genus and if both homothallic and heterothallic species exist, then one might question the choice of such organisms for experimentation. These considerations must also be kept in mind during investigations of the problem of morphological variability.

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## Nitrous Acid Mutation of Transforming DNA: Consideration of Mode of Action

Abstract. *Apparently nitrous acid produces genetic alterations, expressed as antibiotic-resistant markers, directly on heat-denatured transforming DNA of* Haemophilus influenzae, *rather than producing DNA which acts as a nonspecific mutagen. The markers which arise as a result of treatment with nitrous acid behave similarly to naturally occurring antibiotic markers. In addition, data comparing the expression and replication of induced markers to natural markers suggest that the nitrous acid–induced markers express and multiply in the same fashion as do "normal" markers. Therefore, mutations which require additional time to produce a functional DNA by a base-pair substitution, or by replication of the introduced DNA, are not responsible for the mutants observed.*

Nitrous acid is a well-known mutagenic agent and has been employed successfully in a variety of systems (*1*). Horn and Herriott (*2*) showed that nitrous acid was mutagenic for transforming DNA obtained from *Haemophilus influenzae* when the DNA was heat-denatured prior to treatment and renatured after treatment. In that work (*2*) it was not clear whether nitrous acid produced new genetic information in the DNA or whether the treated DNA itself acted as a mutagenic agent in the host's genome.

We have attempted to decide between these alternative hypotheses and have investigated the mechanism of mutagenesis by nitrous acid treatment in the transforming system of *Haemophilus*. The occurrence of interspecific transformations between *H. influenzae* and *H. parainfluenzae* provided us with a useful tool (*3*). Since the interspecific transformation frequencies of certain markers were already known (*3*), we compared these with the transformation frequencies yielded by DNA treated with nitrous acid.

The competent cultures of *H. influenzae* (Rd) were prepared by the Cameron modification of the method of Goodgal and Herriott (*4*); this strain was obtained originally from Alexander and Leidy (*5*).

The *H. parainfluenzae* wild type was originally obtained from Leidy and was made competent by the procedure of Nickel and Goodgal (*3*). At times a 4-hour anaerobic incubation was used in place of an 8-hour period.

Transforming DNA's were prepared and purified according to the procedure of Marmur (*6*). Thermal denaturation, nitrous acid treatment, and transformation of *H. influenzae* have been described (*2*). Assays of the transformation of *H. parainfluenzae* have been reported (*3*).

Horn and Herriott (*2*) found that nitrous acid did not produce mutations in "native" DNA of *H. influenzae*. However, denatured DNA was modified by nitrous acid so that after renaturation and subsequent uptake of DNA by recipient cells, mutations were observed. Nitrous acid was not mutagenic for "native" *H. parainfluenzae* DNA. If the parainfluenzae DNA was heat-denatured before treatment and then renatured, there was new transforming activity upon introduction of this DNA into competent *H. parainfluenzae* cultures. Thus, *H. parainfluenzae* DNA behaves like *H. influenzae* DNA in its mutagenic response to nitrous acid.

The frequency of interspecific transfer of genetic markers between *H. influenzae* and *H. parainfluenzae* depends upon the markers transferred and the direction of transfer (*3*), but the amount of DNA transferred interspecifically does not differ from the amount transferred intraspecifically (*7*). It was, therefore, possible to test whether or not the mutagenicity of DNA treated with nitrous acid was a function of the amount of treated DNA taken up by a population of bacteria or a function of the specific marker integrated by a population undergoing transformation.

In Table 1 are shown the results of a typical experiment in which the recipient is the *H. influenzae* and treated DNA from either species is the donor. With *H. influenzae* DNA, that is homologous DNA, as the donor, there is a significant rise of streptomycin- and erythromycin-resistant colonies with the duration of nitrous acid treatment (Table 1). When the donor is *H. parainfluenzae* DNA treated with nitrous acid, however, there are no transformations arising from this DNA; the number of colonies remains essentially the same, representing only spontaneously occurring mutations.

If nonspecific mutagenic DNA were responsible for the observed mutations, heterospecific transformations should yield similar frequencies of mu-

