiazine did not require prolonged incubation in drug-free medium, but occurred in SULF agar during the time that the intracellular concentration of critical metabolic products was being depleted (23, 41). A sample of the recipient cell plus DNA reaction mixture was added to HIY-1 soft agar containing sulfadiazine and was overlaid in 5-ml volumes on five plates of hard agar of similar composition. Cultures were incubated (35 C, humidified air atmosphere) for 3 to 6 days before colonies were counted, growth of Sul-r/$Met^-$ transformants being much slower than Sul-r $Met^+$ on media containing 100 μg and especially 200 μg of sulfadiazine per ml.

The lowest concentration of sulfadiazine, 20 μg/ml, used routinely in transformation tests was selected after considerable preliminary study. Colony formation by the recipient strain Ne 15 on HIY-1 agar was partially inhibited at a concentration of 5 μg of sulfadiazine per ml, the number inhibited being inversely related to population size. Bacteriostasis was nearly complete at 10 μg/ml, but the fraction of Sul-r mutants was unduly high. Even in 20 μg of sulfadiazine per ml, spontaneons mutants were always encountered. To determine their number accurately, a control reaction mixture (recipient cells plus deoxyribonuclease-inactivated DNA) was incubated and sampled in parallel with the transformation tests. In calculating percentage transformation, the number of mutant colonies was subtracted from the number of colonies on transformant plates at corresponding sulfadiazine concentrations. The percentage of spontaneous mutants on sulfadiazine (20 μg/ml)-agar commonly ranged from 0.001 to 0.0004, and on sulfadiazine (100 μg/ml)-agar from 0.0003 to 0.00005. Mutant colonies were never observed on sulfadiazine (200 μg/ml) control plates. Of 41 spontaneous mutants picked from sulfadiazine (20 μg/ml)-agar of one control test, 13 were $Met^+$ and 28 were $Met^-$; all of 33 colonies from agar containing 100 μg of sulfadiazine per ml were $Met^-$.

The method for assay of total colony-forming units, details concerning the recipient cell plus DNA reaction mixture, and various precautions have been described before (2–4).

Transformation to prototrophy of methionine-requiring strains was examined by use of defined medium. $Met^-$ recipient cells were grown for 12 to 14 hr on agar medium, usually defined agar supplemented with 0.05 mM methionine, or occasionally HIY-1 agar. Cells were removed with a loop and suspended in defined fluid, prewarmed to 35 C. Equal volumes of cellular suspension (with no intervening incubation) and transforming DNA (diluted in defined fluid) were mixed and allowed to react at 35 C for 20 to 30 min. $Met^+$ transformants were assayed by alternative methods. Method A provided more quantitative results, but was more time-consuming. Deoxyribonuclease was added to terminate uptake of DNA. A sample of the reaction mixture was added to 20 ml of methionine-free defined soft agar at 44 C, which was immediately overlaid in 5-ml volumes on four defined hard agar plates. Method B, which gave only semiquantitative results, was used to expedite sampling of

larger numbers of reaction mixtures. For this, deoxyribonuclease was not added. Single samples (0.05 ml) of each reaction mixture were spread over the surfaces of methionine-free defined hard agar plates, with the aid of sterile bent glass rods. A deoxyribonuclease-inactivated DNA control reaction mixture was included in experiments with every recipient suspension. For determination of the total number of treated cells (expressed as colony-forming units), dilutions in defined fluid were plated in triplicate (by the overlay method) in defined agar containing 0.1 mM methionine.

*Replica plating.* Cells from a DNA reaction mixture, suitably diluted, were spread with a sterile glass rod over the surface of HIY-1 agar containing 20 $\mu$g of sulfadiazine per ml. After incubation (35 C, 20 to 40 hr), the transformant colonies on this "master" plate were sampled by pressing gently against sterile velvet stretched over a flat circular form. Cells were transferred, by similar pressure, from the velvet to the surfaces of as many as eight sterile agar media. Faithful replicas of colonies on the "master" plate were obtained by using damp velvet cloth (circles in large petri dishes were autoclaved within a few hours of use) and agar plates which had been judiciously dried (retained at room temperature long enough to eliminate subsequent development of surface moisture, which displaces the cells, but not so long as to become wrinkled, a deterrent to growth).

*PAB.* Two methods were used to determine the concentration of PAB or compounds having PAB-equivalent activity. The first employed PAB Assay Medium (Difco) with slight modification of the method described (*Difco Supplementary Literature*, Difco Laboratories, Detroit, Mich.). Stock strains of *Acetobacter suboxydans* ATCC 621 exhibited filamentous cells and flocculent growth which tended to adhere to glass. More homogeneous growth was obtained in the assay medium by using a small inoculum of cells selected from smooth colonies. Cultures (10-ml volumes aseptically distributed in 125-ml screw-cap flasks) were shaken continuously during incubation (30 C, 60 hr). Readings of optical density were made at 650 m$\mu$ (Spectronic-20 colorimeter) after diluting each culture with an equal volume of water and mixing thoroughly. Characteristics of this assay system (e.g., appreciable growth in the absence of added PAB) were such as to make quantitative aspects of the test dubious (22).

Therefore, an independent method was devised to compare the sulfadiazine-antagonizing activities of defined culture filtrates from various strains of *N. meningitidis*. For this, defined agar with Fisher glutamate (5) containing 0.6 $\mu$g of sulfadiazine per ml was pipetted aseptically in 20-ml volumes into flat-bottom Pyrex petri dishes. Left overnight at room temperature, the agar became dry enough to accept a 4-ml overlay of soft defined agar (with sulfadiazine, 0.6 $\mu$g/ml, and an inoculum of *N. meningitidis* Ne 15 Sul-s cells, $10^5$ to $10^6$/ml) without subsequent development of surface moisture. Sterile discs (diameter, 6.5 mm), punched from absorbent Millipore filter support pads, were saturated with test fluid and dropped on the solidified inoculated agar. Culture filtrates were tested in duplicate, and PAB standard solutions (diluted in defined fluid) were tested in triplicate at concentrations of 4, 2, and 1 $\mu$g of PAB/ml. Growth of the meningococcus was inhibited by the sulfadiazine except in zones peripheral to the discs where sulfadiazine antagonizing compounds had diffused. Zones were clearly defined and their diameters measurable after incubation at 35 C for 2 days. A linear relationship was found between the mean response and the logarithm of PAB concentration.

