drug was also variable. For example, the whole brain values for 5-HT increase by 30 percent for Hormone Assay rats and 35 percent for Simonsen rats. However, the brain area changes range from an increase of + 13 percent for medulla of the Hormone Assay rats to + 57 percent for the hypothalamus of these rats. In the case of NE, the increases in whole brain levels are + 18 percent for the Hormone Assay rats and + 9 percent for the Simonsen rats. In contrast, the changes in NE in brain areas range from no change (actually a 1 percent decrease) in medulla of the Simonsen rats to an increase of + 45 percent in the cerebellum of Simonsen rats. The whole brain values most closely compare to the effects in cerebral hemispheres, the largest portion (by weight) of the whole brain.

These results contain implications of considerable significance to workers examining interactions of drugs with brain biogenic amines, and may serve to explain some of the apparent discrepancies that exist in the literature. As previously mentioned (*3*), differing amine levels have been reported in various species and strains of animals. However, no attention has been paid to possible differences within the same strain. For example, these results would seriously question the validity of extrapolating data obtained on biochemical effects of drugs influencing brain 5-HT and NE from one supply of rats to another, even though both are from the same strain. Similar questions might well be raised regarding attempts to relate biochemical data on the amines obtained in one laboratory with drug effects on animal behavior obtained in another.

The precise reason for the differences in basal levels is as yet unknown and will require further study. However, the similarity in response to pargyline suggests similarity in rates of biosynthesis and degradation of the amines. Thus, the marked differences in basal levels may reflect differences in storage sites.

FRANCIS P. MILLER
RAYMOND H. COX, JR.
ROGER P. MAICKEL

*Laboratory of Psychopharmacology, Departments of Pharmacology* and *Psychology, Indiana University, Bloomington 47401*

**References and Notes**

1. B. M. Twarog and I. H. Page, *Amer. J. Physiol.* **175**, 157 (1953); D. F. Bogdanski, A. Pletscher, B. B. Brodie, S. Udenfriend, *J. Pharmacol. Exp. Ther.* **117**, 82 (1956).
2. U. S. Von Euler, *Acta Physiol. Scand.* **12**, 73 (1946); M. Vogt, *J. Physiol. (London)* **123**, 451 (1954); P. A. Shore and J. S. Olin, *J. Pharmacol Exp. Ther.* **122**, 295 (1958).
3. D. F. Bogdanski, L. Bonomi, B. B. Brodie, *Life Sci.* **2**, 80 (1963); G. R. Pscheidt, *Comp. Biochem. Physiol.* **24**, 249 (1968).
4. R. P. Maickel, R. H. Cox, Jr., J. Saillant, F. P. Miller, *Int. J. Neuropharmacol.* **7**, 275 (1968).
5. Supported by PHS grants MH-06997 and MH-14658.

24 July 1968

## Prophage S2 Mutants in Haemophilus influenzae: A Technique for Their Production and Isolation

Abstract. *A procedure utilizing nitrosoguanidine has been developed to produce defective and temperature-sensitive mutants of prophage (S2) in lysogenic* Haemophilus influenzae. *The system should be generally applicable to all temperate phage systems. At saturating concentrations of phage DNA, more than 25 percent of recipient mutant lysogenic bacteria can be transformed to the wild type.*

Mutants of virulent bacteriophage are ordinarily obtained by treating free phage (*1*) or infected cells (*2*) with mutagen. The mutants so obtained fall mainly into three categories—(i) plaque-morphology mutants, (ii) mutants having distinct physical characteristics (such as an altered bouyant density), and (iii) conditional-lethal mutants (including temperature-sensitive and host-range mutants). Totally lethal mutations cannot be obtained (except perhaps transiently) by mutations originating in the phage unless the lethal mutant is not dominant, and unless it can be separated from a complementary phage after mixed infection (*3*). However, it is possible to propagate all types of mutations, including lethals, in temperate bacteriophage if the mutations are introduced into the prophage, providing only that the ability to be maintained as a prophage is not lost. This report describes a technique by which lysogenic cells of *Haemophilus influenzae* are mutagenized with nitrosoguanidine and the mutant S2 prophage is selected by replica plating of the lysogenic cells onto plates overlaid with indicator bacteria (lawn plates). Additional advantages of this technique are the potent mutagenicity of nitrosoguanidine in cells (compared with its feeble effects on free phage); quick and easy scanning for mutants, yielding many kinds of mutants in large numbers; and the general applicability of the technique to all temperate phage systems, including those for which no host suppressor mutants are yet known.

A stock of mutated lysogenic cells was prepared according to the following procedure [which is an adaptation of the procedures of Galinski and Goodgal, and Michalka and Goodgal (*4*)].