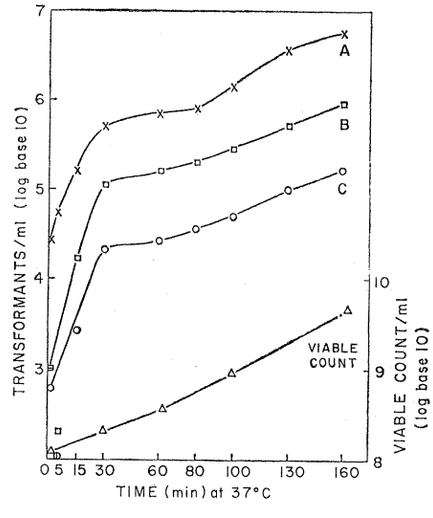


Fig. 1. Comparison of the expression of nitrous acid–induced transformants and "normal" transformants. *Haemophilus influenzae* competent cells ($1 \times 10^8$/ml) were exposed to (*A*) 0.2 μg of DNA per milliliter, from streptomycin-resistant cells, (*B*) 0.5 μg/ml of denatured and renatured DNA from streptomycin-resistant cells, and (*C*) 0.5 μg/ml of nitrous acid–treated DNA from streptomycin-sensitive cells. Samples were taken, diluted, and plated immediately with 8 μg/ml of streptomycin agar. Background has been subtracted.

Table 1. Assay of nitrous acid–treated DNA from *H. influenzae* (wild type) and *H. parainfluenzae* (wild type), respectively, as donors in competent recipient cells of *H. influenzae*. *Haemophilus influenzae* competent cells ($3 \times 10^8$/ml) were exposed to DNA (0.5 μg/ml) treated with nitrous acid for the times indicated, incubated at 37°C for 2 hours, diluted and plated with antibiotic containing agar (streptomycin at 8 μg/ml, and erythromycin at 6 μg/ml). Col, colonies; Trans, transformants.

| $HNO_2$ treatment (min) | Streptomycin-resistant colonies | | | Erythromycin-resistant colonies | | |
|---|---|---|---|---|---|---|
| | Col/plate* | Minus zero sample | Trans/ml† | Col/plate* | Minus zero sample | Trans/ml† |
| | | | Treated DNA, *H. parainfluenzae* | | | |
| 0‡ | 107 | 0 | $<10^2$ | 517 | 0 | <5 |
| 10 | 533 | 426 | $4.3 \times 10^4$ | 1490 | 973 | $4.9 \times 10^3$ |
| 30 | 1062 | 955 | $9.6 \times 10^4$ | 1996 | 1479 | $7.4 \times 10^3$ |
| 50 | 1384 | 1277 | $1.3 \times 10^5$ | 2206 | 1689 | $8.5 \times 10^3$ |
| 120 | 1432 | 1325 | $1.3 \times 10^5$ | 1780 | 1263 | $6.3 \times 10^3$ |
| 300 | 1610 | 1503 | $1.5 \times 10^5$ | 1661 | 1144 | $5.7 \times 10^3$ |
| | | | Treated DNA, *H. influenzae* | | | |
| 0 | 104 | 0 | NSI§ | 476 | 0 | NSI§ |
| 10 | 115 | 11 | NSI | 446 | −30 | NSI |
| 30 | 100 | −4 | NSI | 541 | +65 | NSI |
| 60 | 112 | 8 | NSI | 380 | +96 | NSI |
| 120 | 112 | 8 | NSI | 500 | +24 | NSI |
| 300 | 110 | 6 | NSI | 366 | −110 | NSI |

* Average number from two plates. † Numbers after zero sample is subtracted are multiplied by the dilution factor of the transformation mixture. This was $1 \times 10^2$ for streptomycin and $5 \times 10^9$ for erythromycin. ‡ Zero sample represents spontaneous mutation rate (*2*), which is subtracted from each sample. § No significant increase.

tants. However, as already shown, heterologous DNA is not effective in the production of streptomycin- and erythromycin-resistant transformants in *H. influenzae*, an argument against nonspecificity of mutation during the transformation process.

The frequencies of transfer of interspecific markers between *H. influenzae* and *H. parainfluenzae* vary. The frequencies of transfer of *H. parainfluenzae* streptomycin and erythromycin markers into *H. influenzae* represent values of the order of 1 percent of homospecific transfer, and the transfer of *H. influenzae* streptomycin markers to *H. parainfluenzae* is of the same order of magnitude (0.2 to 1 percent). In the case where *H. influenzae* erythromycin factor is transferred to *H. parainfluenzae* the frequency observed is approximately 10 percent of homospecific transfer. It was possible to evaluate the production of nitrous acid mutations as a function of the amount of material integrated for a specific region of the bacterial chromosome. This relation was determined from the numbers of mutations to streptomycin and erythomycin resistance produced by nitrous acid treatment of *H. influenzae* DNA as measured on *H. influenzae* and *H. parainfluenzae*.

With homologous *H. parainfluenzae* DNA as the donor, a significant number of transformations to both kinds of antibiotic resistance are produced (Table 2). With heterologous influenzae DNA, there appear to be no transformants to streptomycin resistance. On the other hand, in *H. parainfluenzae* recipient cells there is an increase in the appearance of erythromycin mutants obtained in *H. influenzae*. Here, then, is evidence which supports the notion that nitrous acid acts directly on the denatured DNA to produce mutations. The spontaneous rate of mutations is great enough to mask an increase in mutations of the order of 1 to 2 percent of the number seen homospecifically (*2*).

