Studies on Depurination and Alkylation of DNA's of Different Base Compositions

STEPHEN ZAMENHOF AND SHIGEYO ARIKAWA

Department of Medical Microbiology and Immunology, Department of Biological Chemistry, and Molecular Biology Institute, University of California, Los Angeles, California 90024

(Received July 13, 1966)

SUMMARY

DNA's from four different sources, varying in G + C content from 36% to 72%, were subjected to depurination by heating at neutral pH (100°, 24 hr) or to alkylation (dimethyl sulfate, 37°); the extent of alkylation was measured by total release of 7-*N*-methylguanine by heating at neutral pH (100°, 4 hr). The fraction of guanine or adenine released by depurination and the fraction of guanine alkylated were found to be independent of G + C content, i.e., independent of the average base distribution or base densities along the DNA molecule. These two reactions (depurination at neutral pH and alkylation) have been implicated as causes of spontaneous and induced mutability. If, indeed, these reactions under the conditions studied are representative of the mutagenic processes *in vivo*, then causes other than variations in base distributions must be sought to explain the fact that observable mutabilities vary along the molecule of DNA.

INTRODUCTION

The causes of differences in mutabilities of individual structural genes and, more precisely, of individual sites within these genes, are still largely undetermined [for review see (1)]. The apparent differences in mutabilities, as detected in phenotypes, may be partially due to the functional and structural characteristics along the *protein* molecules coded by these genes (2): A change in a critical part of the protein molecule is likely to be scored more frequently ("high mutability" or "hot spot") than a change of the same magnitude in a noncritical part ("low mutability" or "cold spot"). When the differences are due to DNA itself, they are likely to be caused either by separate mutator genes (special cases), or by the hypothetical influences inherent in the structural genes themselves [more general case; for review see (3)]. It has been suggested that in this latter case, these influences are bases (base sequences) neighbor to the sites whose mutabilities are being considered (3–5): Thus, different distributions of particular bases along the DNA molecule ought to result in different patterns of mutabilities.

The purpose of the present work is to investigate whether pronounced differences in base composition (and therefore in the average base distributions) result in different chances for any adenine or guanine molecule to participate in reactions that have been implicated as causes of spontaneous and of induced mutabilities, respectively: *depurination* at neutral pH (6–8) and *alkylation* of guanine (9, 10).

MATERIALS AND METHODS

Isolation of DNA

DNA of calf thymus was obtained from Gallard-Schlesinger Mfg. Corp.; it was isolated by previously described methods (11).

DNA's of *Escherichia coli*, strain 15S (12), *Sarcina lutea* (A.T.C.C. 381) and *Streptococcus faecalis* (A.T.C.C. 8043) were isolated by the previously described

method (13) from the cells grown in Difco Penassay broth (*E. coli* and *S. faecalis*) or on Difco nutrient agar (*S. lutea*). The preparations were freed from protein by the method of Sevag (14) and from RNA by the method of Marmur (15), as described in (8).

Analyses

DNA and RNA contents were assayed as described previously (16); the latter were lower than 1% of DNA for calf thymus, *E. coli*, and *S. faecalis*, and 3.5% for DNA of *S. lutea*.

Chromatographic analysis was performed on Whatman No. 3MM paper essentially as described previously (8) using as solvent butanol-NH_3 (base analysis of intact or alkylated DNA) or butanol-H_2O (analysis of purines liberated by heat). Commercial guanine (Schwarz BioResearch, Inc.), adenine (CalBiochem.) and 7-*N*-methylguanine (Sigma Chemical Co.) were used as reference standards.

Depurination

Depurination of intact DNA preparations was performed essentially as described previously (8). One-milliliter aliquots of solution containing 500–550 μg of DNA per milliliter of 0.005 M phosphate buffer, pH 6.8, were heated in totally immersed sealed tubes for 24 hr at 100°. Solid NaCl was then added to obtain final concentration 0.1 M, and DNA was precipitated by addition of 2 volumes of 95% ethanol. The resulting amorphous precipitate was removed by centrifugation, and the supernatant was evaporated to dryness (nitrogen current, 23°). The sediment was redissolved in 0.1 ml of 0.1 N H_2SO_4 (23°) and subjected to chromatographic analysis as described above.

