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Estimation of the amount of 5-methylcytosine in *Drosophila melanogaster* DNA by amplified ELISA and photoacoustic spectroscopy

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We have previously reported a sensitive immunochemical method for detecting 5-methylcytosine in DNA which involves spotting DNA samples on nitrocellulose paper and detection of 5-methylcytosine, if any, by a combination of the double antibody method and a staining reaction brought about by biotin-avidin and peroxidase. We report here a linear relationship between the concentration of 5-methylcytosine in DNA and staining intensity, as recorded by photoacoustic spectroscopy. It appears possible to obtain, by this method, reliable quantitative estimates of 5-methylcytosine in nanogram quantities of intact DNA. When *Drosophila melanogaster* DNA was assayed for the presence of 5-methylcytosine by this method, a faint but clearly positive reaction was obtained. When the photoacoustic intensity of this stained spot is compared with a calibration plot derived from ϕX174 DNA whose 5-methylcytosine content is known, we obtain, for *D. melanogaster* DNA, one 5-methylcytosine residue in ~12 500 bases or 0.008 mol% methylation.
Key words: biotin-avidin cross-linking/DNA methylation/DNA-protein interaction/gene regulation/photoacoustic spectroscopy/X-chromosome inactivation

Introduction

5-Methylcytosine (5mC) occurs in the DNA of most organisms (Ehrlich and Wang, 1981). When present in place of cytosine in DNA, 5mC can influence DNA-protein interactions (Razin and Riggs, 1980) as well as DNA conformation (Behe *et al.*, 1981). 5mC has been implicated in many cellular functions such as the regulation of gene activity (Holliday and Pugh, 1975; Razin and Riggs, 1980), recombination (Korby and Hays, 1982) and maintenance of the inactive state of the mammalian X chromosome (Liskay and Evans, 1980; Mohandas *et al.*, 1980). We have previously reported a sensitive immunochemical method which permits the detection of femtomol (10^{-15} mol) quantities of modified nucleotides in nanogram quantities of DNA (Achwal and Chandra, 1982). This method appears to provide greater sensitivity than conventional methods, including h.p.l.c. and nearest neighbour analysis (Achwal *et al.*, 1983). Briefly, the method involves spotting DNA on nitrocellulose paper, incubation of the paper strip with the antibody solution and treatment with biotinylated anti-antibody. The paper strip is then incubated with a complex of avidin-DH and biotinylated peroxidase and stained with diaminobenzidine tetrahydrochloride (DABT) and hydrogen peroxide (Hsu *et al.*, 1981a, 1981b). This set of reactions yields a coloured spot on the paper if the DNA contains 5mC. DABT is preferred over other peroxidase-staining agents because the oxidised products of DABT seems to remain bound to the enzyme even after extensive washing, yielding a coloured spot. The application of photoacoustic spectroscopy for measuring the staining intensity of these spots and the quantitative estimation of 5mC in DNA by this method are described here. In particular, we have attempted to obtain quantitative data on the amount of cytosine methylation in *Drosophila melanogaster* DNA (Achwal *et al.*, 1983).

Results

DNA from a species of mealybug belonging to the genus *Planococcus* (provisionally identified as *P. lilacinus* Cockerell: Coccoidea; Homoptera; Insecta) which appears to contain very high amounts of 5mC (~2.3 mol%) (Deobagkar *et al.*, 1982) gave a strong staining reaction. On the other hand, the same concentrations of DNA from the phage ϕX174, which contains one 5mC residue in a genome of 5386 nucleotides (Sanger *et al.*, 1978), gave a weakly stained spot. This observation suggested to us that there may be a correspondence between staining intensity of a spot and the amount of 5mC contained in it.

Very few methods are, however, available for the accurate measurement of the intensity of a staining reaction on solid substrates, in this case nitrocellulose paper. Attempts to quantitate the avidin-biotin staining reaction by the use of ELISA (enzyme-linked immunosorbent assay) readers and gel scanners were not successful in our hands largely because the resolving power of these instruments was not sufficient for our purposes. We therefore attempted to estimate the staining intensity of the spots by examining them in a photoacoustic spectrometer (Rosencwaig, 1975). Our choice of photoacoustic spectroscopy was prompted by reports of successful application of this method in quantitating chromophores present in thin layer chromatograms, paper chromatograms and such other materials which are difficult to handle by conventional spectroscopic methods (Castleden *et al.*, 1979; Rosencwaig, 1980; Ferguson, 1983). The results given below demonstrate the greater sensitivity of this method compared with several other conventional methods, especially when solid substrates are involved.

