# ON THE INTERACTION OF N-2-FLUORENYLHYDROXYLAMINE WITH NUCLEIC ACIDS IN VITRO*

E. Kriek

Department of Biochemistry, Antoni van Leeuwenhoek-Huis: the Netherlands Cancer Institute, Amsterdam, the Netherlands.

Received August 23, 1965

The N-hydroxylation of aromatic amines is likely to play an essential role in the carcinogenic properties of a number of these compounds, since N-hydroxy-derivatives of N-2-fluorenylacetamide (Miller et al.,1961a), 4'-phenylacetanilide (Miller et al.,1961b), 4'-styrylacetanilide (Anderson et al.,1963) and 2-naphthylamine (Boyland et al.,1963) are more potent carcinogens than are the parent compounds under certain experimental conditions. Recently Marroquin and Farber (1962) observed binding between N-2-fluorenyl-9-($^{14}$C)acetamide and nucleic acids of rat liver in vivo. Although suggestive evidence was obtained that the binding was covalent in nature, the manner of binding is still unknown. In the present study N-OH-FA**, the most reactive metabolite of N-2-fluorenylacetamide, was reacted with isolated nucleic acids in order to elucidate the nature of the bound material.

---

* These results were communicated in part at the 2nd Meeting of the Federation of European Biochemical Societies, Vienna, April 1965.

** Abbreviation: N-OH-FA = N-2-Fluorenylhydroxylamine (Chemical Abstracts nomenclature).

Methods and Materials - N-OH-FA was prepared from 2-nitrofluorene by the method of Poirier et al.(1963). It was found to be 96-97 % pure by titration with $TiCl_3$ (Horner and Steppan,1957; see also Poirier et al.1963). 2-Nitrosofluorene was prepared by oxidation of N-OH-FA with ferric ammonium sulphate (Miller, personal communication).

Yeast-s-RNA (Monier et al.,1960), purified by chromatography on DEAE-cellulose (Serva,Germany; 0.4 meq./g), or DNA from calf thymus (Kay et al.,1952) was incubated with N-OH-FA in buffered aqueous ethanol for different periods of time under the conditions mentioned below. All reaction mixtures were flushed with nitrogen in order to counteract oxidation of N-OH-FA. Controls were treated in the same way except for the addition of N-OH-FA. After purification by extraction with phenol, amyl alcohol and precipitation with ethanol, the U.V.-spectra of the reacted nucleic acids were measured in 0.14 M NaCl-0.015 M citrate buffer, pH 7.2. Sedimentation constants were determined in the same buffer in a Spinco E analytical ultracentrifuge equipped with an U.V.-recording system. The nucleotide composition of s-RNA was estimated by the method of Katz and Comb (1963) after hydrolysis in 0.3 N KOH at 37° for 18 h; the base composition of DNA was determined by the method of Wyatt and Cohen (1953).

Results and Discussion - After purification by extraction with phenol and precipitation with ethanol, the reacted nucleic acids show an absorption band between 300 and 360 mμ (fig.1) and an increased $A_{280}/A_{260}$ ratio as compared with control material. These absorption changes resist prolonged dialysis and chromatography on DEAE-cellulose,

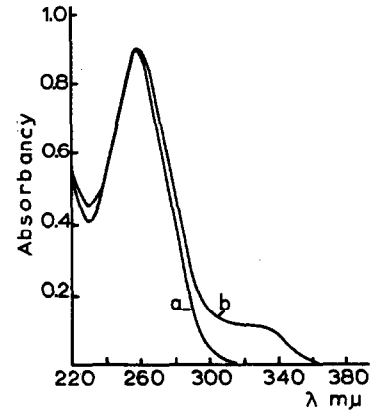



Fig.1 - Absorption spectra of control s-RNA (curve a) and of s-RNA treated with N-OH-FA (curve b) in 0.14 M NaCl-0.015 M citrate buffer, pH 7.2.

suggesting a covalent binding between the nucleic acids and N-OH-FA. N-OH-FA-treated s-RNA is adsorbed more strongly to the DEAE-cellulose than is control-s-RNA. Since the sedimentation constant of this material ($s_{20,w}$ = 3.5 S as compared with 4.0 S of control s-RNA) is only slightly lower, the DEAE adsorption effect may be due to loss in secondary structure of the reacted s-RNA.

