ABNORMAL BASE PAIRING UNDER THE INFLUENCE OF NITROGEN MUSTARD

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1. Introduction

Cyclophosphamide, frequently used in tumor therapy, has proved to be mutagenic [1–4], but so far no clear evidence has been obtained in the host-mediated assay as to whether the substance causes point mutations [5]. Therefore we decided to approach the problem biochemically.

Ludlum [6, 7] has recently shown that the methylation of cytidine-diphosphate, or poly(C), and – to a lesser degree – that of the corresponding deoxy-derivatives, alters the pairing characteristics of this base in such a way that in the transcription and/or polymerization process not only guanine, but also – though to a smaller extent – uracil and adenine are incorporated into the resulting polymer. Similar observations regarding the misreading of chemically altered poly(C) have been made by Singer and Fraenkel-Conrat [8, 9] and also by Means and Fraenkel-Conrat [10]. On the basis of the results they obtained with several mutagens, these authors suggest that the substitution of position 3 of cytosine by an alkyl group might well represent a mutagenic event, inasmuch as methylated poly(C) shows appreciable ambiguity, thus leading to abnormal base pairing (with simultaneously diminished capacity for normal pairing with guanine).

Since cyclophosphamide as such is not an alkylating agent, we utilized β, β'-dichlordiethylamine (nor-nitrogen mustard) (I) in our experiments. This compound has been postulated to be one of the active substances formed in the body after application of cyclophosphamide [11]. Therefore, because of the alkylating and cross-linking properties of I [12], a strong diminution in the template function of poly(C) after incubation with I is by no means surprising. What could not be foreseen, however, was whether the treatment of poly(C) with such a relatively large molecule (large in comparison with methyl groups) would lead to abnormal base pairing. In fact, our findings have shown a very slight tendency towards the incorporation of UMP, significantly higher than the "spontaneous" rate of abnormal base pairing with UMP, and increasing linearly with the time of incubation.

2. Materials and methods

All reagents used were A-grade. Poly(C), bovine serum albumin, and unlabelled ribonucleoside triphosphates were obtained from Serva Laboratories (Heidelberg), [^{3}H]GTP and [^{3}H]UTP from the New England Nuclear Corporation. RNA polymerase (EC 2.7.7.6) from *M. lysodeikticus* (activity 762 units/mg protein) was a product of Miles (Elkhart, Indiana). β, β'-Dichlordiethylamine hydrochloride was a gift of ASTA (Brackwede).

Phosphorus was determined according to Chen et al. [13].

All readings were performed with the Zeiss PMQ spectrophotometer. Radioactivity was measured with the Tricarb liquid scintillation spectrometer (Packard). Statistical substantiation was assured by application of the Wilcox Test [14].

Incubation of poly(C): 5–10 μmoles (as nucleotide-P) were dissolved in 2 ml 0.15 M KNO_3, 0.015 M K-phosphate, pH 7.5, and mixed with 24 ml of 1.4×10^{-4} M β, β'-dichlordiethylamine hydrochloride, previously warmed to 37° for 45 min in the same buffer in order to form the active immonium ion of the

amine [15]. Incubation was for 4 hr according to Ruddon and Johnson [16]. Since we had no radioactive I, we were unable to define the exact amount of binding to poly(C)*.

The reaction mixture was dialyzed against 0.1 × SSC (15 mM NaCl – 1.5 mM trisodium citrate, pH 6.8) until no alkylating agent could be detected in the outer fluid with 4, 4′-nitrobenzyl-pyridine according to Friedman and Boger [17].

The content of the bag was then concentrated by lyophilization to an appropriate volume and the amount of the polymer determined by phosphorus analysis as well as by absorbance measurement, using the extinction coefficients at pH 7.5 for poly(C):

$\epsilon_{260} = 5.3 \times 10^3$ and $\epsilon_{269} = 6.3 \times 10^3$ (Ludlum, [7]).

The assays for template activity were performed in general as previously described [8]. The reaction mixture contained in 0.5 ml: 0.1 M Tris buffer, pH 7.55; 1.5 mM $MnCl_2$; 2×10^{-4} M dithiothreitol; 1.0 mM KCl; 0.2 μmole UTP and GTP, respectively, one labelled with tritium (approx. 7.5×10^3 cpm nmole); and template polynucleotide as specified. The reaction was started at 37° by the addition of 10 μl RNA polymerase, corresponding to 20 μg protein.