*Cross-resistance.* Sulfadiazine was the only sulfonamide examined in quantitative tests. However, qualitative tests were performed to determine whether resistance of the *N. meningitidis* strains applied to other sulfonamides as well. HIY-1 agar was used: 20-ml bottom layers and 3-ml overlays of soft agar inoculated with not more than $10^6$ cells from a 16- to 18-hr HIY-1 agar culture. Commercially available paper discs (generously provided by BBL) containing 0.25 mg of each compound were placed on the solidified agar. Plates, after incubation for 24 hr, were examined for significant inhibition of bacterial growth (zones with a radius greater than 1 mm) peripheral to the discs. The meningococcal strain Ne 15 used as recipient in transformation tests was susceptible to the following compounds: (a) sulfadiazine, (b) sulfathiazole, (c) sulfamerazine, (d) sulfisoxazole, (e) sulfisomidine, (f) sulfamethoxypyridazine, (g) sulfachloropyridazine, (h) 2,4-dimethoxy-6-sulfanilamido-1,3-diazine, (i) sulfamethylthiadiazole, (j) sulfaethylthiadiazole. Strains 64-84, 64-129, and NM-131 were insusceptible to compounds a–g and susceptible to compounds h–j. Transformant clones picked from HIY-1 agar containing 200 $\mu$g of sulfadiazine per ml showed similar patterns of resistance, except that some remained susceptible to compound g.

## RESULTS

*Sulfadiazine resistance transformation frequencies.* DNA preparations from a variety of sulfadiazine-resistant strains of *Neisseria* had transforming activity for the sulfadiazine-susceptible meningococcal strain Ne 15 (Table 2). Genetic information which evoked resistance to 20 $\mu$g of sulfadiazine per ml or to 500 $\mu$g of DHS/ml (reference marker) was introduced separately but at frequencies which did not differ greatly, except for the DNA examined in test 10. This DNA produced twice as many transformants resistant to sulfadiazine (20 $\mu$g/ml) as to DHS, owing to the fact (established by replica plating, and discussed below) that two different kinds of transformant colonies were being counted on agar containing 20 $\mu$g of sulfadiazine per ml.

DNA preparations from *N. gonorrhoeae* and *N. perflava* elicited transformations at lower frequencies than those typical of *N. meningitidis* DNA, as expected of inter-specific transformation (3).

The sulfadiazine resistance of DNA donor strains could be inferred in relative terms from the

TABLE 2. *Transformations of Neisseria meningitidis Ne 15 by DNA from various Neisseria*[a]

| Test no. | DNA prepn[b] | Transformation (%) | | | | |
|---|---|---|---|---|---|---|
| | | Resistance to sulfadiazine | | | | Resistance to DHS (500 μg/ml) |
| | | 20 μg/ml | 50 μg/ml | 100 μg/ml | 200 μg/ml | |
| 1 | *N. meningitidis*, I | 0.196 | 0.165 | 0.167 | 0.152 | 0.156 |
| 2 | *N. meningitidis*, II | 0.197 | 0.158 | 0.141 | 0.138 | 0.134 |
| 3 | *N. meningitidis*, III | 0.224 | 0.181 | 0.180 | 0.172 | 0.187 |
| 4 | *N. meningitidis*, IV | 0.213 | 0.161 | 0.153 | 0.154 | 0.137 |
| 5 | *N. gonorrhoeae*, V | 0.037 | 0.0024 | <0.00001 | <0.00001 | 0.039 |
| 6 | *N. gonorrhoeae*, VI | 0.039 | 0.033 | 0.030 | 0.002 | 0.042 |
| 7 | *N. meningitidis* Ne 15 Sul-r20 Met$^{+}$16 | 0.169 | 0.157 | <0.00001 | <0.00001 | SD[c] |
| 8 | *N. meningitidis* Ne 15 Sul-r/Met$^{-}$ Str-r 64 | 0.280 | 0.236 | 0.230 | 0.003 | 0.271 |
| 9 | *N. perflava* Ne 16 Sul-r/Met$^{-}$ Str-r | 0.013 | 0.011 | 0.010 | (no test) | 0.021 |
| 10 | *N. meningitidis* (Sul-r I) Met$^{-}$ Str-r | 0.327 | 0.309 | 0.291 | 0.134 | 0.176 |

[a] Reaction mixtures in HI-1 broth contained recipient cells (suspension prepared from 12-hr HIY-1 agar culture) with 5.0 μg of DNA/ml. Reaction at 35 C was terminated by deoxyribonuclease after 30 min. A single recipient cell suspension, $7.1 \times 10^6$ colony-forming units/ml was used for tests 1–8; 9 and 10 were separate.

[b] Details, Table 1.

[c] DNA donor strain and transformants (0.056%) were streptomycin-dependent (2).

transformation frequencies. Thus, the four *N. meningitidis* clinical isolates possessed the genetic potential for resistance to 200 μg of sulfadiazine per ml, as shown by the transforming activities of their DNA (I-IV). Three of the strains had been recorded (18) as having this degree of phenotypic resistance, and, indeed, all four were capable of growth on HIY-1 agar containing 200 μg of sulfadiazine per ml.

In comparison, the two *N. gonorrhoeae* strains were revealed as having greater and unequal susceptibility. DNA VI evoked resistance to 20 and 100 μg of sulfadiazine per ml at nearly the same frequencies, but to 200 μg/ml at a lower frequency. DNA V evoked resistance to 20 μg of sulfadiazine per ml at a frequency similar to that of DNA VI. However, DNA V transformants formed colonies only rarely on agar containing 100 μg of sulfadiazine, and never on agar with 200 μg of sulfadiazine per ml. The degree of resistance of the gonococci themselves could not be determined on the same medium because of inadequate growth. However, introduction of the relevant genes into the uniform genetic background of Ne 15 resulted in Sul-r transformants which clearly differed from those produced by DNA preparations I-IV. A further difference was reflected in the smaller size of DNA VI transformant colonies on agar containing 100 μg of sulfadiazine per ml after incubation for 2 days. On agar with 200 μg of sulfadiazine per ml, they were barely visible in 2 days, and tended to remain very small even after a 6-day incubation. They were counted as transformants, however, because no colonies were ever detected on corresponding control plates (i.e., agar containing 200 μg of sulfadiazine per ml inoculated with cells treated with deoxyribonuclease-inactivated DNA). This growth characteristic of the DNA VI transformants could not be attributed merely to the fact that the genetic factor originated in *N. gonorrhoeae*, as comparable transformants also were produced by the DNA preparations examined in tests 8 and 9.