For standardisation of the photoacoustic procedure, we used mealybug DNA and DNA from the phage ϕX174. Mealybug DNA has been shown, by dinucleotide analysis, to contain high amounts of 5mC (Deobagkar *et al.*, 1982). The staining intensity obtained with increasing concentrations of mealybug DNA is shown in Figure 1. Visual examination indicated a proportionality between the expected amount of 5mC in a DNA sample and the staining intensity of the spot.

When the photoacoustic signal due to the spot was plotted against the estimated amount of 5mC in each sample (Figure 2), a straight line was obtained. When ϕX174 DNA was used as standard, the results were as shown in Figure 3. With in-

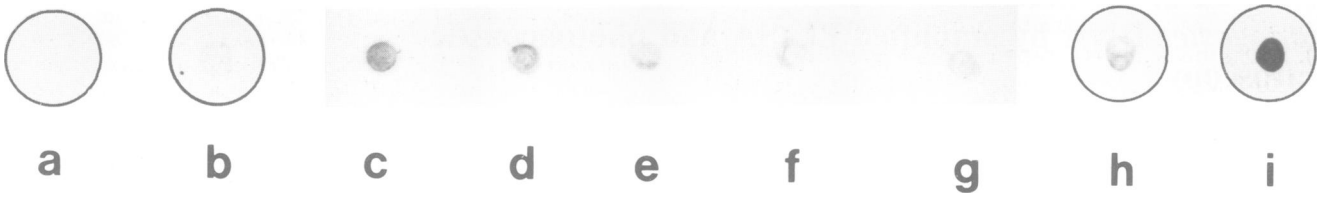

Fig. 1. Immunochemical spot assay for 5mC in DNA. DNA was spotted on nitrocellulose paper and stained for the presence of 5mC by the double antibody method as described in the text. **(a)** Non-methylated DNA from phage λ (100 ng); **(b)** DNA from *D. melanogaster* (100 ng); **(c–g)** DNA of the mealybug, *P. lilacinus*, in decreasing concentrations: **(c)** 4 ng (0.38 pmol); **(d)** 2.5 ng (0.24 pmol); **(e)** 1.6 ng (0.15 pmol); **(f)** 1.2 ng (0.12 pmol); **(g)** 0.6 ng (0.058 pmol); **(h)** DNA from a human diploid cell line, MRC-9, ATCC, CCL-212 (6 ng); **(i)** *P. lilacinus* DNA, 16 ng (1.56 pmol). In the case of mealybug DNA, the numbers in parentheses (pmol) represent the expected amount of 5mC in each DNA sample based on the assumption that this DNA contains 2.3 mol% 5mC (Deobagkar *et al.*, 1982).

