Biochimica et Biophysica Acta 867 (1986) 24–30
Elsevier

BBA 91580

Relationship between DNA acid-solubility and frequency of single-strand breaks near apurinic sites

Suzanne Bricteux-Grégoire [a], Michel Liuzzi [b], Myriam Talpaert-Borlé [b], Martial Winand [c] and Walter G. Verly [a,*]

[a] *Biochemistry Laboratory, Faculty of Sciences, Liège University, Sart Tilman B6, 4000 Liège (Belgium),*
[b] *Biochemistry Laboratory, Biology Group, CEC Joint Research Centre, Ispra (Italy) and*
[c] *National Institute of Radioelements, Fleurus (Belgium)*

(Received November 26th, 1985)

Key words: DNA damage; DNA alkylation; Single-strand break; Apurinic site; Strand-break frequency

Using [^{32}P]DNA alkylated with [^{3}H]methyl methanesulfonate, depurinated by heating at 50°C for various periods, then treated with sodium hydroxide, a table was constructed giving the DNA fraction soluble in 5% perchloric acid at 0°C as a function of the frequency of strand breaks. The alkaline treatment placed a break near each apurinic site; the apurinic sites were counted in two ways which gave consonant results: by the loss of [^{3}H]methyl groups and by reaction with [^{14}C]methoxyamine. The ^{32}P label of DNA was used to measure the acid-solubility.

Introduction

Paquette et al. [1] used DNA alkylated with methyl methanesulfonate and partially depurinated as substrate to assay for an AP endonuclease activity. After incubation with the enzyme, the DNA fraction that became soluble in 5% $HClO_4$ was measured; a linear relationship was found between the DNA acid-soluble fraction and the amount of enzyme in the incubation assay as long as the acid-soluble fraction remained lower than half the value given by an alkaline treatment placing a break near each apurinic site so that the enzyme could operate at maximum velocity. Later work, which has not been published, showed that the linear relationship did not exist when the amount of AP endonuclease was low, and that extrapolation of the quasi-linear part of the experimental curve indicated a negative acid-soluble fraction when there was no enzyme.

Indeed, proportionality should not be expected between the amount of enzyme and the acid-soluble fraction, but rather, between the amount of enzyme and the amount of product, i.e., the breaks near the AP sites. It is thus important to have the precise relationship between acid-solubility and break frequency in methylated-depurinated DNA. This relationship is also necessary in order to have an enzyme unit defined relative to the number of breaks produced in DNA strands per unit time.

The problem has been solved experimentally using [^{3}H]methylated [^{32}P]DNA that was submitted to various extents of depurination before measuring the number of apurinic sites and determining, after an alkaline treatment, the acid-soluble ^{32}P. Two methods were used to count the apurinic sites: the loss of [^{3}H]methyl groups; the quantitative and specific fixation of [^{14}C]methoxyamine on the apurinic sites.

* To whom correspondence should be addressed.

Materials and Methods

[^{3}H]Methylated [^{32}P]DNA

DNA, randomly labelled with ^{32}P, was prepared from *Escherichia coli* B41 cells grown in the presence of [^{32}P]phosphate using the method of Marmur [2]. The [^{32}P]DNA was purified by treatments with T1 (PL Biochemicals) and T2 (Sigma) RNAases, then with proteinase K (Boehringer). The shape of the thermal denaturation curve and the hyperchromic jump on the one hand, the absorbance ratios 260 nm/280 nm and 260 nm/230 nm on the other hand, indicated that the [^{32}P]DNA contained negligible RNA or protein contaminations.

1.5 mg [^{32}P]DNA in 2 ml 0.5 M potassium phosphate (pH 7.0) and 50 μl of [^{3}H]methyl methanesulfonate (IRE, Fleurus) were mixed and incubated 1 h at 37°C. This treatment was followed by an extensive dialysis at 4°C against 0.15 M NaCl/0.015 M sodium citrate (pH 7.0) (SSC), for several days, changing the bath every day, until no more ^{3}H could be detected in the dialysate.

Determinations of the specific radioactivities of [^{3}H]methyl and [^{32}P]DNA

[^{3}H]Methyl iodide was used simultaneously for the synthesis of [^{3}H]methyl methanesulfonate and [^{3}H]methyl *p*-toluenesulfonate. The specific radioactivity of the latter compound was determined on the peak given in a HPLC chromatography; it was found to be $1.399 \cdot 10^7$ and $1.221 \cdot 10^7$ dpm/μmol, in two different preparations, relative to a standard that was used throughout this work. The [^{3}H]methyl specific radioactivity in [^{3}H]methylated [^{32}P]DNA, prepared with the [^{3}H]methyl methanesulfonate, was assumed to be the same as that of the [^{3}H]methyl *p*-toluenesulfonate.