Alkylation

Alkylation of intact DNA preparations was performed essentially as described previously (9). To 1-ml aliquots of solution containing 300–400 μg of DNA in 0.1 N aqueous sodium citrate of pH 7.5, dimethyl sulfate (DMS; Eastman Organic Chemicals) was added to obtain a final concentration of 0.13 M. The solution was incubated at 37° for various lengths of time, with shaking. To avoid precipitation of citrate, pH was then adjusted to 4.5 by careful addition of 1 N HCl, and DNA was precipitated by addition of 2 volumes of 95% ethanol. The threadlike precipitate was removed by centrifugation, washed in 2 volumes of 75% ethanol, and redissolved in 1 ml of 0.005 M phosphate buffer, pH 6.8.

Alkylated purines were released from DNA by heating for various lengths of time at 100°, as described above. In some experiments, as indicated, after alkylation, DNA was redissolved in 0.1 ml of 0.1 N H_2SO_4 instead of buffer, and alkylated purines were released by heating for 1 hr at 100°. Preliminary experiments indicated that neither of these treatments caused any destruction of 7-methylguanine that would be detectable by chromatography and ultraviolet absorption spectrum.

RESULTS AND DISCUSSION

The results of depurination of intact DNA at neutral pH are represented in Table 1. It can be seen that despite considerable differences between the G + C contents (72–36 mole %) or G:A ratios (2.57–0.56) of the DNA's studied, the fraction of guanine released by heat (ratio

TABLE 1

Release of purines from DNA's of different G + C contents upon heating

Heating conditions: 100°, 24 hr, 0.005 M phosphate buffer pH 6.8.

Sources of DNA	Intact DNA G + C[a] (mole %)	Purines released		
		G[b]	A[b]	G:A[b]
Sarcina lutea	72	13.8	10.1	1.37
Escherichia coli	51	13.0	9.5	1.37
Calf (thymus)	43	13.6	9.9	1.37
Streptococcus faecalis	36	14.9	10.8	1.38

[a] As determined in this work. Values reported in the literature are in close agreement.

[b] As per cent of total guanine or adenine present in intact DNA; average of 5 determinations for each DNA.

of guanine released to total guanine present in intact DNA) is essentially the same. Adenine shows a similar relationship. In purines released by heat, the ratio of these two fractions is therefore constant (1.37), regardless of the G:A in intact DNA. Thus, there is no evidence that the *average* chance of a release of a purine by heat depends on the content of G-C pairs and on the average density of adenine or guanine along the DNA molecule. However, the influence of local peculiarities of base distribution on the release of purines in some *individual* cases cannot be excluded at present. It is also to be noted that in these experiments the heating was not performed in biological conditions and therefore may not be representative of depurination that could lead to mutations.

This and the previous study (8) also reveal that the fraction of guanine released by heat is always higher than the fraction of adenine so released. This circumstance may somewhat facilitate the release of guanine after alkylation (attack on 7-*N* position of guanine; see below).

As can be seen from Table 2, in the conditions used, 45 min incubation with DMS was sufficient to obtain the maximum yield of 7-*N*-methylguanine. This length of incubation was therefore adapted for further experiments.

TABLE 2
Production of 7-N-methylguanine in calf thymus DNA upon treatment with dimethyl sulfate[a]

Duration of alkylation treatment (min)	7-*N*-methylguanine[b]
15	22.9
45	32.6
60	28.1
240	25.9
480	21.7
1440	21.2

[a] Alkylating conditions: 0.13 M dimethyl sulfate, 37°. Release of 7-*N*-methylguanine: 1 hr, 100°, 0.1 M H_2SO_4.

[b] As per cent of total guanine present in intact DNA.

It is known that alkylated purines are slowly and incompletely released from DNA at neutral pH and 37° (17–19). In the present work, methylated guanine was rapidly and completely released by heating at neutral pH at 100°.

As Table 3 shows, in the conditions used, 4 hr of heating at 100° at neutral pH was sufficient to obtain maximum release of alkylated guanine. This length of heating was therefore adopted for further experiments. Such heating at neutral pH gives practically the same (total) release of methylated guanine as the heating in acid (Tables 2 and 3); heating at neutral pH was used in further experiments because the total release of 7-*N*-methylguanine with only small release of adenine and guanine (5–7% of total, for each base, after 4 hr) was more convenient for chromatographic analysis.

TABLE 3
Release of 7-N-methylguanine from alkylated calf thymus DNA at 100° and neutral pH[a]

Duration of heat treatment (hr)	7-*N*-methylguanine[b] released
2	28.1
4	29.7
6	29.2
24	29.5

[a] Alkylating conditions: 0.13 M dimethyl sulfate, 37°, 45 min. Release of 7-*N*-methylguanine: 0.005 M phosphate buffer, pH 6.8.

