THE ACTION OF NITROUS ACID ON TRANSFORMING DESOXYRIBONUCLEIC ACIDS

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Received November 6, 1962

The absence of any mutagenic activity of nitrous acid upon transforming H. influenzae DNA, ascertained in our (see table II, part A) as well as in other laboratories (Stuy 1961) (Horn and Herriott 1962), is in apparent conflict with its renowned mutagenic activity upon a wide variety of organisms, as different as plant or animal viruses, bacteriophage[+] and bacteria (Kaudewitz 1959), as well as upon "in vitro" treated RNA (Gierer and Mundry 1958) and DNA from D. pneumoniae (Litman and Ephrussi Taylor 1959) and B. subtilis (Anagnostopoulos and Crawford 1961). The mutagenic, as well as the lethal (or inactivating) activity of nitrous acid has been ascribed to the deamination of certain nucleic acid bases : the deamination of adenine to hypoxanthine and cytosine to uracil having a mutagenic effect, while the deamination of guanine to xanthine is believed to lead only to lethal consequences (Vielmetter and Schuster 1960) (Litman 1961).

Recent evidence (Geiduscheck 1961) has shown that, besides deaminating nucleic acid bases, nitrous acid induces the formation of covalent cross-links between the two strands of the DNA double helix. We have thus undertaken to evaluate more closely the im-

+ for references, see Horn and Herriott (1962).

portance of each of these two types of modifications of H. influenzae transforming DNA on its behaviour upon nitrous acid treatment.

In order to determine whether cross-linkage does occur between the two strands of H. influenzae DNA under conditions of rather mild inactivation of its transforming activity, nitrous acid treated DNA was subjected to acid denaturation. As shown in table I, the transforming activity of nitrous acid treated DNA shows a greater resistance to acid denaturation than the control sample, and is renatured more extensively when brought slowly back to neutral pH under proper conditions. These results can be interpreted as indicating that nitrous acid treatment induces the formation of cross-links between DNA strands, which prevent to some extent the acid induced disorganisation of its double-helical structure and which, under renaturation conditions, will provide nucleation centers (Geiduscheck 1962) to insure proper matching of base pairs along the disordered segments of DNA strands. This interpretation of the resistance of the transforming activity of H. influenzae DNA to denaturation, brought about by nitrous acid treatment, is further supported by results obtained by treating the same DNA with U. V., which is also thought to produce cross-links between DNA strands (Marmur and Grossman 1961). As seen in table I, UV treatment also enhances the resistance of transforming activity of H. influenzae DNA to denaturation.

There are some data - such as: 1) the "overlapping" effect of the lesions (a lesion in one marker also inactivates the adjacent linked marker) (Stuy 1961) (Luzzati and al. 1961) and, 2) the non-integration of inactivated markers into the recipient cell genome (Stuy 1961) - which make it already difficult to ascribe the inactivation of the transforming activity of H. influenzae DNA to a mere deamination of

DNA bases. These data indicate that, even if inactivation of transforming activity follows first order kinetics (which is agreed to mean that one lesion is enough to inactivate one marker carried by one DNA molecule), the effect of that lesion extends over a rather broad segment of the DNA molecule.

Table I

Transforming activity of H. influenzae DNA (novobiocin^{+} marker) after various treatments

Treatment (min.)		1 Activity after treatment (%)	2 Activity after denaturation (% of 1)	3 Activity after renaturation (% of 1)
Nitrous acid	0	100	1.9	19.1
	15	100 (6,7)	16.2	57.5
Ultraviolet	0	100	0.9	"
10.000 ergs		100 (2,2)	3.9	"
Succinic peroxide	0	100	0.4	
	50	100 (18)	4.3	

Nitrous acid : DNA 250 μ g in $NaNO_2$ 1 M, acetate buffer 0,25 M pH 4,3 at 22°. Neutralisation at indicated time, dialysis against NaCl 0.05 M, citrate 5 x 10^{-3} M. Denaturation at 20 μ g/ml in the last solvent, by 0,1 M acetic acid (pH 2,8). Renaturation by dialysis (24 h. at 22°) against NaCl M, citrate 0,01 M, pH 7.