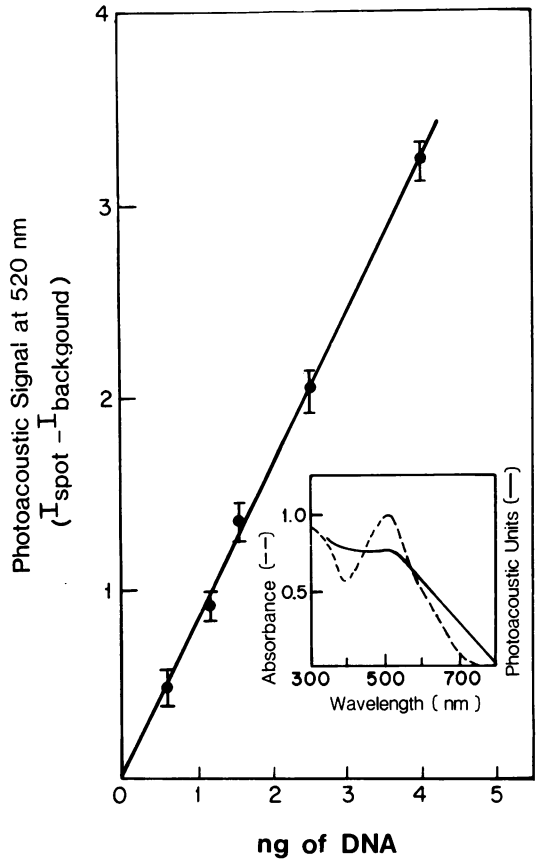

Fig. 2. Intensity of the photoacoustic signal of the spot ($I_{spot} - I_{background}$) plotted against increasing concentrations of mealybug (*P. lilacinus*) DNA (solid circles). Each point represents the average value of at least three measurements. **Inset**: the photoacoustic spectrum of DABT (solid line). The transmission spectrum of DABT remaining in solution after staining the nitrocellulose paper is also shown (broken line). The intensity of the photoacoustic signal is expressed in arbitrary units.

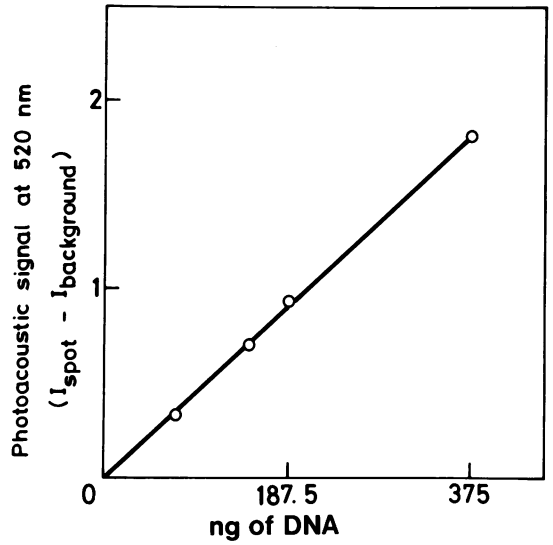

Fig. 3. Intensity of photoacoustic signal of the spot ($I_{spot} - I_{background}$) plotted against increasing concentration of φX174 DNA (observed values and the standard deviations are given in Table I).

Table I. Estimated total cytosine methylation in DNA from various sources based on the calibration plot derived from φX174 DNA (Figure 3)

Source of DNA	Amount of DNA	Photoacoustic signal at 520 nm ($I_{spot} - I_{background}$)	Estimated methylation, in mol%
Phage φX174	75 ng	0.3 ± 0.02	
	150 ng	0.75 ± 0.02	0.02
	187 ng	0.85 ± 0.02	
	385 ng	1.8 ± 0.02	
Mealybug[a]	0.6 ng	0.45 ± 0.05	1.75
	1.2 ng	0.9 ± 0.05	1.818
D. melanogaster	100 ng	0.3 ± 0.02	0.008
Human	6 ng	1.5 ± 0.05	1.22
Phage λ (not-methylated)	400 ng	0 ± 0	Not detectable

[a]Provisionally identified as *Planococcus lilacinus* Cockerell.

creasing concentrations of phage DNA the intensity of the photoacoustic signal again showed a linear increase, as in the case of mealybug DNA. The data from mealybug DNA and φX174 DNA were then used as calibration plots for estimation of 5mC content in DNA samples from other sources. Non-methylated DNA from the phage lambda, which is used as a negative control, did not show any staining and, as expected, also did not show a discernible photoacoustic signal (Figure 1; Table I).

Drosophila melanogaster

It is generally believed that *D. melanogaster* DNA does not contain detectable amounts of 5mC (Urieli-Shoval *et al.*, 1982; Doerfler, 1983, review). However, when 100 ng of *D. melanogaster* DNA was treated with antibody to 5mC, a faint but clearly positive reaction was visible (Achwal *et al.*,

1983). These spots, when examined in the photoacoustic spectrometer, gave a discernible signal (0.3 photoacoustic units). When this signal is compared with the calibration plot derived for mealybug DNA (Figure 2), we obtain an estimate of one 5mC residue in ~15 000–20 000 nucleotides of *D. melanogaster* DNA. On the other hand, when the intensity of this signal is compared with the calibration plot derived from φX174, we obtain a methylation level of 0.008 mol% or one 5mC residue in ~12 500 nucleotides. This difference in the level of methylation as estimated by the two standards may be due to the following reasons: the estimated level of cytosine methylation in mealybug DNA is based on our earlier investigation of this DNA by nick translation followed by dinucleotide analysis (Doebagkar *et al.*, 1982). A significant degree of variation between experiments was noted in this earlier study. When the level of methylation in mealybug DNA is estimated from the φX174 calibration plot, we obtain a methylation level of 1.75–1.81 mol% of 5mC (Table I). It is therefore possible that our earlier estimation of 2.3 mol% methylation in mealybug DNA is a slight overestimate.