After the times indicated in the tables, 0.2 ml of saturated pyrophosphate (pH 7.2) and 300 μg of serum albumin, as well as 300 μg unlabelled triphosphates in 0.2 ml were added, and the mixture – after cooling in ice – precipitated with 4 ml of 6% (w/v) TCA. After standing in ice for 30 min, the insoluble material was filtered on Whatman GFC glass fiber filters, followed by 5-fold washing with 5 ml portions of ice-cold 5% TCA. The last washings contained 100 μg ^{12}C-UTP/ml. Filters were dried in vacuo, placed in 10 ml of toluene scintillator fluid, and counted. In cases in which the incorporation of radioactivity was very low, another procedure was also employed: 100–200 μl of the reaction mixture, after addition of sodium pyrophosphate and ^{12}C-UTP, were cooled in ice, rapidly applied to strips of Whatman 3MM paper (18 × 3 cm), and eluted for 10 hr with a mixture of 1 M ammonium acetate and ethanol (1:1, v/v), the pH having been adjusted to 4.2 with acetic acid. The strips were washed twice with cold ethanol, once in ethanol ether (2:1), and dried in a warm stream of air. The spots were localized in UV, cut out, and counted as described. This procedure diminishes the background to less than 20 cpm and allows determination of very low incorporation rates.

Table 1
Dependence of [^{3}H]GMP incorporation rate on template concentration.

Template (nmoles)	Control (cpm)	Treated (cpm)
–	227	–
7.25	45,300	5,560
14.5	75,200	6,219/5,800
29.0	116,900	5,860
58.0	144,915	6,413
116.0	133,679	7,285

The reaction mixture contained in 0.5 ml various amounts of treated and non-treated poly(C) (nucleotide-P) (isolated after a 4 hr treatment at 37° with 7 moles/mole β, β′-dichlordiethylamine hydrochloride at pH 7.4); 0.2 μmole [^{3}H]GTP (specific activity 6×10^3 cpm/mμmole); 0.2 μmole UTP; and 20 μg polymerase protein. Incubation was for 25 min at 37° and was terminated by the addition of 0.2 ml saturated sodium pyrophosphate and 300 μg albumin with 4 ml 6% TCA. The insoluble material was processed as described in the text (GFC filter method).

It has been postulated [11, 19–24] that nitrogen mustard, or its aziridine derivative, is one of the forms of activated cyclophosphamide, but so far this has not been substantiated convincingly.

According to our results, obtained with labelled cyclophosphamide after activation by microsomal hydroxylation (to be published elsewhere), we conclude that, in addition to I, the asymmetric phosphate diamide – β, β′-dichlordiethylamidophosphamide – is formed. There can be no doubt, however, that the effect of activated cyclophosphamide is associated with the alkylating properties of the β-chlorethyl side chains, present in nitrogen mustard [11].

Besides the expected reduction of the normal template function of poly(C) for guanine, there is a slight – but statistically proven – tendency towards abnormal base pairing with uridine. This abnormal pairing

* Incubation for 4 hr at 37° with the 12-fold amount of cyclophosphamide (radioactivity labelled in the side-chain), previously activated by microsomal oxydative hydroxylation, gave a 1:20 incorporation rate, based on polynucleotide phosphorus.

Table 2
Abnormal base pairing with poly(C) as template before and after treatment with β, β'-dichlordiethylamine.