Ultraviolet : DNA 20 μ g/ml in NaCl 0,1 M phosphate buffer 1 x 10^{-2} M. Irradiation with Phillips germicide lamp in a quartz cuvette, light path 1 cm. Denaturation by heating 5' at 100°, immediate cooling on ice.

Succinic peroxide : DNA 250 μg/ml, in succinate buffer 0,2 M, pH 7 ; succinic peroxide 2 mg/ml, 37° ; reaction stopped by dilution 1/10 into Levinthal broth ; dialysis against NaCl 0,15 M, phosphate buffer 1 x 10^{-2} M, pH 7. Denaturation by heat (5 min, at 100°) cooling on ice. (In brackets : remaining activity, relative to control).

+ Novobiocin = cathomycin

These two manifestations ("overlapping" effect of the lesion and non-integration into the recipient cell genome of inactivated markers) of the extension, along a broad segment of the H. influenzae DNA molecule, of the effect of one lesion, are also displayed by two other inactivating agents : UV (Stuy 1962) and succinic peroxide (Luzzati and al. 1961) (Bach and Luzzati 1962). Interestingly enough, these last two agents seem also to produce cross-linkages between DNA strands under the very conditions where they do inactivate transforming activity of DNA from this species, as evidenced by their ability to enhance the resistance of treated DNA to denaturation (table I). The correlation, for these three agents, of these two sets of data - one indicating an extended effect of the lesion and the other an ability to cross-link DNA strands - is striking. It has led us to speculate that the formation of cross-links between two DNA strands could produce an inability of the molecule, or rather, segments of it, to undergo the changes of structure required for the integration into the recipient cell genome, thereby eliminating from integration relatively large segments of the DNA molecule. This hypothesis could account for the extended effect of the lesion, as well as for the observed absence of mutagenic activity of any of these three agents on H. influenzae DNA : if the altered bases are mostly included in such unintegratable segments, they will have a very low probability of being integrated into the recipient cell genome, and of carrying into the cell their mutational potentialities.

This hypothesis can be tested by preventing the formation of cross-links during nitrous acid treatment and testing for its mutagenic activity under these conditions. This has recently been achieved (Horn and Herriott 1962) by treating heat-denatured H. influenzae DNA with nitrous acid. We have used a technique similar to theirs : as shown

in table II, part B, nitrous acid treatment of heat denatured DNA is indeed mutagenic.

Table II

Mutagenic action of nitrous acid on native and heat denatured H. influenzae DNA

Acquired resistance for	Duration of nitrous acid treatment (min.)	resistant cells/ plate	resistant cells/10^4 novobiocin resistant cells
A) NATIVE DNA			
Viomycin 120 μg/ml	0	83	"
	30	53	"
	60	46,6	"
Kanamycin 4 μg/ml	0	0	"
	30	0	"
	60	0	"
Streptomycin 25 μg/ml	0	0	"
	30	0,3	"
	60	6,6	"
Erythromycin 5 μg/ml	0	"	"
	30	2	"
	60	0,3	"
Novobiocin 2 and 4 μg/ml	0	0	"
	30	0	"
	60	0,3	"
Streptomycin 25 μg/ml	0	1	1,4
	6	0	0,3
	10	0,3	1,8
B) HEAT DENATURED DNA			
Viomycin 150 μg/ml	0	6	1,3
	5	4	0,8
	10	164	80
	15	166	180
Kanamycin 4 μg/ml	0	0	0
	5	14	5,5
	10	32	17
	15	34	38
Streptomycin 25 μg/ml	0	2	0,4
	5	2	0,4
	10	12	5
	15	14	15,8
Erythromycin 5 μg/ml	0	105	14
	5	35	14
	10	156	102
	15	160	110

Native (A) and heat denatured (B) DNA (5 min, at 100°) were subjected to nitrous acid treatment as indicated in table I. DNA bearing

only the oleandomycin resistance marker was used for the A experiments ; DNA bearing only the novobiocin-resistance marker (N) was used for the B and A^+ experiments.

Inactivation of N marker by nitrous acid treatment (remaining transforming activity) A^+ : 6 min, 56 % ; 10 min, 26 % ; B : 5 min, 56 % ; 10 min, 36 % ; 15 min, 19 %.