The time curves at different pH values (fig.2) show the catalytic effect of $H^+$ ions; at 37° the reaction proceeds much faster than at 21°. After hydrolysis of the variously treated nucleic acids and analysis of the nucleotides, a decrease in guanylic acid content was consistently observed, as illustrated in fig.3. Since the characteristic absorption of the reacted nucleic acids is lost on hydrolysis, the reaction product of N-OH-FA and guanine is apparently destroyed by hydrolytic action. From the decrease in guanylic acid content the degree of binding has

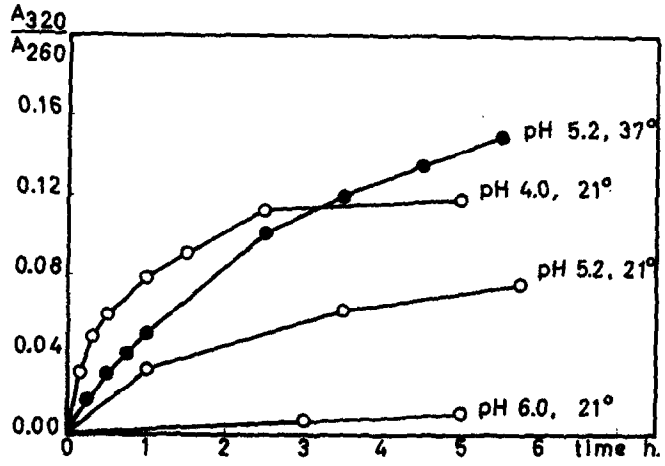



Fig.2 - pH- and temperature-dependence of the reaction of s-RNA with N-OH-FA.
s-RNA (0.09 mmole RNA-P) was treated with N-OH-FA (0.03 mmoles) in 60 ml of 0.005 M acetate buffer-ethanol = 4:1 (v/v) at 21 and 37° in an atmosphere of $N_2$. After different periods of time aliquot portions of 10 ml were withdrawn for analysis.

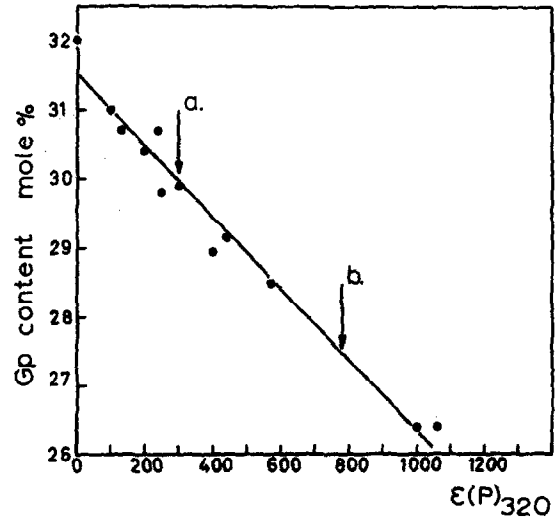



Fig.3 - Guanylic acid content (mole per cent of total nucleotide after alkaline hydrolysis) of s-RNA treated for different periods of time (see fig. 2) with N-OH-FA.
$\varepsilon(P)_{320}$ = molar absorption coefficient at 320 mμ per mole RNA-P per l.
Points a and b are referred to in the text.

been calculated in two cases, based on the assumption that one mole of N-OH-FA reacts with one mole of guanine, giving 2 and 4 fluorene residues (fig.3, points a and b, respectively) per s-RNA molecule of 80 nucleotides (Cantoni et al., 1962). In these cases, about 6 and 12 mole per cent of added N-OH-FA has been bound to s-RNA.

An identical reaction was observed for native DNA under the same conditions; after hydrolysis with 98 % formic acid at 175° and base analysis, a decrease in guanine content was observed (see Table I).

TABLE I

BASE COMPOSITION OF DNA TREATED WITH N-OH-FA

DNA (0.045 mmoles DNA-P) and N-OH-FA (0.015 mmoles) in 30 ml of 0.005 M acetate buffer, pH 5.2 - ethanol = 4:1(v/v) were incubated at 40° for 3 h in an atmosphere of $N_2$. Control DNA was treated in the same way except for addition of N-OH-FA.

| | $\frac{A_{320}}{A_{260}}$ | $\frac{A_{280}}{A_{260}}$ | Base composition, mole % | | | |
|---|---|---|---|---|---|---|
| | | | A | G | C | T |
| DNA + N-OH-FA | 0.20 | 0.60 | 29.9 | 15.4 | 23.2 | 31.5 |
| DNA control | 0.00 | 0.53 | 27.9 | 20.9 | 22.6 | 28.6 |

2-Nitrosofluorene did not react with nucleic acids.