To determine the ^{32}P specific radioactivity of the [^{3}H]methylated [^{32}P]DNA, aliquots were mineralized and divided in two. One part was used to measure the radioactivity (the yield of the scintillation counter was assumed to be 100%); phosphorus content was determined on the other part according to Chen et al. [3] with reference to a calibration curve constructed with KH_2PO_4 solutions of known concentrations. These measurements were done in triplicate; the ^{32}P specific radioactivity of the [^{3}H]methylated [^{32}P]DNA was $8.63 \cdot 10^4$ and $1.57 \cdot 10^5$ dpm/μmol phosphorus in two different preparations. A solution of [^{32}P]phosphate was kept to correct all results for radioactive decay.

Depurination

The [^{3}H]methylated [^{32}P]DNA in SSC (200 μg/ml) was heated at 50°C for 24 h. Aliquots were taken at 0, 1, 2, 3, 6 and 24 h and used to measure: (1) the ^{3}H/^{32}P ratio in DNA; (2) the ^{14}C/^{32}P ratio in DNA after reaction with [^{14}C]methoxyamine; (3) the fraction of ^{32}P directly soluble in 5% $HClO_4$ at 0°C; (4) the fraction of ^{32}P soluble in 5% $HClO_4$ at 0°C after an alkaline treatment placing breaks near all AP sites. All these determinations were done in triplicate.

^{3}H/^{32}P ratio in [^{3}H]methylated-depurinated [^{32}P]-DNA

To a 20 μl aliquot from the depurination solution was added 100 μl SSC containing 200 μg calf thymus DNA and 240 μl 95% ethanol. After 24 h at −30°C, the DNA was collected by centrifugation, rinced twice with 80% ethanol, dried under vacuum, then hydrolyzed in 600 μl 1 M HCl at 50°C for 1 h, vortexing from time to time to ease the dissolution, then at 65°C for 30 min. To 500 μl hydrolysate was added 5 ml Aquassure (New England Nuclear) and the ^{3}H and ^{32}P radioactivities were assayed in a scintillation counter. Each channel was calibrated with the ^{3}H and ^{32}P standards already mentioned; corrections were made for the contribution of one radionuclide in the channel used to count the other.

The ^{3}H/^{32}P ratio was used to calculate the abundance of methyl groups in the [^{3}H]methylated-depurinated [^{32}P]DNA. From the [^{3}H]methyl and [^{32}P]phosphorus specific radioactivities, one calculates that 1 methyl group per 1000 nucleotides corresponds to a ratio of $:1.399 \cdot 10^7/8.63 \cdot 10^7 = 0.162$ in experiment I and $1.221 \cdot 10^7/1.57 \cdot 10^8 = 0.0778$ in experiment II.

Counting AP sites with [^{14}C]methoxyamine

Talpaert-Borlé and Liuzzi [4] have given the conditions to have a specific and quantitative reaction of [^{14}C]methoxyamine with AP sites in DNA. The amount of ^{14}C retained by DNA can

thus be used to count AP sites.

The specific radioactivity of the [^{14}C]methoxyamine was determined by mass spectrometry. The mass 47 and 49 peaks were measured. Taking into account the contributions at mass 49 of species other than $^{14}C^{1}H_3{}^{16}O^{14}N^{1}H_2$, the ratio $^{14}C/^{12}C$ could be calculated and, from that, the specific radioactivity of the [^{14}C]methoxyamine. It was found to be: $1.37 \cdot 10^7$ dpm/μmol, in good agreement with the value of 6.25 Ci/mol ($1.387 \cdot 10^7$ dpm/μmol) given by the manufacturer.