*Replica plating.* The transformants elicited by the DNA examined in test 10 (Table 2) were of two types. One formed small colonies on agar containing 100 μg of sulfadiazine per ml, like those of tests 6, 8, and 9. Colonies of the other type were large, like DNA I transformants. The sulfadiazine resistance of the DNA-donor strain itself was of mixed origin: resistance introduced into Ne 15 by transformation with DNA I, and additional resistance obtained by spontaneous mutation. Replica plating of transformant colonies which had grown on agar containing 20 μg of sulfadiazine per ml revealed a nutritional difference. The type which produced small colonies on agar containing sulfadiazine was invariably methionine-requiring (Fig. 1–7). Methionine-free defined agar (Fig. 1) supported growth of the large colonies only. Visible but limited growth of the small type occurred on defined agar containing 0.005 mM methionine

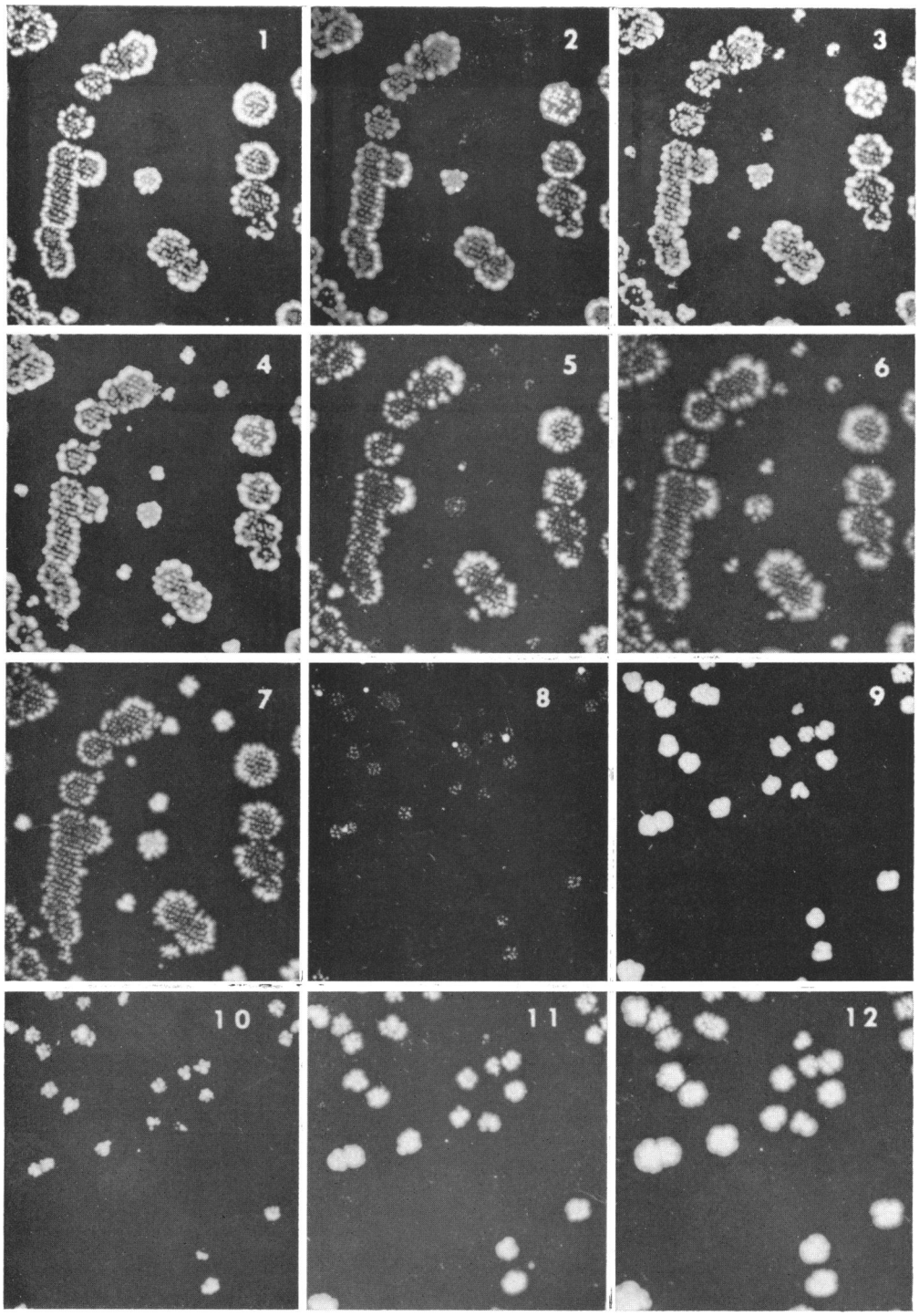


FIG. 1–12. *Sulfadiazine-resistant transformant colonies on various media, incubated for 2 days. Figures 1–7 are corresponding areas of replica plates made in the order given from a single sulfadiazine (20 μg/ml) transformation plate (not shown) from test 10, Table 2; Fig. 8–12 are a series of replicas from a single transformation plate (20 μg/ml; not shown) from test 6, Table 2. Growth responses of cells from identical clones can be compared on defined agar lacking methionine (Fig. 1) or with methionine at concentrations of 0.005 mM (Fig. 2, 8), 0.05 mM (Fig. 3), 0.1 mM (Fig. 4, 9); HIY-1 agar with 100 μg of sulfadiazine per ml (Fig. 5, 10), with 20 μg/ml (Fig. 6, 11), or sulfadiazine-free (Fig. 7, 12). Photographs are not magnified and not retouched.*

(Fig. 2). Progressively higher concentrations of methionine (0.05 mM, Fig. 3; 0.1 mM, Fig. 4) promoted proportionally better growth of these colonies. Their growth after 2-day incubation on HIY-1 agar with 100 $\mu$g of sulfadiazine per ml (Fig. 5), as expected, was less than on 20 $\mu$g/ml (Fig. 6) or on drug-free agar (replicated last, Fig. 7). The methionine content of HIY-1 agar was sufficient for optimal growth of the methionine-requiring transformants in the absence of sulfadiazine. Thus, when growth on HIY-1 agar was initiated from isolated cells, Sul-r Met$^+$ colonies and Sul-r/Met$^-$ colonies were indistinguishable. Differences observed in Fig. 7 reflect the differing size of the original transformant colonies on the sulfadiazine (20 $\mu$g/ml) "master" plate, the velvet pile design being due to the relatively dry agar which restricted growth.