Test for "in vitro" mutagenesis : 1) Transformation mixture : (per ml) : 3×10^8 precompetent Rd cells - sensitive to all listed antibiotics - DNA 1 μg, NaCl 0,15 M ; 30 min at 37°. 2) Expression of transformants : liquid medium (1/10 dilution of mixture 1) in Levinthal broth, 1,5 hours at 37°. Acquired resistance for the antibiotic listed, tested by spreading 0,1 ml of suspension 2) on antibiotic containing Levinthal-agar plates. Number of plates plates per experimental point : A experiments, 3 - B and A^+ experiments, 5.

The mutagenic activity of nitrous acid when acting on native D. pneumoniae or B. subtilis transforming DNA contrasts with its inability to produce mutations when acting on native (but not on denatured) H. influenzae DNA. This could be tentatively ascribed to a greater susceptibility of the DNA of the later species to the cross-linking ability of nitrous acid (an alternative hypothesis being a difference in the integration - recombination pattern between these transformable species). The first hypothesis was tested by comparing the extent of cross-linkage produced in these three different species of DNA by a standard nitrous acid treatment. We used Geiduscheck's technique (Geiduscheck 1961) ; that is, we determined spectrophotometrically the "reversibility" of the thermal denaturation of the treated DNA. After a 40 minute nitrous acid treatment (under the conditions indicated in fig. 1, 2 and 3), B. subtilis and D. pneumoniae DNA are just as "irreversible" as the untreated controls, but H. influenzae DNA shows a complete reversibility to its former optical density[+]. In the case of D. pneumoniae, such complete "reversibility" can only be obtained after a much longer nitrous acid treatment (120 min.).

+ Absorbance of the treated samples, measures at the elevated temperatures ("standard" melting curves) is very similar to that of control.