Human DNA

The 5mC content of human DNA (isolated from a diploid fibroblast cell line), as estimated from the mealybug calibration plot (Figure 2), is 0.8 mol%. When the 5mC content of this DNA was estimated from the φX174 calibration plot, we obtain a methylation level of 1.22 mol%. Both these values are consistent with those obtained by other methods (Ehrlich *et al.*, 1982).

Discussion

The procedure outlined here appears to provide a relatively simple and rapid method for estimation of 5mC in a given sample of intact DNA. The stained spot on the nitrocellulose paper does not fade for at least 6 months and the intensity of staining remains unaffected on examination by photoacoustic spectroscopy. In addition, this method permits detection of extremely low levels of DNA methylation in nanogram quantities of DNA. In this respect it appears to offer advantages over h.p.l.c. and nearest neighbour analysis (Gruenbaum *et al.*, 1981; Singer *et al.*, 1979). H.p.l.c. is said to permit detection of 0.1 mol% of 5mC in DNA whereas the lower limit of detection by nearest neighbour analysis appears to be one 5mC residue in 10 000 nucleotides (Urieli-Shoval *et al.*, 1982). The immunochemical method, on the other hand, appears to permit detection of one 5mC residue in ~12 000–20 000 nucleotides, as judged by analysis of the staining intensity of *D. melanogaster* DNA in relation to that of known concentrations of φX174 and mealybug DNA. By visual examination of the spots on nitrocellulose paper it is possible to detect staining of even 10 ng of φX174 DNA but this low level of staining is not detected by the photoacoustic spectrometer when a 200 W Tungsten lamp is used in it (see Materials and methods).

It may be possible to improve the sensitivity of the photoacoustic method by an order of magnitude or more by substituting a 1000 W, high-pressure Xenon arc lamp for the 200 W Tungsten filament lamp used in the present study.

Materials and methods

Preparation of DNA

DNA from gravid females of the mealybug *Planococcus lilacinus* was isolated as described by Deobagkar *et al.* (1982). *D. melanogaster* DNA was isolated as described by Singh *et al.* (1981). Care was taken to avoid contamination from DNA of yeasts which are a common constituent of the food in which *Drosophila* are grown (Achwal *et al.*, 1983). Extensive RNase treatment was done to remove any contaminating RNA from DNA samples. λ DNA ('non-methylated') was obtained from Sigma Chemicals. It is isolated from phages grown in *dcm*$^-$ and *dam*$^-$ *Escherichia coli* and as a result is completely digested by *Eco*RIII, *Xba*I, *Mbo*I and *Bcl*I. This DNA was used as a negative control for the immunochemical assays. Human DNA was isolated from a diploid fibroblast cell line (MRC-9, ATCC, CCL-212). The cell line was maintained at the National Institute of Virology, Pune. It was originally derived from female 'Caucasian' foetus.

Preparation of antibodies

The elucidation and characterisation of rabbit antibodies to 5mC has been described previously (Achwal and Chandra, 1982). The 5mC-bovine serum albumin conjugate was injected into the foot-pad and back muscle of rabbits at 10-day intervals. Serum from these animals was prepared and purified by ammonium sulphate fractionation and DEAE column chromatography. The antibodies had a *K*a of $1.56 \times 10^9\ M^{-1}$. The reaction of antibodies with DNA was not inhibited by addition of 6-methyladenine, 7-methylguanine, thymine or cytosine at 10^{-5} M (Achwal and Chandra, 1982).