The transformant colonies produced by gonococcal DNA VI (test 6, Table 2) were shown by replica plating to be all of the methionine-requiring type. Their response to methionine was the same as that of other Sul-r/Met$^-$ transformants, as to degree of growth on the defined agar supplemented with low concentrations of methionine (0.005 mM, compare Fig. 8 with Fig. 2), and optimal growth at a concentration of 0.1 mM (Fig. 4, 9). Various transformant clones differed somewhat in their capacity to tolerate sulfadiazine, as revealed by the decreased number of transformants with increased concentration. An example is provided by the colony, located close to the numeral in Fig. 12, which produced no growth on 100 $\mu$g of sulfadiazine per ml (Fig. 10) and only limited growth on 20 $\mu$g/ml (Fig. 11) during the 2-day incubation period.

A few Met$^+$ papillae appeared on Met$^-$ colonies on defined agar containing low concentrations of methionine (Fig. 8). Very few occurred on methionine-free agar, and they were present only rarely at corresponding sites on companion replica cultures. The replica-plating technique (25) thus established that they originated as separate events occurring after the bacteria were deposited on the replica agar. If a genetic event leading to production of the Sul-r Met$^+$ phenotype had occurred in the DNA reaction mixture, the colony produced on the sulfadiazine-agar "master" plate must be wholly Met$^+$. Therefore, Met$^+$ clones must appear on all methionine-deficient agar replicas at a topographically identical site.

These criteria were fulfilled for Sul-r transformants obtained with DNA V. Hundreds of colonies were examined by replica plating and all were Met$^+$. Similarly, the transformants obtained in test 7 (Table 2) were Met$^+$. This DNA, obtained to serve as a model for comparison with DNA V, was from a spontaneous mutant (isolated from Ne 15) which was resistant to 20 $\mu$g of sulfadiazine per ml, susceptible to 100 $\mu$g/ml, and Met$^+$. It resembled DNA V except with respect to the ratio of Sul-r50 to Sul-r20 transformants.

In summary, transformants of tests 1–4 (Table 2) were highly resistant to sulfadiazine and exhibited no added nutritional requirements (Met$^+$). Those of tests 5 and 7 showed only a limited increase in sulfadiazine resistance, and were Met$^+$. Transformant colonies of tests 6, 8, and 9 were Met$^-$, and, though moderately resistant, were characterized by a tendency for small size on elevated concentrations of sulfadiazine.

*PAB production.* Many investigators have found that PAB is synthesized in larger amounts by some sulfonamide-resistant clinical isolates than by susceptible strains of the same bacterial species. The availability of additional PAB either inside or outside the cells acts at the phenotypic level to decrease bacterial susceptibility to sulfonamides, as mentioned previously. It is questionable, however, whether the two to five times higher concentrations of PAB reported for *Neisseria* (18, 24) provide the genetic basis for 200 times greater sulfonamide resistance. This question can be examined critically by transformation. As relatively short nucleotide sequences appear to be integrated typically in transformation, only limited genetic information is introduced into the genome of the recipient cell. Physiological differences between the Sul-s recipient strain and the Sul-r transformants, therefore, should be restricted to those directly associated with the resistance.

Strain Ne 15 and various transformant clones (isolated colonies picked from agar containing 20 $\mu$g of sulfadiazine per ml in tests similar to those of Table 2) were cultivated in defined fluid (with 0.05 mM methionine, if required). The cultures (25-ml volumes in 500-ml flasks), incubated in air at 35 C on a rotary shaker, were examined periodically for 3 days. Population densities of $3 \times 10^9$ to $6 \times 10^9$ colony-forming units/ml were attained within 36 hr. Thereafter, viability declined and considerable autolysis occurred. The concentration of PAB (or compounds that could replace it as growth factor for *A. suboxydans*) increased progressively during the 3-day incubation (samples sterilized by Millipore membrane filtration). For cultures of the same age and population density, PAB-equivalent concentration differed by less than a factor of 2.

The possibility remained, however, that the values determined on filtrates might not represent total cellular PAB. As PAB is heat-stable (24, 40), 62-hr cultures were subjected to heat extraction (121 C, 10 min). The supernatant fluids were as-

sayed by two methods: (i) growth stimulation for *A. suboxydans*, and (ii) sulfadiazine-antagonizing activity (disc test). Again, differences between cultures were not greater than twofold, although method ii apparently determined compounds additional to those which possessed growth-promoting activity in i. Values obtained for the Sul-s strain Ne 15, expressed as micrograms of PAB equivalents per milliliter, were 0.43 by method i and 2.5 by method ii. Corresponding values for six Sul-r transformant clones (picked from agar containing 20 μg of sulfadiazine per ml of tests, as enumerated in Table 2) were: no. 1, 0.43, 3.2; no. 2, 0.35, 3.0; no. 3, 0.52, 3.5; no. 5, 0.42, 2.5; no. 7, 0.42, 2.5; no. 9, 0.5 and 5.1. Although quantitative aspects of both methods are open to criticism, the assays should have detected gross differences of PAB or of compounds having sulfadiazine-antagonizing activity, had such differences existed.

The conclusion appears justified, therefore, that PAB production of recipient strain Ne 15 is not significantly affected when these sulfonamide resistance factors are introduced by transformation.

*Gonococci of the sulfonamide era.* Transformants elicited by DNA from a gonococcal strain (7764/45) isolated in Denmark during the period of therapeutic use of sulfathiazole (31) differed nutritionally from those obtained with DNA from a more recent isolate (1197/61). It was of great interest, therefore, to examine five additional Danish strains of sulfathiazole-resistant *N. gonorrhoeae* (Table 1) which also had been isolated during the sulfonamide era. Retained in the lyophilized state during the intervening years, these strains were received after the nutritional difference between Sul-r transformants was recognized. Therefore, precautions were taken to avoid laboratory manipulations which might affect the genetic constitution of these gonococci. They were cultivated on drug-free chocolate-agar exclusively; only a few subcultures were made, and these were initiated from a considerable number of typical colonies.