Some characteristic properties of transformed cells are (i) the time required to express the property transformed, (ii) the onset of duplication, and (iii) the rate of duplication of the transformed cell. The first of these properties, expression, cannot be precisely determined for antibiotic-resistance factors, but can be used to give a qualitative measure of expression by determining what fraction of a cell population has acquired the ability to grow in the presence of a selected concentration of antibiotic. To help elucidate the nature of the nitrous acid–induced mutation, we compared the expression of the "normal" transformants and that of nitrous acid–produced markers. The *H. influenzae* DNA (streptomycin-sensitive) was treated to produce mutations to streptomycin resistance, and its expression curve was compared to that of transformants resulting from a streptomycin-marked DNA which was derived from a nitrous acid–induced mutation (Fig. 1). There appears to be no significant difference with regard to the expression curves of the "normal" transformants and the transformants resulting from mutations induced by nitrous acid, that is, expression is complete within 30 minutes.

In addition to determining the expression of markers, it is possible to allow complete expression to occur and to examine the time and rate of duplication of newly transformed cells. If there is any delay in the time of replication of the nitrous acid–treated DNA, or if the treatment places restrictions on the rate of duplication, then these should become evident by

Table 2. Assay of nitrous acid–treated DNA from *H. influenzae* (wild type) and *H. parainfluenzae* (wild type), respectively, as donors in competent recipient cells of *H. parainfluenzae*. The procedure followed was the same as that for the experiment in Table 1 except for the use of $6.6 \times 10^8$ competent *H. parainfluenzae* cells and an incubation time of 3 hours before plating (streptomycin, 8 μg/ml; erythromycin, 4 μg/ml).

| $HNO_2$ treatment (min) | Streptomycin-resistant colonies | | | Erythromycin-resistant colonies | | |
|---|---|---|---|---|---|---|
| | Col/plate* | Minus zero sample | Trans/ml† | Col/plate* | Minus zero sample | Trans/mil/ |
| | | | Treated DNA, *H. influenzae* | | | |
| 0‡ | 16 | | | 21 | 0 | <5 |
| 10 | 47 | 31 | $3.1 \times 10^3$ | 170 | 149 | $7.5 \times 10^2$ |
| 30 | 130 | 114 | $1.1 \times 10^4$ | 356 | 335 | $1.7 \times 10^3$ |
| 60 | 171 | 155 | $1.6 \times 10^4$ | 486 | 465 | $2.3 \times 10^3$ |
| 120 | 152 | 136 | $1.4 \times 10^4$ | 472 | 451 | $2.3 \times 10^3$ |
| 300 | 202 | 186 | $1.9 \times 10^4$ | | | |
| | | | Treated DNA, *H. parainfluenzae* | | | |
| 0 | 20 | 0 | NSI§ | 22 | | |
| 10 | 19 | −1 | NSI | 45 | 23 | $1.2 \times 10^2$ |
| 30 | 26 | 6 | NSI | 90 | 68 | $3.4 \times 10^2$ |
| 60 | 21 | 1 | NSI | 78 | 56 | $2.8 \times 10^2$ |
| 120 | 25 | 5 | NSI | 39 | 17 | $8.5 \times 10^1$ |
| 300 | 13 | −7 | NSI | | | |

* Average number from two plates. † Numbers after zero sample is subtracted are multiplied by the dilution factor of the transformation mixture. This was $1 \times 10^2$ for streptomycin and $5 \times 10^9$ for erythromycin. ‡ Zero sample represents spontaneous mutation rate (*2*), which is subtracted from each sample. § No significant increase.

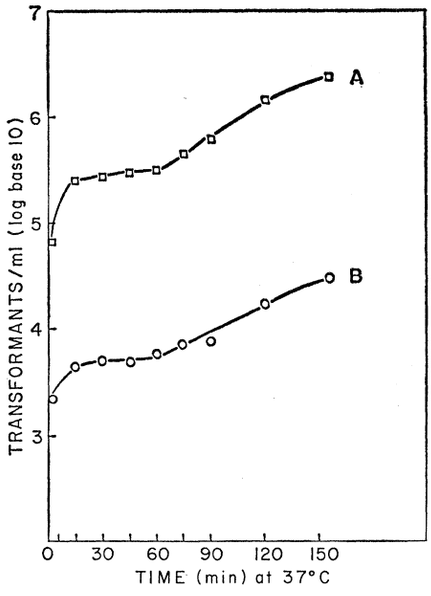


Fig. 2. Comparison of the replication of nitrous acid–induced transformants and "normal" transformants. *Haemophilus influenzae* competent cells ($1 \times 10^8$/ml) were exposed to (*A*) DNA (0.2 μg/ml) from streptomycin-resistant cells and (*B*) nitrous acid–treated DNA (0.5 μg/ml) from streptomycin-sensitive cells. Samples were taken, diluted, and plated with antibiotic agar and incubated for 2 hours at 37°C. Then agar containing streptomycin was added to select for streptomycin-resistant cells. The final concentration of streptomycin was 8 μg/ml. Background has been subtracted.