1) Wild-type lysogenic cells were grown to about $10^9$ per milliliter (measured by turbidity) at 37°C in 75 ml of sBHI [brain-heart infusion broth (Difco) supplemented with 10 $\mu$g of hemin and 2 $\mu$g of nicotinamide adenine dinucleotide (NAD) per milliliter].

2) The cells were washed once in saline, and resuspended in 1/20th volume (3.75 ml) of saline-acetate buffer (which consists of 1 volume of 0.1*M* sodium acetate, *p*H 5.0, added to 5 volumes of saline).

3) A solution of nitrosoguanidine (0.75 ml) [2 mg of *N*-methyl-*N*-nitroso-*N'*-nitro-guanidine (*5*) per milliliter in saline-acetate buffer] was added to 3.75 ml of a cell suspension in saline-acetate buffer. The final *p*H of this suspension is about 6.0.

4) Suspended cells were incubated with mutagen for 10 minutes at 37°C, and the reaction was terminated by the addition of an equal volume of ice-cold BHI broth (no hemin or NAD). The cells were centrifuged and washed with more cold BHI broth.

5) Following resuspension in 100 ml of fresh sBHI broth, cells were incubated with shaking at 31°C for 2½ hours (four to five generations).

6) The cells were centrifuged, resuspended in BHI broth plus 15 percent glycerol, and stored at − 70°C in several small portions.

7) Working stocks of mutated cells were prepared as needed from the above by thawing a portion, diluting into fresh sBHI broth, and growing for four or five generations at 31°C. These cells were resuspended in fresh BHI broth plus 15 percent glycerol and stored in small portions at −70°C.

Mutant prophages were identified by replica plating from "working stock" as follows: (i) Plates of sBHI agar were spread with diluted working stock of mutated lysogenic cells so that each plate received about 150 to 300 viable cells. (ii) The plates were incubated at 31°C for 24 hours, or until colonies reached about 1 to 2 mm in diameter.

(These plates were the "masters," and could be stored at room temperature for a day or two before use.) (iii) The replica pad consisted of brushed felt stretched over a wooden support. Four sheets of Whatman No. 1 filter paper (circular) under the felt helped to absorb excess moisture and improved the clarity of the replicas. (iv) The pads were pressed into the master and imprinted as follows: Print No. 1, onto an sBHI agar plate (incubated at 31°C); print No. 2, onto an sBHI agar plate overlaid with $5 \times 10^7$ indicator bacteria in 4 ml of 0.6 percent BHI agar (incubated at 40°C); and print No. 3, onto an sBHI agar plate overlaid with indicator as above (incubated at 31°C). After overnight incubation the plates were compared. Clear areas (plaques) found on the plates overlaid with indicator cells corresponded to the position of bacterial colonies on the sBHI agar plate without indicator bacteria. The resolution of the colony images on the lawn plates was sufficiently good to allow rapid scanning of replicas from plates having up to about 300 colonies. Temperature-sensitive prophage mutants produce a corresponding plaque at 31°C, but not at 40°C; defective prophage mutants produce no corresponding plaque at either temperature. No mutants were found that produce plaques at 40°C, but not at 31°C—temperature reactivated defectives—although such mutants are conceivable. Colonies for which plaques were missing at either one or both temperatures were picked for further examination.

A preliminary verification of the mutants was performed by stabbing the mutant colony with a sterile toothpick and transferring it to the surface of two sBHI agar plates overlaid with indicator bacteria. These plates were incubated at 31° and 40°C, respectively. Colonies that gave the original mutant response were repicked from single colonies and tested a third time in the same way. The mutants obtained by this procedure might be expected to fall into three categories—mutants whose loci are within the prophage region itself; mutants whose loci are outside the prophage region, but which influence the induction of the prophage (*6*); and mutants in which the bacteria have been cured of their prophage.

Temperature-sensitive mutants are obviously excluded from the third category. In order to demonstrate that such mutants fall into the first category, it is sufficient to show that the free phage produced by temperature-sensitive prophage mutants is temperature sensitive. All 20 of these mutants that we obtained do produce temperature-sensitive phage.