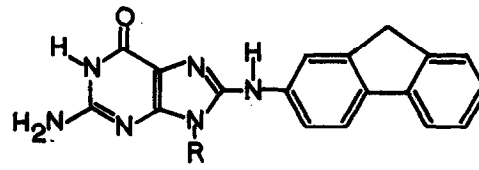
From the results obtained it is concluded that under the conditions employed, the mechanism proposed by Heller, Hughes and Ingold (1951) may be operative:

$$Ar\text{-}\underset{\underset{H}{|}}{N}\text{-}OH \xrightleftharpoons{H^+} Ar\text{-}\underset{\underset{H}{|}}{\overset{\overset{H}{|}}{\overset{+}{N}}}\text{-}OH \longrightarrow Ar\text{-}\underset{II}{\overset{+}{N}}\text{-}H \xrightarrow[phile]{Nucleo-} \text{products}$$

I

Loss of water from ion I would yield ion II in which the nitrogen atom has only a sextet of valence electrons. If this ion is sufficiently resonance-stabilized, as might be

the case if Ar = fluorenyl or biphenyl, it could react partly in an electrophilic substitution reaction at C-8 of guanine, which is the most likely to be attacked by an electrophilic agent (Pullman and Pullman,1963), giving (tentative structure):



4-Biphenylhydroxylamine and 2-naphthylhydroxylamine did not react at pH 5.2, but after 4 h at pH 4.0 and 40° a slight reaction was observed, the degree of binding being about 40 and 10 per cent of that of N-OH-FA, respectively.

Further work on the identification of the reaction product and the biological properties of nucleic acids containing fluorene residues is in progress. Preliminary experiments have already shown that the ability to incorporate amino acid of rat-liver s-RNA which contains 2-3 fluorene molecules per chain, is almost completely lost.

Acknowledgements - The author is indebted to Dr. P.Emmelot for his encouragement throughout the investigation, to Dr. J.A.Miller (McArdle Memorial Laboratory for Cancer Research, Madison, Wisconsin) for advice on the synthesis of N-OH-FA and 2-nitrosofluorene, to Mr. W.S. Bont for experiments in the analytical ultracentrifuge and to Mrs. G. Wind-Slump for expert technical assistance.

## REFERENCES

Anderson,R.A., Enomoto,M., Miller,J.A. and Miller,E.C., Proc.Am.Assoc.Cancer Res.4(1963)2.
Boyland,E., Dukes,C.E. and Grover,P.L., Brit.J.Cancer 17 (1963)79.

Cantoni,G.L., Gelboin,H.V., Luborsky,S.W., Richards,H.H. and Singer,M.F., Biochim.Biophys.Acta 61(1962)354.
Heller,H.E.,Hughes,E.D. and Ingold,C.K.,Nature 168(1951)909.
Horner,L. and Steppan,H., Annalen 606(1957)24.
Katz,S. and Comb,D.G., J.Biol.Chem.238(1963)3065.
Kay,E.,Simmonds,N. and Dounce,A.,J.Am.Chem.Soc.74(1952)1724.
Marroquin,F. and Farber,E., Biochim.Biophys.Acta 55(1962)403.
Miller,E.C., Miller,J.A. and Hartmann,H.A., Cancer Res.21 (1961a)815.
Miller,J.A., Wyatt,C.S., Miller,E.C. and Hartmann,H.A., Cancer Res.21(1961b)1465.
Monier,R., Stephenson,M.L. and Zamecnik,P.C., Biochim. Biophys.Acta 43(1960)1.
Poirier,L.A., Miller,J.A. and Miller,E.C. Cancer Res.23 (1963)790.
Pullman,B. and Pullman,A., Quantum Biochemistry, Interscience 1963, pp.226-230.
Wyatt,G.R. and Cohen,S.S., Biochem.J.55(1953)774.