Transforming activities of DNA preparations from all the strains of *N. gonorrhoeae* were determined, and transformant colonies which developed on sulfadiazine (20 μg/ml)-agar (300 or more for each DNA) were examined by replica plating. The frequencies of transformation obtained with DNA preparations VI-XI were not all alike (Table 3), and different degrees of resistance were elicited. Nevertheless, the transformants obtained were uniformly methionine-requiring. A few Met$^+$ colonies were present on transformation plates containing sulfadiazine (20 μg/ml)-agar, but their number was not greater than the number of Sul-r Met$^+$ spontaneous mutants, which were found on corresponding control plates spread with cells treated with deoxyribonuclease-inactivated DNA. Transformants of DNA V, examined for control purposes, were Met$^+$ as before. The tests did not show mixtures of Met$^-$ and Met$^+$, and thus failed to reveal different mechanisms of resistance, such as were found for the DNA donor strain examined in test 10 (Table 2). It was inferred, therefore, that resistance of all six gonococcal strains isolated during the period 1940–1946 was exclusively of the type Sul-r/Met$^-$.

TABLE 3. *Transformation of Neisseria meningitidis Ne 15 by DNA from N. gonorrhoeae*[a]

| DNA prepn[b] | Transformation (%) of resistance to sulfadiazine | | | |
|---|---|---|---|---|
| | 20 μg/ml | 100 μg/ml | 150 μg/ml | 200 μg/ml |
| VI | 0.0492 | 0.0402 | 0.0212 | 0.0009 |
| VII | 0.0613 | 0.0499 | 0.0237 | 0.00022 |
| VIII | 0.0024 | 0.0018 | 0.0016 | 0.00002 |
| IX | 0.0060 | 0.0041 | 0.0035 | 0.00008 |
| X | 0.0071 | 0.0040 | 0.0026 | 0.00009 |
| XI | 0.0438 | <0.000005 | <0.000005 | <0.000005 |
| V[c] | 0.0380 | <0.000005 | <0.000005 | <0.000005 |

[a] Reaction mixtures in HI-1 broth contained recipient cells ($2.0 \times 10^7$ colony-forming units/ml; suspension prepared from 12-hr HIY-1 agar culture) with 10.0 μg of DNA/ml. Reaction at 35 C was terminated by deoxyribonuclease after 20 min.

[b] Details, Table 1.

[c] Str-r transformation (0.032%) obtained in this test.

*Examination of Sul-r/Met$^-$ strains of Ne 15.* Results described for this class of transformants suggested that the two phenotypic properties, sulfonamide resistance and requirement for methionine, were acquired concurrently. To obtain insight into the relationship, various Sul-r/Met$^-$ strains were examined to determine which step in the biosynthetic pathway was affected. Also, the Met$^-$ strains were used as recipients in transformation tests to see whether their biosynthetic defects could be corrected by genetic information derived from various other Met$^-$ strains. Recovery of Met$^+$ transformants in such crosses testified to the nonidentity of the genetic alterations underlying the methionine requirement. Furthermore, the Met$^+$ transformants so obtained were tested on sulfadiazine-agar to determine whether loss of the methionine requirement was accompanied by loss of resistance.

*Growth response to methionine and possible precursors.* Methionine biosynthesis by various members of *Enterobacteriaceae* involves a series of reactions leading from homoserine and cysteine

to cystathionine. Cleavage of cystathionine yields homocysteine. Three genetic loci govern the structures of three enzymes which catalyze these reactions. Mutations affecting any of these loci result in a nutritional requirement for methionine, which can be replaced by homocysteine (14, 20, 36). The final reaction of the biosynthetic sequence involves the methylation of homocysteine to yield methionine. This step in *E. coli* and *Salmonella typhimurium* is a complex one which involves methyltetrahydrofolates as the immediate sources of the transferred methyl group (6). Auxotrophic mutants blocked in the performance of the terminal step show a methionine requirement which cannot be replaced by any of the previous compounds in the series (6, 14, 36).

The following Sul-r/Met$^-$ transformants of Ne 15 were tested: isolates from sulfadiazine (20 $\mu$g/ml)-agar of tests 8, 9, 10 (Table 2), and from tests with DNA VI (isolates g2, g52), VII (g54), VIII (g22), IX (g7), X (g55), and XI (g58). Also, six Ne 15 Sul-r/Met$^-$ spontaneous mutants were examined: M1, M33, M54, M58, M61, M69. Simultaneous tests were run with the parent strain to reveal possible growth inhibition by the various compounds. Light suspensions in defined fluid were streaked on defined agar separately supplemented with L-methionine (5.0 to 0.0005 mM), L-homoserine (0.5, 0.05 mM), L-cysteine (0.5, 0.05 mM), DL + allo cystathionine (0.5, 0.05 mM), L-homocysteine (0.5, 0.2, 0.1, 0.05 mM), L-homocystine (0.5 to 0.02 mM), L-cystine (0.5, 0.05 mM), vitamin $B_{12}$ (0.005, 0.00005 mM), or *S*-adenosyl-L-methionine (0.2, 0.1, 0.02 mM).

Of these supplements, only methionine promoted significant growth of Sul-r/Met$^-$ colonies on defined agar. Trace activity observed for *S*-adenosylmethionine was attributed to methionine, arising by decomposition. Furthermore, PAB, vitamin $B_{12}$, and folic acid (diffusing from separate discs saturated with 1 mg/ml solutions) did not increase the growth of Sul-r/Met$^-$ strains on defined agar with 0.005 mM methionine, a suboptimal concentration.

Some of the compounds restricted or delayed growth of the Met$^+$ strain. After 24-hr incubation, colonies were either absent or small on the highest concentrations listed of cysteine (also cysteine plus homoserine, each at 0.25 mM), homocysteine, homocystine, and *S*-adenosylmethionine. No effect was found with lower concentrations. Methionine did not affect colonial growth of Ne 15 Met$^+$ at concentrations below 0.05 mM; however, above 0.5 mM, a temporary inhibition was evident. This inhibitory effect, which was marked with 5.0 mM methionine, resembles the regulation of methionine biosynthesis in *E. coli*, in which methionine represses the formation of enzymes required for its own synthesis (21, 33).