To 60 μl from the depurination solution was added 15 μl 25 mM [^{14}C]methoxyamine/10 mM NaCl/10 mM sodium phosphate (pH 7.2). After 30 min at 50°C, three 20 μl aliquots were placed on Whatman GFC fiber-glass filters which were immersed in 1 M HCl at 0°C. Each filter was passed in four such baths successively, remaining 1 h in each with occasional shaking. The filter was then introduced in a vial containing 1 ml 1 M HCl and heated 1 h at 50°C to hydrolyze the DNA. After cooling, 10 ml Aquassure was added and the mixture was vortexed vigorously. The vial contents were analyzed in two channels (^{14}C and ^{32}P) of a scintillation counter. The yields in each channel for ^{3}H and ^{32}P were measured with the standards already described; for ^{14}C, we used an absolute standard of [^{14}C]hexadecane obtained from Amersham. A computer program enabled us to calculate the ^{14}C and ^{32}P radioactivities.

Three identical controls were carried out in a similar way except that the 60 μl of depurination solution were replaced by 60 μl SSC. The mean ^{14}C radioactivity of the controls was used to correct the ^{14}C values given by the samples containing [^{3}H]methylated-depurinated [^{32}P]DNA.

The ^{14}C (corrected)/^{32}P ratio was used to calculate the abundance of AP sites in the [^{3}H]methylated-depurinated [^{32}P]DNA. From the [^{14}C]methoxyamine and [^{32}P]DNA specific radioactivities, one calculates that 1 AP site per 1000 nucleotides corresponds to a ratio of :$1.387 \cdot 10^7/8.63 \cdot 10^7 = 0.161$ in experiment I and $1.387 \cdot 10^7/1.57 \cdot 10^8 = 0.0884$ in experiment II.

Acid-soluble ^{32}P from [^{3}H]methylated-depurinated [^{32}P]DNA with and without a preliminary alkaline treatment

To 20 μl from the depurination solution was added 20 μl 0.4 M NaOH, and the mixture was incubated at 37°C for 15 min. This treatment enables to place a break near each AP site [1] without releasing inorganic phosphate. SSC (100 μl) containing 200 μg calf thymus DNA, and then 5.8% $HClO_4$ (860 μl) were successively added. After 10 min at 0°C, the tube was centrifuged at $16\,000 \times g$ for 10 min. One-half of the supernatant (500 μl) was removed and added to 5 ml Aquassure; the ^{32}P radioactivity was measured (A cpm). The tube, still containing the acid-insoluble ^{32}P and one-half of the acid-soluble ^{32}P, was heated with occasional vortexing for 1 h in order to hydrolyze the polynucleotides. To the hydrolysate was added 5 ml Aquassure and the ^{32}P radioactivity was determined (B cpm). The ^{32}P acid-soluble fraction $2A/(B + A)$ was thus obtained from a single aliquot and was consequently independent of any error on the amount of sample used for analysis.

The same procedure was followed but without an alkaline treatment in order to estimate the acid-soluble fraction that might be due to breaks present in the [^{3}H]methylated-depurinated [^{32}P]-DNA strands before the alkaline treatment. The 20 μl 0.4 M NaOH was replaced by 20 μl SSC. These controls gave ^{32}P values in the supernatants indistinguishable from the background, showing that the [^{3}H]methylated-depurinated [^{32}P]DNA contained practically no parasitic breaks.

Results

Determination of the number of AP sites in the [^{3}H]methylated [^{32}P]DNA

Depurination already occurred during the treatment of the [^{32}P]DNA with the [^{3}H]methyl methanesulfonate and during the long dialysis used to eliminate the radioactive alkylating agent. It was thus imperative to know how many AP sites were already in the methylated DNA before the depurination step.

This number has been determined with [^{14}C] methoxyamine. One difficulty is that, to have a complete reaction of AP sites with [^{14}C]methoxyamine, a minimal 10-min heating at 50°C is needed and that depurination occurs in the meantime. To count AP sites with [^{14}C]methoxyamine, we proceeded as described in Materials and Methods

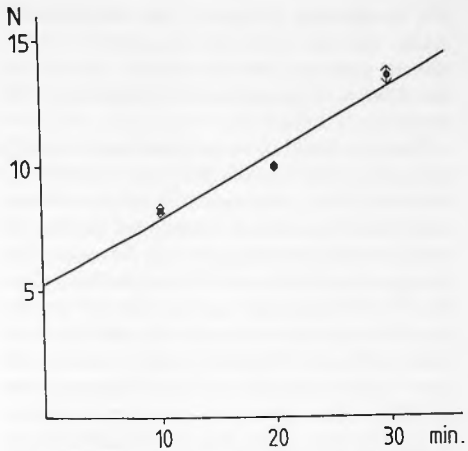

Fig. 1. Number of AP sites in the [^{3}H]methylated [^{32}P]DNA. The [^{3}H]methylated [^{32}P]DNA was incubated with [^{14}C]methoxyamine at 50°C for 10, 20 or 30 min before measuring the $^{14}C/^{32}P$ ratio. The number, N, of AP sites per 1000 nucleotides, calculated from these ratios, is plotted against time. The frequency of AP sites in the starting [^{3}H]methylated [^{32}P]DNA is obtained by extrapolation at zero-time incubation with [^{14}C]methoxyamine. The graph relates to Expt. 1 (see Table I); the measurements were done in triplicate; the mean ratios are given together with the standard errors.