allowing the transformed cells to grow and measuring the increase in the number of transformants as a function of time. After the samples were taken and plated, time was allowed for all the transformants to express fully before they were challenged with the antibiotic. Transformants from both the untreated streptomycin-marked DNA and nitrous acid–induced mutants began to replicate after about 60 minutes (Fig. 2). Therefore, no additional time was required for the nitrous acid–induced characteristic to be replicated. The same results were found with the antibiotic markers novobiocin (0.5 μg/ml) and dalacin (8 μg/ml). In addition to the time of replication, the rate of replication was the same as the rate of growth on the whole cell population, that is, a generation time of about 30 minutes during the logarithmic phase of growth.

Thus, genetic markers are produced directly on the DNA in vitro during exposure to nitrous acid. During transformation these markers closely resemble the behavior of "normal" genetic markers. The expression curves and the time and rates of multiplication of transformants of "normal" and nitrous acid–induced markers are essentially identical. This evidence places certain restrictions on the base-transition hypothesis of nitrous acid mutagenesis in the present system. If we assume that transitions take place, these transitions must not delay the incorporation process and the replication of the incorporated entity; the transforming molecule containing deaminated bases recombines with the recipient DNA and produces a marker which is functional and normal in subsequent replications.

One possibility is that the mutations observed here are all deaminations of cytosine to uracil which, in turn, may behave like thymine with regard to its pairing function and its ability to transcribe. On the other hand, a deamination of adenine, resulting in hypoxanthine, would probably not be consistent with the results reported since hypoxanthine would be expected to function more like adenine than guanine. If such mutations occur in the system we have tested, they must be selected against.

It seems unlikely that the nitrous acid mutations represent deletions produced in solution, since these deletions would break denatured DNA and greatly lower the efficiency of renaturation. In addition, mutations to low levels of streptomycin, which have been tested, are revertible (*8*).

The finding that mutations or the change leading to mutations occur on heat-denatured DNA, which is presumably single stranded, and the fact that the onset and rate of replication of mutants and transformants are the same, lead to the conclusion that only one of the two strands of DNA is sufficient in the transformation process. A more extensive series of analogous experiments with heat-denatured and renatured DNA's lead to the same conclusion (*9*). These results agree with similar conclusions derived in completely different ways (*10*).

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## Tryptamine Oxidation by Extracts of Pea Seedlings: Effect of Growth Retardant β-Hydroxyethylhydrazine

Abstract. *β-Hydroxyethylhydrazine forces flowering of pineapple plants, possibly by reducing auxin concentration. A specific and sensitive assay showed that β-hydroxyethylhydrazine ($3.3 \times 10^{-7}$M) inhibits noncompetitively and almost completely the oxidation of tryptamine to indoleacetaldehyde by extracts of pea-seedling* (Pisum sativum *L. var. Alaska). The relative rate of oxidation of putrescine to $\Delta^1$-pyrroline compared to that of tryptamine by these extracts and the inhibition of both reactions by β-hydroxyethylhydrazine suggest that diamine oxidase is inhibited. β-Hydroxyethylhydrazine is a potent inhibitor in vivo of animal diamine oxidase.*

In 1955 Gowing and Leeper (*1*) found that β-hydroxyethylhydrazine (BOH) induces flowering in pineapple. The first action of this compound was retardation of stem elongation (*2*); then flowering was initiated. It has been suggested that this compound induces flowering by reducing the concentration of native auxin (*3*).

Although the pathways for biosynthesis of indoleacetic acid are not clearly established, one possible route in certain plants, such as pineapple, is by decarboxylation of tryptophan to tryptamine, which in turn may be oxidized to indoleacetaldehyde by diamine oxidase (*4*). Clarke and Mann (*5*) have found that tryptamine is converted to indoleacetaldehyde by diamine oxidase from pea seedlings.

The inhibitory effects of hydrazines on animal amine oxidases have been studied, yet little is known concerning the effects of these inhibitors on plant amine oxidase. However, Hill and Mann (*6*) reported changes in the absorption spectrum of purified pea-seedling diamine oxidase after addition of hydra-