Growth of Sul-r/Met$^-$ strains on defined agar was optimal with methionine at 0.1 to 0.5 mM. As with the parent strain, their growth was inhibited progressively by concentrations higher than 1.0 mM. At suboptimal concentrations, the size of colonies was a sensitive indicator of available methionine [including that amount which might be introduced as a contaminant of "pure" amino acids (9, 27)]. Colonies on defined agar with 0.0005 mM methionine were barely visible after incubation for 24 hr, and thereafter increased in size only slightly if at all. No isolated colonies were macroscopically visible in 24 hr on unsupplemented defined agar cultures of any Sul-r/Met$^-$ strain, but after longer incubation microcolonies could be detected with 4 $\times$ magnification. The strains differed from one another only slightly in regard to the size of these microcolonies, although their sulfadiazine resistances differed considerably.

In a further attempt to discern some relation between amount of methionine required and concentration of sulfadiazine tolerated, strains of differing resistance were streaked on defined agar lacking serine but containing five different concentrations of methionine alone or in combination with sulfadiazine (0.05 and 0.1 $\mu$g/ml, separate series). A concentration of 0.05 $\mu$g of sulfadiazine per ml in the defined agar, either with or without methionine, was sufficient to inhibit growth of the parent strain. (At lower concentrations of sulfadiazine, methionine exerted an antagonizing action, as expected.) All of the Sul-r/Met$^-$ strains could tolerate 0.05 and 0.1 $\mu$g of sulfadiazine per ml. (Some were inhibited by 0.5 $\mu$g/ml.) Their colony size was determined, as on drug-free medium, by time of incubation and concentration of methionine. Thus, the challenge of a low dose of sulfadiazine did not affect the degree of response to methionine of the various Met$^-$ strains, though they differed in degree of resistance.

*Transformation of Met$^-$ strains.* To obtain basic knowledge of the particular *met* locus which controls the Sul-r/Met$^-$ phenotype, a preliminary study was made of spontaneous mutants of strain Ne 15. Colonies, picked from sulfadiazine-containing HIY-1 agar, were subcultured on sulfadiazine-free agar and screened for methionine requirement and genetic stability. DNA was extracted from six of these Sul-r/Met$^-$ mutants. M1 and M33 were strains which had been isolated from agar containing 20 $\mu$g of sulfadiazine per ml, M54 and M58 were from 50 $\mu$g/ml, and M61 and M69 were from 100 $\mu$g/ml. All six DNA prepara-

tions had transforming activity for the parent strain Ne 15; as expected, transformants were exclusively Sul-r/Met$^-$.

Each DNA was examined in quantitative transformation tests (method A, described in Materials and Methods), with suspensions of each of the six Sul-r/Met$^-$ strains serving as recipient. Such tests should yield prototrophic recombinants if the *met* locus involved is genetically complex, as, for example, has been shown for other *met* loci (36) and the *amiA* region (13). Thus, information needed to correct the genetic defect and restore the biosynthetic function of one Sul-r/Met$^-$ mutant can be supplied by transforming DNA from a differently defective Sul-r/Met$^-$ strain, but not from an identical mutant. The results (Table 4) confirmed this expectation. Each DNA showed transforming activity for practically all recipients except the one from which the DNA was obtained. That is, DNA from one mutant provided no information to repair the defect of the same mutant because the genetic alteration was identical; however, it did furnish information to other phenotypically similar mutants, indicating the nonidentity of their mutant sites.

Two additional DNA preparations examined in other tests (7, 8, Table 2) were included in all these experiments (Tables 4, 5). The *str* gene provided a reference marker outside the *met* locus for measuring competence of the strain. DNA from the Sul-r20 Met$^+$ strain was used to determine the efficiency of the process of genetic elimination of the mutant site by the wild-type *met* locus. The frequency of transformation of Met$^-$ recipients by this preparation (one-point cross) was always higher than that found in tests of the DNA from Met$^-$ strains (two-point crosses), implying some degree of linkage between the mutant *met* sites of donor and recipient (13). Close linkage of the mutant sites of M54 and M58 was indicated by the very low frequencies of reciprocal transformation. However, nonidentity of these sites was revealed by other tests with a Sul-r/Met$^-$ recipient (M113) which was transformed by DNA from M54 but not from M58.

Strain Ne 15 Sul-r/Met$^-$ Str-r 64 was a multistep Sul-r mutant; that is, its high degree of sulfadiazine resistance was the result of multiple independent mutations. Its responses as recipient ("64") are given in Table 4. Its DNA, which transformed all strains to streptomycin resistance, likewise elicited Met$^+$ transformants from M1, M33, M54, and M58. However, Met$^+$ transformants of M69 were very rare, and they were not detected with M61, suggesting that the altered sites of these mutants might lie close to or overlap those of "64." Furthermore, Met$^+$ transformants of "64" were not detected after treatment with DNA (result not tabulated) from a multistep Sul-r/Met$^-$ strain of *N. perflava* Ne 16 (the DNA used in test 9, Table 2). Prototrophic transformants were elicited, however, in the approximately reciprocal test performed with "64" DNA and the recipient strain p8(16), which originated as a

TABLE 4. *Numbers of prototrophic transformants obtained in tests of various Neisseria meningitidis Ne 15 Sul-r/Met$^-$ recipients treated with DNA from the same strains*

| DNA prepn | Recipient strains[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | M1 | M33 | M54 | M58 | M61 | M69 | "64" | p8(16) |
| M1 | 0 | 80 | 16 | 53 | 122 | 40 | 4 | 32 |
| M33 | 34 | 0 | 36 | 91 | 147 | 76 | 20 | 76 |
| M54 | 14 | 120 | 0 | 0.4 | 113 | 37 | 5 | 79 |
| M58 | 16 | 80 | 0.4 | 0 | 105 | 45 | 12 | 78 |
| M61 | 34 | 114 | 11 | 62 | 0 | 7 | 0 | 32 |
| M69 | 25 | 143 | 17 | 89 | 26 | 0 | 0 | 28 |
| Ne 15 Sur-r/Met$^-$ Str-r 64 | 101<br>3,514[b] | 641<br>4,246[b] | 34<br>1,611[b] | 234<br>3,615[b] | 0<br>1,960[b] | 0.2<br>2,260[b] | 0<br>—[c] | 596<br>2,196[b] |
| Ne 15 Sul-r20 Met$^+$ 16 | 1,568 | 1,816 | 456 | 2,010 | 1,125 | 646 | 692 | 3,269 |

[a] Each column gives results (expressed as number of transformants/$10^6$ treated cells) of tests of one recipient strain with eight DNA preparations. Reaction mixtures in methionine-free defined fluid contained cells plus 5 $\mu$g of DNA/ml; deoxyribonuclease was added after 30 min.