Immunochemical assay

The desired amounts of mealybug DNA in 0.6 μl of Tris-EDTA (Tris-HCl, 10 mM, pH 7.2, EDTA 2 mM) were spotted onto BA85 nitrocellulose paper. The paper was baked at 60°C for 4–6 h and the assay was carried out as described previously (Achwal and Chandra, 1982). The paper was placed in a Petri dish and flooded with 10 ml of Tris-buffered saline containing 100–120 μg of antibodies to 5mC. The paper was left in this solution at room temperature for 6–8 h and then washed extensively, also in Tris-buffered saline (Tris-HCl, pH 7.5, 10 mM and 0.14 M NaCl). It was then incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), and subsequently in a complex of biotinylated peroxidase and avidin-DH (Vector Laboratories). Many biotin molecules can be conjugated to a protein molecule. Each molecule of avidin can bind four biotin molecules with very high affinity. The biotin-avidin link is essentially irreversible. Avidin cross-links biotinylated peroxidase into a three-dimensional array such that although the biotin is bound by avidin, some sites remain free for binding to biotinylated anti-antibody (Hsu *et al.*, 1981a, 1981b). The paper was then washed extensively and stained for peroxidase using a substrate solution containing 0.1% DABT and 0.02% H_2O_2 (Hsu *et al.*, 1981a, 1981b). The paper strip was then washed and dried. DABT stains the peroxidase in this complex. Because of the high degree of signal amplification, it appears that even small amounts of 5mC become detectable.

In an initial set of experiments, the nitrocellulose paper to which DNA had been bound was pre-incubated with 400 μg of normal human IgG to reduce any non-specific binding of the antibody to the paper. The staining intensity of the blot and surrounding areas remained unaffected whether or not the paper was pretreated in this manner. Thus, non-specific binding of antibodies to nitrocellulose paper seems to be negligible under the conditions employed in this assay.

Photoacoustic spectroscopy

Photoacoustic spectroscopy involves illumination of the sample with monochromatic light which is 'chopped' at a fixed frequency. The resultant pressure oscillation in the gas phase of the photoacoustic cell (when the sample returns to ground state non-radiatively) is picked up by a microphone as an acoustic signal of the same frequency. A quantitative assay is possible with the photoacoustic spectrometer as long as saturation effects are absent. These effects occur when the optimal absorption length, μ_β becomes less than the thermal diffusion length of the sample, ($\mu_\beta = 1/\beta$, where β = optical absorption coefficient). Nevertheless, for adsorbed layers, when the thickness of the sample is likely to be less than the thermal diffusion length of the sample, saturation effects are expected to be absent (McClelland and Kniseley, 1976; Lin and Dudek, 1979). The components responsible for the observed staining of 5mC in DNA appear to form a three-dimensional array (Hsu *et al.*, 1981a, 1981b), but the overall thickness of this complex is apparently not large enough to cause saturation effects. In any case, our investigations were confined to a range of concentrations within which no saturation effects were observed. Quantitative estimates of the chromophores present could therefore be obtained by measuring the intensity of the photoacoustic signal at the absorption maximum. Under these conditions the photoacoustic intensity is proportional to the absorption coefficient and hence, for a fixed wavelength, to the concentration of the chromophore.

The spectra of the spots of DNA stained by the immunochemical procedure were obtained in a single-beam photoacoustic spectrometer built at the Indian Institute of Science, Bangalore (Ganguly and Rao, 1981). The illuminating source is a 200 W Tungsten lamp. The chopping frequency employed for this study was 23 Hz. The magnitude of the photoacoustic signal was obtained by adjusting the quadrature component to zero and then recording the in-phase

component. By use of appropriate optics and manipulation of slit widths, the entire stained spot was illuminated for purposes of measurement.

When examined in the visible range, by a transmission spectrometer (Shimadzu, Model UV 200, Kyoto, Japan) the absorption maximum of the DABT which remained in the solution phase after staining the nitrocellulose paper was 520 nm. The spots on the nitrocellulose paper when examined in a photoacoustic spectrometer also gave an absorption maximum of 520 nm, indicating that bound DABT is the agent primarily responsible for the photoacoustic signal. The intensity of the photoacoustic signal from the stained spot (I_{spot}) was obtained as an average of three measurements of the same spot. The unstained part of the nitrocellulose paper also contributed to the photoacoustic signal at the same wavelength. Hence, signals from three areas adjacent to the stained spot were also recorded ($I_{background}$), again as an average of three measurements. Subsequently, the photoacoustic signal due to the background was subtracted from the signal obtained from the spot itself. The difference ($I_{spot} - I_{background}$) was taken as the photoacoustic signal from the spot.

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