INACTIVATION OF TRANSFORMING DEOXYRIBONUCLEIC ACID BY NITROUS ACID

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The mutagenic action in vitro of nitrous acid on viruses has now been well established. Mundry and Gierer (1958) found that nitrous acid treatment of ribonucleic acid from tobacco mosaic virus lead to a specific mutation. Litman and Ephrussi-Taylor (1959) reported that the transforming activity of Diplococcus pneumoniae deoxyribonucleic acid (DNA) was inactivated at almost entirely exponential rates. A mutagenic action of nitrous acid was also demonstrable but it seemed rather specific since not all the mutations could be induced for which assay techniques were available (in fact, only two instances were mentioned).

We have done similar experiments with transforming DNA of Haemophilus influenzae but we have not been able to detect any mutagenic action with respect to four different antibiotic resistances. The reason for this seemed clear upon further study of the mechanism of marker inactivation.

EXPERIMENTAL - Strains, media, and transformation techniques have been fully described (Stuy, 1961a, 1961b). The wild type sensitive strain Rd of H. influenzae was obtained from Dr. Grace Leidy (New York). It served as the (recipient) test strain. Antibiotic-resistant (mutant) strains (which carried the corresponding genetic markers) are here designated Rd/SM, Rd/Ka_1, and so on while their DNA extracts are termed DNA(SM), DNA(Ka_1) etc. DNA was incubated at 37 °C in 0.2 M

sodium nitrite dissolved in 0.2 M sodium citrate-citric acid buffer with a pH of 4.8.

RESULTS - (1) Inactivation. The streptomycin (SM), kanamycin (Ka_1), and cathomycin (Ca) resistance markers in the DNA studied were all located on one particle (Goodgal, pers. comm.) (Fig. 1). For a detailed description of this marker system, see Stuy (1961a).

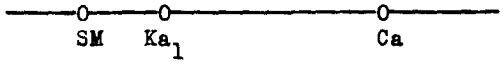

Fig. 1. The three-marker system

Inactivation of the three markers and all possible combinations proceeded almost exponentially (Fig. 2). Marker complexes were more sensitive than single markers. The sensitivity of the small complex SM-Ka_1 (slope of its inactivation curve) was smaller than the sum of the sensitivities of the SM and Ka_1 markers. This "overlapping" effect indicates that the induced damage can extend its influence over DNA regions greater than that between the two markers (or rather, mutated sites). This region is estimated as of the order of a few hundred nucleotide pairs (Stuy, 1961a). The sensitivities of the large complexes Ka_1-Ca and SM-Ca were about equal to the sums of the sensitivities of the component markers. This means that little or no chain breakages had occurred (Stuy, 1961a). The complex SM-Ka_1-Ca was slightly more sensitive than the complex SM-Ca suggesting that a fraction of the inactivated Ka_1 markers was incorporated into the genome of the recipient cells (if there had been no double recombination events).

(2) Mutagenic action. This was studied by treating DNA(Ery6) (1 μg/ml) under the same conditions. The erythromycin resistance marker served as an indicator to the extent of inflicted damage. Samples were withdrawn after intervals (up to 32 minutes) and dialyzed for four hours

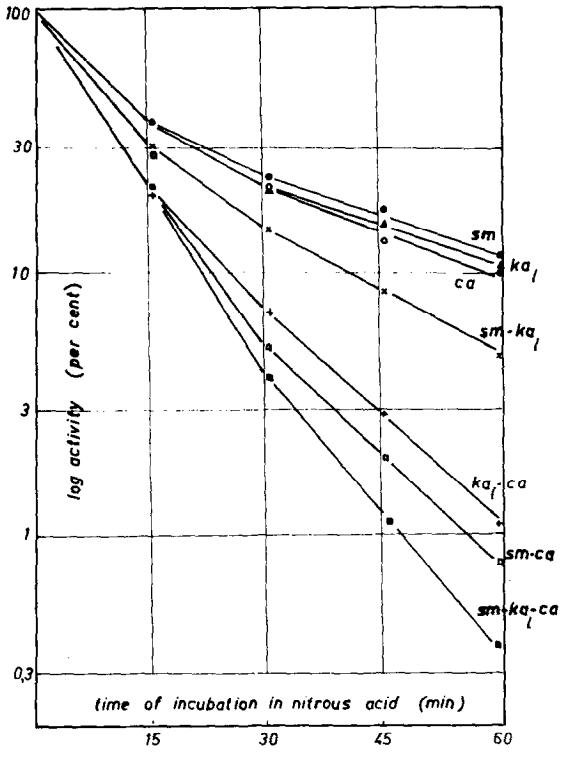

Fig. 2. Decrease in transforming activity of DNA(SM,Ka_1,Ca) due to exposure to nitrous acid. Medium: 0.2 M sodium nitrite in citrate buffer pH 4.8. DNA concentration: 4.2 µg/ml. Incubation at 37 °C. Zero time values in numbers of markers or complexes per ml of reaction mixture, times 10^{-8}; SM: 15; Ka_1: 15; Ca: 15; SM-Ka_1: 13; Ka_1-Ca: 9.0; SM-Ca: 7.0; SM-Ka_1-Ca: 6.3.