[b] As per cent of total guanine present in intact DNA.

The amount of dimethyl sulfate used in alkylation studies was approximately 1000 times higher than the stoichiometric amount needed to alkylate all the guanine molecules in the DNA sample; the concentration of dimethyl sulfate used was not sufficient to fully inactivate the transforming principle of *Bacillus subtilis* (20), and therefore the conditions were not far from biological.

The results are represented in Table 4. It can be seen that, as in the case of depurination of intact DNA, the fraction of guanine methylated is essentially the same for DNA's of different G + C contents. Thus, here too, there is no evidence that the

average action of the alkylating agent used depends on the content of G-C pairs and on the average density of guanine along the DNA molecule. However, again the influence of local peculiarities of base distribution on the attack on position 7 of *individual* guanine molecules cannot be excluded at present.

TABLE 4

Production of 7-N-methylguanine in DNA's of different G + C contents upon treatment with dimethyl sulfate[a]

Source of DNA	G + C (mole %)	7-*N*-methylguanine[b]
Sarcina lutea	72	29.2
Escherichia coli	51	30.8
Calf (thymus)	43	29.6
Streptococcus faecalis	36	32.9

[a] Alkylating conditions: 0.13 M dimethyl sulfate, 37°, 45 min. Release of 7-*N*-methylguanine: 4 hr, 100°, in 0.005 M phosphate buffer pH 6.8.

[b] As per cent of total guanine present in intact DNA; average of 4 determinations for each DNA.

In conclusion, no experimental evidence was obtained to support the view that the chances for any adenine or guanine molecule to participate in depurination or alkylation are influenced by the base composition and the average base distribution or base density along the DNA molecule. If, indeed, depurination or alkylation under the conditions studied is representative of the mutagenic processes *in vivo*, then causes other than variation in base distribution or base densities must be sought to explain the fact that observable mutabilities vary along the DNA molecule.

ACKNOWLEDGMENT

This investigation was supported by grants CA-08128 and HD-01331 from the National Institutes of Health, U. S. Public Health Service, and E-52 from the American Cancer Society.

REFERENCES

1. S. Zamenhof, *Progr. Nucleic Acid Res.* **6**, in press. (1967).
2. E. L. Smith and E. Margoliash, *Federation Proc.* **23**, 1243 (1964).
3. S. Zamenhof, R., de Giovanni-Donnelly and L. H. Heldenmuth, *Proc. Natl. Acad. Sci. U.S.* **48**, 944 (1962).
4. S. Benzer and E. Freese, *Proc. Natl. Acad. Sci. U.S.* **44**, 112 (1958).
5. E. K. F. Bautz and F. A. Bautz, *Proc. Natl. Acad. Sci. U.S.* **52**, 1476 (1964).
6. S. Zamenhof and S. Greer, *Nature* **182**, 611 (1958).
7. S. Zamenhof, *Proc. Natl. Acad. Sci. U.S.* **46**, 101 (1960).
8. S. Greer and S. Zamenhof, *J. Mol. Biol.* **4**, 123 (1962).
9. B. Reiner and S. Zamenhof, *J. Biol. Chem.* **228**, 475 (1957).
10. P. D. Lawley and C. A. Wallick, *Chem. Ind. (London)* p. 633 (1957).
11. S. Zamenhof, *in* "Biochemical Preparations" (C. S. Vestling, ed.), Vol. 6, p. 8. Wiley, New York, 1958.
12. S. Zamenhof, L. H. Heldenmuth and P. J. Zamenhof, *Proc. Natl. Acad. Sci. U.S.* **55**, 50 (1966).
13. S. Zamenhof, B. Reiner, R. De Giovanni and K. Rich, *J. Biol. Chem.* **219**, 165 (1956).
14. M. G. Sevag, *Biochem. J.* **273**, 419 (1934).
15. J. Marmur, *J. Mol. Biol.* **3**, 208 (1961).
16. S. Zamenhof, *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 3, p. 696. Academic Press, New York, 1957.
17. E. Bautz and E. Freese, *Proc. Natl. Acad. Sci. U.S.* **46**, 1585 (1960).
18. P. Brookes and P. D. Lawley, *Biochem. J.* **77**, 478 (1960).
19. P. Brookes and P. D. Lawley, *Biochem. J.* **80**, 496 (1961).
20. S. Zamenhof, G. Burke, and S. Arikawa, *Bacteriol. Proc.* 227 (1965).