Incubation time (min)	Poly(C)(pmoles incorporated) Control GMP	UMP	(%)	Treated GMP	UMP	(%)	Difference (μmoles)
10	10,000	1.3	0.013	1,400	1.8	0.128	0.50
20	15,600	2.65	0.017	1,975	3.42	0.163	0.77
35	23,900	4.74	0.020	2,750	6.2	0.225	1.46
50	32,200	5.50	0.017	3,400	8.3	0.244	2.80

The reaction mixture contained 50–55 μmoles template poly(C) after 4 hr treatment with I, as specified under Materials and methods. Individual runs for each incubation time with treated and untreated template were carried out simultaneously. Several 100 μl samples per run were applied to Whatman 3MM strips and processed as described. The results are the average from two closely agreeing experimental series, and are statistically substantiated with regard to their tendency to exceed the corresponding controls. ($T = 0$; $\mu = 39$; $\sigma_T = 12.75$; $Z = 3.059$; $P \approx 0.001$). The incorporation figures contain a relatively high, but constant background (deducted here) corresponding to about 18 pmoles nucleotide, probably due to unspecific retention of radioactivity on enzyme protein or albumin. The figures for GMP incorporation are taken from a separate run (not shown), recording the time dependence of GMP incorporation under the same conditions as above.

rate amounts to 0.13–0.24% of the rate obtained for guanine under analogous conditions. It is therefore possible that cyclophosphamide gives rise to abnormal amino acid incorporation *in vivo*. Triphosphate incorporation *in vitro* (with modified templates) can, of course, only simulate true *in vivo* replacement and gives reliable results only with poly(C) derivatives as templates (poly(C) shows an ordered structure over a wide range) [25].

It should be added that the incorporation of triphosphates has proved to be a much more responsive method for the detection of mutagenic reactions than the incorporation of amino acids in the translation process *in vitro* [9].

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References

[1] D. Brittinger, Humangenetik 3 (1966) 156.
[2] K.E. Hampel, Int. J. Clin. Pharmacol. 1,4 (1968) 349.
[3] E. Gebhart, in: Chemical Mutagenesis in Mammals and Man, eds. F. Vogel and G. Röhrborn (Springer Verlag, Berlin, Heidelberg, New York, 1970) p. 367.
[4] G. Röhrborn, in: Chemical Mutagenesis in Mammals and Man, eds. F. Vogel and G. Röhrborn (Springer Verlag, Berlin, Heidelberg, New York, 1970) p. 294.
[5] P. Propping, G. Röhrborn and W. Buselmaier, Molec. Gen. Genet. 117 (1972) 197.
[6] D.B. Ludlum, Biochim. Biophys. Acta 213 (1970) 142.
[7] D.B. Ludlum Biochim. Biophys. Acta 247 (1971) 412.
[8] B. Singer and H. Fraenkel-Conrat, Biochemistry 8 (1969) 3260.
[9] B. Singer and H. Fraenkel-Conrat, Biochemistry 9 (1970) 3694.
[10] G.E. Means and H. Fraenkel-Conrat, Biochim. Biophys. Acta 247 (1971) 441.
[11] H.M. Rauen and U. Dirschka, Arzneimittel Forschg. 14 (1964) 159.
[12] M.D. Mamet-Bratley, Biochim. Biophys. Acta 195 (1969) 422.
[13] P.S. Chen. T.Y. Toribara and H. Warner, Anal. Chem. 28 (1958) 1756.
[14] S. Siegel, in: Nonparametric Statistics for the Behavioral Sciences, ed. S. Siegel (McGraw-Hill Book Co., Inc., New York, Toronto, London, 1956) p. 150.
[15] W.G. Verly and L. Brakier, Biochim. Biophys. Acta 174 (1969) 674.
[16] R.W. Ruddon and I.M. Johnson, Mol. Pharmacol. 4 (1968) 258.
[17] O.M. Friedman and E.A. Boger, Anal. Chem. 33 (1961) 906.
[18] O.L. Klamerth and M. Kopun, European J. Biochem. 21 (1971) 199.
[19] H. Arnold and F. Bourseaux, Angew. Chem. 70 (1958) 539.
[20] H. Arnold and F. Bourseaux, Nature 181 (1958) 931.
[21] N. Brock and H. J. Hohorst, Nat. Wiss. 49 (1962) 610.
[22] N. Brock and H.J. Hohorst, Arzneimittel-Forschg. 13 (1963) 1021.
[23] N. Brock and H.J. Hohorst, Cancer 10 (1967) 900.
[24] O.M. Friedman, Cancer Chemotherapy Reports 51 (1967) 327.
[25] G.D. Fasman, C. Lindlow and L. Grosman, Biochemistry 3 (1964) 1015.