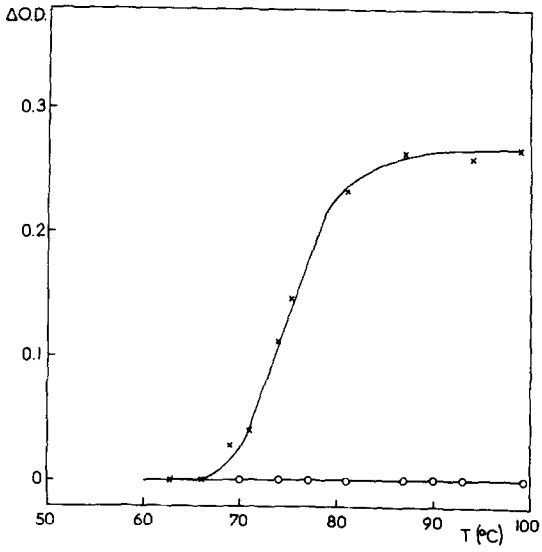


Fig. 1 H. influenzae DNA

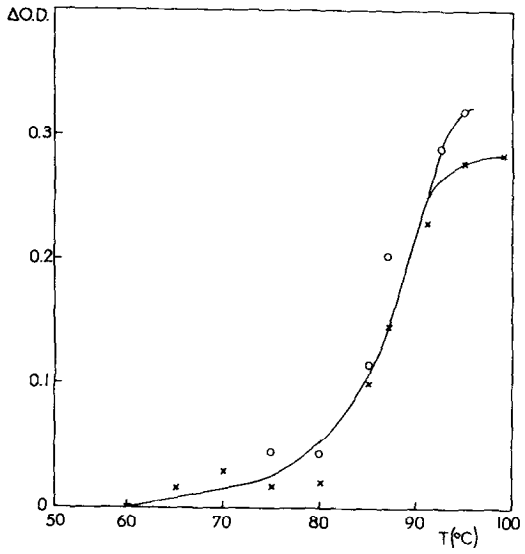
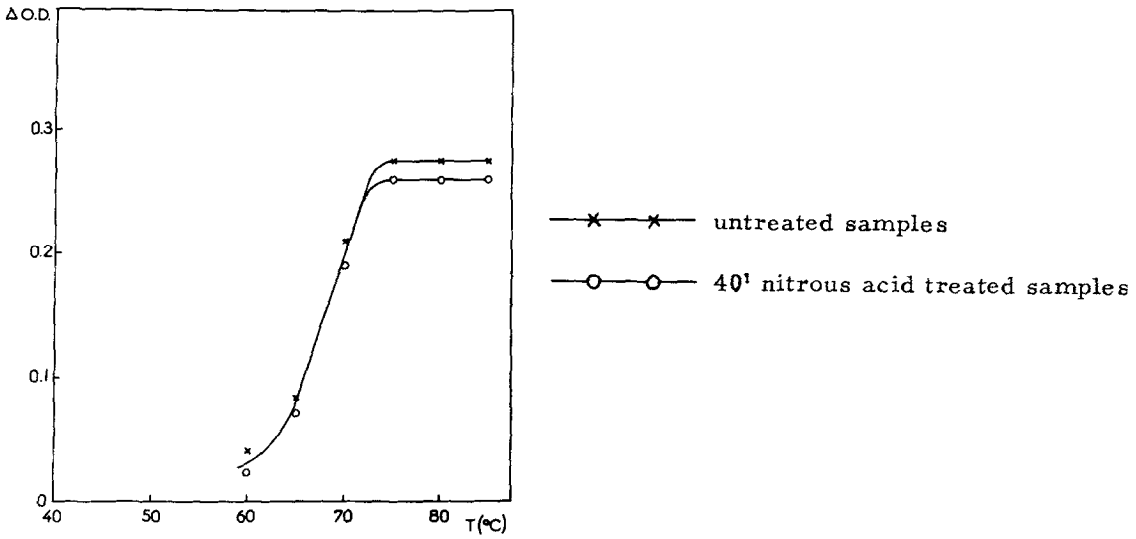


Fig. 2 B. subtilis DNA



"Reversibility" of nitrous acid treated DNA

Nitrous acid treatment : as in table I. Treated and control samples are then diluted in NaCl 0,01 M, citrate 0,001 M, phosphate buffer 0,001 M and heated to the indicated temperatures for 10 minutes, cooled on ice and their optical density read at 260 mμ (22°). Δ O.D. = O.D.$_t$ - O.D.$_{22°}$/O.D.$_{22°}$.

As the overall base composition of these three DNAs is very similar - even identical in the case of D. pneumoniae and H. influenzae (Marmur and Doty 1962) - the differences in their susceptibility to be cross-linked by nitrous acid, as shown by the experiments described above, cannot be ascribed to a difference in the amount of base pairs susceptible to cross-linkage, but rather to the amount and recurrence of certain types of sequences of base pairs along the DNA double helix. Research is currently progressing along these lines.

Acknowledgements. We wish to thank Dr. Sicard and Mr. G. Balassa, who provided us, respectively, with the D. pneumoniae and the B. subtilis DNA samples used in this work, as well as Miss M. L. Greth for her able technical assistance.

References

Anagnostopoulos, C. and Crawford, I. P., Proc. Nat. Ac. Sc. 47, 378, (1961).

Bach, M. L. and Luzzati, D. (unpublished experiments (1962)).

Geiduscheck, E. P., Proc. Nat. Ac. Sc. 47, 950, (1961).

Geiduscheck, E. P., J. Mol. Biol., 4, 467, (1962).

Gierer, A. and Mundry, K. W., Nature, 182, 1457, (1958).

Horn, E. E. and Herriott, R. M., Proc. Nat. Ac. Sc., 48, 1409, (1962).

Kaudewitz, F., Z. für Naturforschung, 146, 528, (1959).

Litman, R. M. and Ephrussi-Taylor, H., C. R. Ac. Sc., Paris, 249, 838, (1959)

Litman, R. M., J. Chimie Phys., 58, 997, (1961).

Luzzati, D., Schweitz, H., Bach, M. L. and Chevallier M. R., J. Chimie Phys., 58, 1021 (1961).

Marmur, J. and Grossman, L., Proc. Nat. Ac. Sc., 47, 778, (1961).

Marmur, J. and Doty, P., J. Mol. Biol., 5, 109, (1962).

Stuy, J. H., Biochem. Biophys. Res. Com., 6, 328, (1961).

Stuy, J. H., J. Photochem. and Photobiol., 1, 41, (1962).

Vielmetter, W. and Schuster, H., Biochem. Biophys. Res. Com., 2, 324, (1960).