except that three incubation times were used (10, 20 and 30 min) before measuring the $^{14}C/^{32}P$ ratio in the DNA. Each determination was done in triplicate. The $^{14}C/^{32}P$ ratios were converted in AP sites per 1000 nucleotides. The number of AP sites at zero-time incubation with [^{14}C]methoxyamine was obtained by extrapolation (Fig. 1) and found to be 5 per 1000 nucleotides of the [^{3}H]methylated [^{32}P]DNA in two independent experiments.

The loss of methyl groups and the appearance of AP sites during the depurination of the [^{3}H]methylated [^{32}P]DNA

The [^{3}H]methylated [^{32}P]DNA was heated at 50°C for 24 h as described in Material and Methods and aliquots were taken at 0, 1, 2, 3, 6 and 24 h to determine the $^3H/^{32}P$ ratio in DNA. These ratios were converted into numbers of methyl groups per 1000 nucleotides (Table I).

Aliquots were taken at the same times to react [^{3}H]methylated-depurinated [^{32}P]DNA with [^{14}C]methoxyamine as described in Material and Methods. There was, of course, an additional de-

TABLE I

LOSS OF METHYL GROUPS, FORMATION OF AP SITES IN METHYLATED DNA HEATED AT 50°C AND ACID-SOLUBILITY OF THE METHYLATED-DEPURINATED DNA AFTER AN ALKALINE TREATMENT

The table gives the results of two independent experiments. [^{3}H]Methylated [^{32}P]DNA in SSC was heated at 50°C. Aliquots were taken at the indicated times to measure: A, the $^3H/^{32}P$ ratio in DNA, from which the number n of methyl groups per 1000 nucleotides could be calculated, and the decrease Δ of this number; B, after reaction with [^{14}C]methoxyamine, the $^{14}C/^{32}P$ ratio in DNA from which the number n' of AP sites per 1000 nucleotides could be calculated; this number was corrected (n' corr) to take account of the depurination occurring during the reaction with the [^{14}C]methoxyamine and the increase, Δ', of the number of AP sites was calculated; D, the ^{32}P fraction soluble in 5% $HClO_4$ at 0°C (A.S.). All figures are averages of triplicates. The error on any of them is smaller then 5%.

Expt.	Time h	A: methyl groups			B: AP sites				D: A.S. %
		$^3H/^{32}P$	n	Δ	$^{14}C/^{32}P$	n'	n' corr	Δ'	
I	0	15.0	92	0	2.17	13.5	5	0	–
	1	13.3	82	10	3.02	18.8	16	11	7.7
	2	12.3	76	16	4.47	27.8	25	20	14.9
	3	11.6	72	20	5.00	31.1	28	23	17.5
	6	8.7	54	38	7.39	46.0	44	39	29.9
	24	3.8	23	69	13.0	80.9	81	76	48.8
II	0	6.71	86	0	1.04	11.7	5	0	1.0
	1	6.12	79	7	1.36	15.4	14	9	5.0
	2	5.43	70	16	1.71	19.3	18	13	10.1
	3	5.29	68	18	2.06	23.3	22	17	14.5
	6	4.37	56	30	2.95	33.4	33	28	23.8
	24	2.21	28	58	4.97	56.3	56	51	40.0

purination during the 30 min heating at 50°C with the labelled reagent. The correction for zero-time depurination has already been calculated (see preceding section); the other data were easily corrected since the rate of depurination, at the same temperature and at each of the chosen times, was known from the data on the loss of methyl groups.

The increase of the number of AP sites during the depurination was calculated (Δ' in Table I) and compared with the loss of methyl groups (Δ in Table I) for the same durations of 50°C heating. The two sets of data were in good agreement in two different experiments.