Table 1. Transformation of mutant prophage S2 by phage and prophage DNA's.

| Source of DNA | Recipient cells (*H. influenzae*) | | | |
|---|---|---|---|---|
| | $S^r$(S2-D1)* | | $S^r$(S2-TS2)* | |
| | PFU as percent of viable cells | C-transformants as percent of viable cells † | PFU as percent of viable cells | C-transformants as percent of viable cells † |
| C 25 (nonlysogenic)† | 0.00 | 1.09 | 0.00 (40°C) | 0.38 (37°C) |
| $S^r$(S2-wt)* | .77 | | .60 (40°C) | |
| S2 (phage) | 12.7 | | 13.3 (40°C) | |
| Corrected PFU‡ | 28.2‡ | | 29.6 (40°C)‡ | |
| No DNA | 0.00 | | 45.0 (31°C)‡ | |
| | | | 0.00 (40°C) | |

* Cells carrying defective mutant phage, $S^r$(S2-D1); cells carrying temperature-sensitive mutant phage, $S^r$(S2-TS2); and cells carrying wild-type prophage, $S^r$(S2-wt). †C 25 DNA is extracted from nonlysogenic cells of *H. influenzae* which are resistant to the antibiotic cathomycin (novobiocin) in concentrations of 25 μg/ml. Transformants with this DNA are scored by challenging with concentrations of the antibiotic at 25 μg/ml. ‡ Corrections are based on the fact that only 45 percent of the prophage from the temperature-sensitive strain are actually induced to form plaques at 31°C in this assay. In other similar assays the wild-type prophage is induced with efficiencies ranging from about 40 to 70 percent of the viable cell number. The prophage is assayed by exactly the same technique as free phage, except that the plate is irradiated with ultraviolet light after 1½ hours of incubation.

The possibility that the defective mutants were cured cells was eliminated by two tests to determine whether (i) the mutant retains its immunity specificity to the bacteriophage, and (ii) the defective prophage is transformable to the wild type with high efficiency by DNA from wild-type phage, temperature-sensitive phage, or other mutant lysogenic bacteria, but not DNA from nonlysogenic bacteria. One of the defective mutants (out of the 50 we obtained) reverts relatively frequently to a temperature-sensitive prophage.

In the *H. influenzae*–S2 system the simplest proof that the locus of the defective mutants is in the prophage region, and not elsewhere in the cell, is the fact that these mutants can be transformed to the wild type at high efficiency by DNA from wild-type phage (S2) or prophage $S^r$ (S2-wt), but not by wild-type, nonlysogenic cell DNA (Table 1). ($S^r$ is the designation for a streptomycin-resistant strain of bacteria.) Nonlysogenic cells also can be transformed to wild-type, plaque-forming units (PFU) by DNA from wild-type phage or prophage, but nonlysogenic cells are transformed to PFU's with less than one-thousandth the efficiency of bacteria carrying a mutant prophage $S^r$ (S2-D1) or $S^r$ (S2-TS2).

Strains bearing mutant prophage may be transformed to the wild type with DNA from wild-type prophage, and with DNA from other mutants. In such transformations, prophage mutants behave exactly as other cell markers and have been used to construct a prophage map by transformation. Details of the prophage map will appear later.

Phage DNA transforms 10 to 20 times as many cells to wild-type prophage as does prophage DNA. This fact is related to the greater homogeneity of phage DNA compared to bacterial DNA. Previous work has shown that competent *H. influenzae* cells take up only five to ten molecules of DNA per cell, and that competition among the heterogeneous fragments of cell DNA reduces the maximum number of cells that are transformed to a given characteristic (*7*). At saturating levels of DNA, phage DNA would experience less competition than bacterial DNA and therefore would produce higher levels of transformation. More than 25 percent of the viable cells have been transformed to wild-type prophage with saturating concentrations of wild-type phage DNA.

JOHN W. BENDLER
SOL H. GOODGAL
*Department of Microbiology,*
*School of Medicine,*
*University of Pennsylvania,*
*Philadelphia 19104*

### References and Notes

1. E. Freeze, E. Bautz-Freeze, E. Bautz, *J. Mol. Biol.* **3**, 133 (1961).
2. E. Freeze, *ibid.* **1**, 87 (1959).
3. A. D. Kaiser and D. S. Hogness, *ibid.* **2**, 392 (1960).
4. J. M. Galinski and S. H. Goodgal; J. Michalka and S. H. Goodgal, unpublished data.
5. K & K Labs., Inc., Plainview, N.J.
6. E. P. Kirby, F. Jacob, D. A. Goldthwait, *Proc. Nat. Acad. Sci. U.S.* **58**, 1903 (1967).
7. S. H. Goodgal and R. M. Herriott, *J. Gen. Physiol.* **44**, 1201 (1961).
8. We gratefully acknowledge the technical assistance of Mrs. Nancy Stanton. This investigation was supported by research grant No. A1-04557 from the National Institute of Allergy and Infectious Diseases. J.W.B. is a Pennsylvania Plan Scholar.

25 July 1968; revised 4 September 1968