[b] Str-r transformants. All other transformants were Met$^+$.

[c] Recipient was Str-r, and was the donor strain for the DNA used in this test.

TABLE 5. *Numbers of prototrophic transformants obtained in tests of various Neisseria meningitidis Ne 15 Sul-r/Met⁻ recipients treated with DNA from Sul-r/Met⁻ Neisseria*

| DNA prepn | Recipient strain | | | | | | |
|---|---|---|---|---|---|---|---|
| | g45(VI) ($6.9 \times 10^6$)[a] | g17(VII) ($7.4 \times 10^6$) | g3(VIII) ($34 \times 10^6$) | g4(IX) ($28 \times 10^6$) | g47(X) ($20 \times 10^6$) | g49(XI) ($29 \times 10^6$) | 1(64) ($7.8 \times 10^6$) |
| VI | 0 | 232 | 0 | 0 | 0 | 483 | 71 |
| VII | 1 | 0 | 0 | 0 | 0 | 32 | 0 |
| VIII | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| IX | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| X | 0 | 5 | 0 | 0 | 0 | 0 | 0 |
| XI | 1 | 125 | 0 | 0 | 0 | 0 | 0 |
| M1 | 15 | 21 | 0 | 0 | 0 | 201 | 236 |
| M33 | 60 | 118 | 0 | 0 | 0 | 452 | 453 |
| M54 | 46 | 79 | 0 | 0 | 0 | 344 | 185 |
| M58 | 60 | 101 | 0 | 0 | 0 | 242 | 203 |
| M61 | 17 | 3 | 0 | 0 | 0 | 10 | 0 |
| M69 | 14 | 14 | 0 | 0 | 0 | 78 | 0 |
| Ne 15 Sul-r/Met⁻ Str-r 64 | 170<br>11,970[b] | 0<br>20,330[b] | 0<br>17,320[b] | 0<br>19,700[b] | 0<br>13,970[b] | 0<br>12,030[b] | 0<br>12,360[b] |
| Ne 15 Sul-r20 Met⁺ 16 | 7,660 | 13,380 | 2,800 | 3,110 | 3,960 | 3,170 | 7,920 |

[a] Total number of treated recipient cells. Results of tests in defined fluid (containing $10^{-6}$ M methionine) with one cellular suspension, samples of which were treated with the various DNA preparations (10 μg/ml).

[b] Str-r transformants. All other transformants were Met⁺.

Sul-r/Met⁻ transformant of Ne 15 treated with *N. perflava* DNA (as in test 9, Table 2). Thus, multistep Sul-r mutants were not necessarily alike in their ability to receive or to provide information affecting this *met* locus.

Transformation tests were performed by using, as recipients, Sul-r/Met⁻ transformant strains which were isolated in tests (comparable to those of Table 3) of Ne 15 cells with the gonococcal DNA preparations VI-XI. Work with these recipients was complicated by their tendency to lose viability in the methionine-free defined fluid reaction mixtures. The decline of viability began after about 30 to 40 min and appeared to be a function of the initial numbers of cells, as if leakage of nutrients from cells of a large population provided some protection. Therefore, to test one cellular suspension against 15 to 20 DNA preparations without invalidating the transformation results by the lethality reaction, a more rapid, semiquantitative assay was used (method B, Materials and Methods), except for the two control tests (bottom of Table 5) which yielded numerous transformation colonies. Also, a minimal concentration of methionine ($10^{-6}$ M) was included in the defined fluid of the reaction mixtures. Met⁺ spontaneous mutants were not observed on any methionine-free agar plates inoculated with these recipients which had been exposed to deoxyribonuclease-treated DNA.

The transformation pattern shown by each of these Sul-r/Met⁻ recipients in two-point crosses clearly distinguished the genetic defects of DNA VI, VII, and XI (Table 5). DNA VIII, IX, and X were different from the others, but resembled one another. [Two of these gonococcal strains were separate isolates from the same patient (Table 1).] Genetic information was neither given by these gonococcal DNA preparation [except DNA X in the reaction with g17 (VII)], nor received by the Ne 15 transformants which carried their defects. However, as expected, these defects could be eliminated by transformation with DNA having the wild-type locus (Ne 15 Sul-r20 Met⁺ 16).

The pattern of response obtained with recipient "64," discussed in connection with Table 4, was similar to that of recipient 1(64), shown in Table 5. Another recipient 2(64) also was similar. As strains 1(64) and 2(64) were derived by transformation of Ne 15 with DNA from "64" (test 8, Table 2), the *met* lesions of all three should be identical. Such findings provide further justification for using results obtained with transformants as a basis for inference concerning genotypes of DNA donors.

*Restoration of the Sul-s phenotype.* The reaction of *N. meningitidis* Ne 15 cells (Sul-s Met⁺) with *N. gonorrhoeae* DNA VI or DNA VII resulted in transformants resistant to 150 μg/ml or higher

concentrations of sulfadiazine (Table 3), all of which were $Met^-$. Cells transformed by DNA VII served as recipients in a subsequent transformation reaction with DNA VI, and yielded $Met^+$ transformants (Table 5). If the resistance and methionine requirement are dual effects of one underlying genetic change, as proposed, then the return to prototrophy should be associated with a restoration of susceptibility.

To investigate this relationship, $Met^+$ colonies picked from the transformation plates were subcultured once on HIY-1 agar. Diluted suspensions prepared from young cultures were quantitatively plated by the overlay method in sulfadiazine-free HIY-1 agar and in agar containing 20 $\mu$g of sulfadiazine per ml. No growth in the latter (or, at most, rare Sul-r mutants) compared with essentially confluent growth in the former, testified that the prototrophs were susceptible to this level of sulfadiazine. The method was not designed to answer the technically more difficult question whether the level of susceptibility was identical to that of the parent, but rather to obtain unequivocal evidence that the population was susceptible to 20 $\mu$g of sulfadiazine per ml. This was demonstrated, without exception, for over 100 $Met^+$ transformant colonies, representing most of the possible combinations of $Met^- \times Met^-$ indicated in Tables 4 and 5.