Correlation between frequency of nicks near AP sites and acid-solubility of [^{3}H]methylated-depurinated [^{32}P]DNA

Aliquots taken from the depurination solution were submitted to an alkaline treatment sufficient to place a break near each AP site before measuring the fraction of ^{32}P soluble in 5% $HClO_4$ at 0°C as described in Material and Methods. The results were not corrected for controls without alkaline treatment, since the control values were not different from radioactivity background. The results are in Table I.

We are looking for a correlation between nicks and acid-soluble fraction. But, since the alkaline treatment placed a nick near each AP site and that other nicks were almost absent, the number of nicks is equal to the number of AP sites. The average of the number of AP sites calculated from the $^{14}C/^{32}P$ ratio after reaction with [^{14}C]methoxyamine, and the number of AP sites calculated from the loss of [^{3}H]methyl groups corrected for those already present in the [^{3}H]methylated [^{32}P]DNA before the depurination step, was taken as the number of nicks in the [^{3}H]methylated-depurinated [^{32}P]DNA after the alkaline treatment in Fig. 2 and Table II. In Fig. 2, these averages are plotted against the acid-soluble fraction of DNA. Table II gives the numerical figures corresponding to the sigmoidal curve drawn in Fig. 2 through the experimental points.

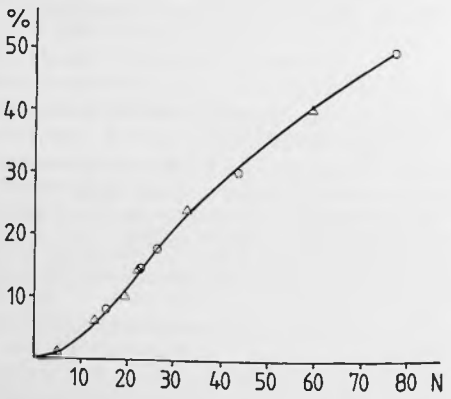

Fig. 2. Single-strand break frequency and acid-solubility of methylated-depurinated DNA. [^{3}H]Methylated [^{32}P]DNA in SSC was depurinated by heating at 50°C. Samples were taken at intervals, incubated at alkaline pH to place a break near each AP site before the solubility (%) of the [^{3}H]methylated-depurinated [^{32}P]DNA was measured. The number, *N*, of single-strand breaks is the average of two independent determinations: loss of [^{3}H]methyl groups corrected for the number of AP sites already in the [^{3}H]methylated [^{32}P]DNA; AP sites counted with [^{14}C]methoxyamine. The results from two different experiments (Table I: ○ = Expt. I; △ = Expt. II) are distributed along a sigmoidal curve.

TABLE II

BREAKS PER 1000 NUCLEOTIDES AS A FUNCTION OF DNA ACID-SOLUBLE FRACTION

Methylated-depurinated DNA was submitted to an alkaline treatment to place a break near each AP site. The break frequency (per 1000 nucleotides) is given together with the corresponding solubility of the methylated-depurinated DNA in 5% $HClO_4$ at 0°C (A.S.). The data are from the sigmoidal curve drawn in Fig. 2.

A.S. %	Breaks per 1000 nucleotides	A.S. %	Breaks per 1000 nucleotides
0	0	26	37
2	7	28	40
4	10	30	43
6	13	32	46
8	16	34	48
10	18	36	51
12	20	38	55
14	23	40	59
16	25	42	62
18	27	44	66
20	29	46	70
22	32	48	74
24	35	50	80

Discussion

The aim of this work was to have a table relating strand-break frequency to DNA acid-solubility. DNA randomly labelled with ^{32}P, was treated with [^{3}H]methyl methanesulfonate and the [^{3}H]methylated [^{32}P]DNA was subsequently heated at 50°C. The appearance of AP sites was monitored at different times in two different ways: by the loss of the [^{3}H]methyl groups; by specific and quantitative fixation of [^{14}C]methoxyamine on the AP sites. At the same intervals, aliquots were submitted to alkaline treatments to nick the strands near all AP sites and the ^{32}P used to determine the acid-soluble DNA fraction.