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against ice-cold citrate-saline (0.015 M and 0.15 M, respectively). Roughly 2 x 10^6 marker equivalents/ml were then mixed with 2 x 10^8 recipient Rd cells. After 30 minutes, 0.3 and 0.03-ml samples were plated and assayed for the presence of bacteria transformed to resistance against SM, Ka, Ca and viomycin. The untransformed cells gave rise to a background of spontaneous mutants: up to 20 colonies per plate depending upon the antibiotic applied. In no experiment were the observed numbers of colonies significantly greater than the back-

ground of mutants on the control plates. The lowest detectable frequencies were about 10^{-5} for the SM and Ca markers and 10^{-4} for the other two. In contrast to this, Litman and Ephrussi-Taylor (1959) reported frequencies up to 10^{-2}.

(3) Fate of damaged markers. Inactivated markers, regarded as induced back-mutations or suppressor-mutations, might be integrated by some recipient cells. This was tested by measuring the residual activity of treated DNA(SM,Ka_1,Ca) on various test strains. If, for instance, every inactivated Ca marker can replace the chromosomal Ca marker in the recipient Rd/Ca cells, then the survival of the complex SM-Ca should be equally low on both test strains (i.e. about 2 per cent). On the other hand, if every inactivated Ca marker is rejected by recipient cells, then the survival of the SM-Ca complex on the strain Rd/Ca should be equal to that of the SM marker alone. The latter case was clearly observed for all three markers (Table I) but the lower survival of the complex SM-Ka_1-Ca with respect to that of the complex SM-Ca still left some uncertainty about the possibility that inactivated Ka_1 markers can be integrated.

In order to obtain conclusive evidence on this matter, 110 SM+Ca-resistant colonies derived from the cross: treated DNA x Rd/Ka_1 (the 1.5 per cent survival group, Table I) were streaked on SM+Ca agar and incubated overnight. They were restreaked on SM+Ka+Ca agar. All 110 cultures did grow on this medium. 12 cultures were grown in broth, lysed by addition of 0.04 per cent sodium lauryl sulphate and their markers checked. All 12 lysates contained equal amounts of the SM, Ka_1, and Ca markers. There seems thus no doubt that the discrepancy in survival of the complexes SM-Ka_1-Ca and SM-Ca was not caused by integration of inactivated Ka_1 markers.

DISCUSSION - The absence of a mutagenic action by nitrous acid cannot be stated absolutely, i.e. after treatment of the DNA certainly less than 10^{-5} to 10^{-4} of the sensitive loci examined had been converted

TABLE I

Fate of damaged markers. DNA(SM,Ka_1,Ca) was treated with nitrous acid during 60 minutes. Survival of the markers and certain complexes was measured on several test strains as described in the text.

Marker or complex	Survival (per cent) measured on			
	Rd	Rd/SM	Rd/Ka_1	Rd/Ca
SM	15	-	18	18
Ka_1	16	20	-	-
Ca	14	-	-	-
SM-Ka_1	8.2	18	16	-
SM-Ca	1.8	-	1.5	22
SM-Ka_1-Ca	0.88	-	0.84	-

into markers active in the transformation process. The reason for this seems clear. The spatial structure of the DNA had been altered by the nitrous acid and as a consequence it did not pair well enough with the bacterial chromosome to give rise to genetic recombination. Rather extensive areas adjacent to the "hit" in the DNA molecule were in this manner rejected by the recipient cells and probably broken down upon further growth. Such DNA regions were thus excluded from being active in transformation and consequently, they were scored as inactivated. The same phenomenon was also found for UV-inactivated DNA (Stuy, 1962) and for DNA heated at sub-melting temperatures (Sluis and Stuy in preparation). We can draw two conclusions from this observation. (1) Nitrous acid causes predominantly the deamination of cytosine, adenine and guanine. Some of those base alterations may be clear mutagenic events. But because of the exclusion phenomenon, none is incorporated and replicated. They cannot be demonstrated. (2) Nitrous acid degrades DNA predominantly in other ways (cross-links between the two strands, depurination, etc.). Such changes are responsible for the exclusion phenomenon. Any mutagenic base alteration in excluded areas cannot manifest itself and it is

thus not observable. Only those which occur outside such areas have a chance of being incorporated and of giving rise to a mutant clone. Of course, their frequencies are greatly reduced in this way. We cannot say at present which of the two conclusions is most probable.

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REFERENCES

Litman, R.M. and Ephrussi-Taylor, H., C.R. Acad. Sci. 249, 838 (1959).
Mundry, K.W. and Gierer, A., Z. Vererbl. 89, 614 (1958).
Stuy, J.H., Doctoral thesis, Utrecht (1961a) (reprints available).
Stuy, J.H., Rad. Res. 15, 45 (1961b).
Stuy, J.H., J. Photochem. Photobiol. in the press (1962).