## DISCUSSION

Pathogenic strains of *Neisseria* have encountered the challenge of sulfonamide treatment of their human hosts on innumerable occasions. Various possible ways were suggested (7) by which bacteria might become resistant to the antibacterial activity of drugs. Two different mechanisms were found among the 11 clinical isolates examined here.

One type of resistance was demonstrated for the six *N. gonorrhoeae* strains isolated during the period 1940–1946 when sulfonamide therapy was standard. Their sulfonamide resistance was the result of genetic alteration of a particular *met* locus. This inference is based on the finding that transforming DNA from each introduces sulfadiazine resistance and a requirement for methionine (Sul-r/$Met^-$) concurrently into a meningococcal recipient, which before transformation was sulfonamide-susceptible and capable of growth in defined medium without supplemental methionine (Sul-s $Met^+$). When a Sul-r/$Met^-$ transformant is used as recipient in a transformation test with DNA from a different Sul-r/$Met^-$ strain, Sul-s $Met^+$ transformants are recovered. The Sul-r/$Met^-$ transformants are enabled to grow in media containing elevated concentrations of sulfadiazine, though not as luxuriantly as in the absence of the drug. Gonococci having these characteristics would be able to incite infection as usual, as the host furnishes required nutritional factors. Though the strains might be considered only partially resistant on the basis of in vitro tests, their survival in the treated host must have been facilitated by the purulent process, which tends to reduce the antibacterial activity of sulfonamide (41). Therapeutic failures were recorded in more than 75% of cases of gonorrhea treated with sulfonamides during 1945–1948 (31, 37). Within a decade of the advent of penicillin therapy, however, sulfonamide-resistant gonococci were reduced to their former low incidence (31, 37) because they were as susceptible to penicillin as were other gonococci.

The four clinical isolates of *N. meningitidis* exhibited a different type of sulfadiazine resistance, which was not associated with increased auxotrophy. Although the Sul-r/$Met^-$ property is acquired readily by meningococci as a result of spontaneous mutation or by transformation, the degree of resistance afforded is less than that characteristic of the four Sul-r $Met^+$ strains isolated from patients with meningitis. These are presumably representative of the highly resistant group B meningococci, which first attracted attention in 1963 (28) and subsequently have become relatively prevalent in some areas of the United States (26).

The enzymatic basis of sulfonamide resistance in *Neisseria* has not been established. However, evidence available from other investigations suggests that folate compounds are ultimately involved in both types of resistance described here. Thus, work with cell-free enzymes of *E. coli* has revealed that sulfonamides inhibit folate biosynthesis by competing with PAB as substrate for the enzyme system that catalyzes the formation of dihydropteroic acid from PAB and 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine (1). Furthermore, enzymes extracted from sulfonamide-resistant mutants combine with sulfonamides less readily than does the corresponding enzyme extracted from the parent strain (30). Sulfonamide resistance arising by mutation in *Diplococcus pneumoniae* also was shown (39) to be due to structural alteration of a PAB-utilizing enzyme, and transformation methods were applied in genetic analysis of the resistant mutants (17). Similarly, the Sul-r $Met^+$ meningococcal strains may possess genetically altered enzymes for catalyzing this step of folate biosynthesis.

In enteric bacteria, the conversion of homocysteine to methionine is a complex reaction which involves the creation of the methyl group as methyltetrahydrofolate, and its subsequent transfer to homocysteine by either of two enzyma-

tic mechanisms (6). The *met* locus affected in *N. meningitidis* resembles the *metF* locus of *S. typhimurium* (36) and *Proteus mirabilis* (14) in that the requirement is satisfied by methionine only. *S. typhimurium metF100* lacks $N^5N^{10}$-methylenetetrahydrofolate reductase, which catalyzes the reduction of $N^5N^{10}$-methylenetetrahydrofolate to $N^5$-methyltetrahydrofolate (6). If the Sul-r/Met$^-$ strains of *Neisseria* are genetically similar to *metF*, their defects likewise must involve alterations of the corresponding reductase.

Whether meningococcal auxotrophs blocked at other steps of the methionine biosynthetic pathway are also sulfadiazine-resistant is not known yet. With *E. coli* mutants auxotrophic for homocysteine or for vitamin $B_{12}$, the minimal inhibitory concentration of sulfathiazole showed a 20-fold increase in the presence of $B_{12}$ or methionine (8). [The relation between vitamin $B_{12}$ and methionine has been clarified recently; *see* Cauthen et al. (6).] Possibly, any genetic event which imposes a requirement for exogenous methionine simultaneously confers some degree of sulfonamide tolerance by exerting a sparing effect on the available single-carbon units. Such a change, simultaneously affecting two properties, is not unique. Recent reports have documented the concurrent appearance of resistance to aminopterin and a specific sensitivity (34), and of resistance to other inhibitors of dihydrofolate reductase concurrently with nutritional requirements for certain end products of biosyntheses involving tetrahydrofolate-containing coenzymes (35).

As a selective agent for auxotropic mutants of *Neisseria*, sulfadiazine appears to be quite specific for spontaneous mutations which affect the particular locus corresponding to *metF* of *Salmonella* (36). This locus of *Neisseria* was revealed to be complex, having a number of sites which undergo recombination. Additional evidence that the number of sites is large is provided by results of transformation tests (to be described elsewhere) with 89 Sul-r/Met$^-$ spontaneous mutants. These were used as recipients in two-point crosses with six DNA preparations (Table 4) from Sul-r/Met$^-$ mutants; the transformation patterns indicated that not more than 3 of the 89 were possibly the same as any of the 6 DNA donor mutants. This *met* locus is of considerable interest, therefore, because it promises to be a powerful tool for study of genetic fine structure of members of *Neisseria*, and of genetic homology. The data presented here (Tables 4 and 5), furthermore, reveal a correspondence of *metF* loci of *N. meningitidis*, *N. gonorrhoeae*, and *N. perflava*.

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