Paquette et al. [1] followed the loss of methyl groups from DNA alkylated with [^{3}H]methyl methanesulfonate to estimate the number of AP sites in the [^{3}H]methylated-depurinated DNA. Such procedure ignores the formation of AP sites during the alkylation treatment and the purification of the [^{3}H]methylated DNA. The AP sites can be counted with [^{14}C]methoxyamine [4], but, when the method is applied to [^{3}H]methylated-depurinated [^{32}P]DNA, an error is introduced which is due to the depurination occurring during the [^{14}C]methoxyamine treatment. In this work, each method was used to correct the other: [^{14}C]methoxyamine was used to count the AP sites already present in the [^{3}H]methylated [^{32}P]DNA before the depurination step, whereas the rate of loss of [^{3}H]methyl groups was used to correct for the depurination that continued during the treatment with [^{14}C]methoxyamine. We finally obtained two sets of results which were in very good agreement in two different experiments (Table I).

To nick the [^{3}H]methylated-depurinated [^{32}P]DNA, a mild alkaline treatment was used sufficient to have a break 3′ to all AP sites [1], but insufficient to induce a second break on its 5′ side [5]. The alkaline treatment catalyzes a β-elimination reaction yielding strand fragments carrying, at their 3′ ends, the AP site with a 2′-3′ double bond. The [^{3}H]methylated-depurinated [^{32}P]DNA did not contain strand breaks; thus, after the incubation at alkaline pH, the only breaks were those produced near AP sites by this treatment. A graph (Fig. 2) could thus be plotted giving the [^{3}H]methylated-depurinated [^{32}P]DNA acid-solubility as a function of break frequency. The curve passing through the experimental points is a sigmoid; the data in Table II were taken from this sigmoidal curve. Table II can be used only for methylated-depurinated DNA used exactly in the conditions that we have described for the acid-solubility assay.

Is this Table II useful for the assay of an AP endonuclease or a catalyst of β-elimination?

The assay of β-elimination catalyst will be discussed first since we have used, in this work, OH^- ions to nick the DNA strands near the AP sites.

For such an assay, the DNA may be methylated as we did but depurination must be during a fixed time; the number of remaining methyl groups and the number of AP sites are both constant and it is the number of nicks near AP sites which varies [1]. This is different from what has been done in this work, where the sum of methyl groups and AP sites was fixed, but the number of methyl groups and AP sites varied inversely with the duration of depurination; moreover, a break was placed near all AP sites. We hope that the presence of an intact AP site instead of a methylated purine does not change too much the acid-solubility of the DNA fragments so that Table II can be used to assess break frequencies in an assay for a β-elimination catalyst causing only partial nicking near the AP sites.

An AP endonuclease hydrolyzes the phosphodiester bond 5′ to the AP sites. The different acid-solubility of the fragments of DNA strands obtained with these enzymes rather with a β-elimination catalyst, is probably not so much that the AP site is at the 5′ end instead of the 3′ end, but rather that the fragments have a terminal 3′-OH and not a 2′-3′ double bond. We cannot repeat our work using an AP endonuclease instead of an alkaline treatment to nick near all AP sites, since some of them are protected by the methyl groups against the action of the enzyme [6]. We hope however, that Table II can be used without too great an error for the assay of AP endonucleases devoid of exonuclease and phosphatase activities.

The sigmoidal shape of the curve in Fig. 2 indicates clearly that, even in conditions of substrate (AP sites) concentration largely above K_m, the amount of enzyme cannot be proportional to

the DNA acid-solubility. Acid-solubility must be first converted in break frequencies which is the true additive function; this is also true for background corrections if the starting methylated-depurinated DNA has already a significant acid-soluble fraction.

Acknowledgements

We thank Dr. S. Facchetti of Ispra, who made the mass-spectrometer analyses for us. M.L. was a C.E.C. fellow. The work was supported by grants from the Fonds Belge de la Recherche Fondamentale Collective, the Fonds Cancérologique de la C.G.E.R., and the Direction Biology, Radiation Protection and Medical Research, DG XII, C.E.C. This publication is contribution 2193 of the Programme Biology, Radiation Protection and Medical Research, Directorate General XII for Research, Science and Education of the Commission of the European Communities.

References

1 Paquette, Y., Crine, P. and Verly, W.G. (1972) Can. J. Biochem. 50, 1199–1209
2 Marmur, J. (1961) J. Mol. Biol. 3, 208–218
3 Chen, P.S., Jr., Toribara, T.Y. and Huber Warner (1956) Anal. Chem. 28, 1756–1758
4 Talpaert-Borlé, M. and Liuzzi, M. (1983) Biochim. Biophys. Acta 740, 410–416
5 Grossman, L. and Grafstrom, R. (1982) Biochimie 64, 577–580
6 César, R. and Verly, W.G. (1983) Eur. J. Biochem. 129, 509–516