

BINDING OF BENZO(a)PYRENE METABOLITE(S) TO MAIN AND SATELLITE CALF THYMUS DNA's *IN VITRO*

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1. Introduction

It has been proved that carcinogens bind to macromolecules of the target tissues [1, 2] and that a positive correlation exists between the extent of binding of hydrocarbons to DNA and their carcinogenic properties [3]. The binding of hydrocarbons is coupled with their metabolic activation [4, 5]. The ultimate metabolite of benzo(a)pyrene (BP) as suggested by Boyland [6] has been isolated as a K-region epoxide by Grover et al. [7]. Since the extent of metabolite(s) binding to DNA is very low (1 hydrocarbon molecule per 20,000 to 50,000 nucleotides), we tried to see if there is a preferential binding on certain species of DNA. Since the nuclear DNA of higher organisms contain repeated nucleotide sequences [8] that were isolated as satellite DNA's [9–12], we studied the binding of BP metabolite(s) to main and satellite calf thymus DNA's *in vitro*. It has been recently shown by Filipisky et al. [13] that it is possible to fractionate calf thymus DNA into a number of components by $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ preparative density gradients. Gelboin [5] described an *in vitro* system in which benzo(a)pyrene, metabolized by microsomal enzymes, binds covalently with calf thymus DNA.

It is shown in this report that, *in vitro*, benzo(a)pyrene metabolite(s) bind throughout the total calf thymus DNA and do not exhibit a preferential binding to one satellite DNA. However the data show that the BP metabolite(s) are bound to all satellite DNA's to a higher extent than to main DNA.

2. Materials and methods

2.1. Binding of benzo(a)pyrene metabolites on calf thymus DNA. Isolation of DNA

Calf thymus DNA was isolated by the method described by Kay et al. [14], deproteinized several times [15] ($s_{20,w} = 13.6 \text{ S}$), precipitated by cold ethanol and dissolved at the final concentration of 1 mg/ml in 0.0385 M EDTA, 0.01925 M sodium phosphate buffer, pH 7.4.

Tritiated benzo(a)pyrene (G) (C.E.A., Saclay) was diluted with cold BP (Schuchardt, Germany) to a specific radioactivity of 226 $\mu\text{Ci}/\mu\text{mole}$.

Microsomes were isolated as described elsewhere [17] from pooled livers of 2 male Wistar rats (70–80 g) which were injected 24 hr before being sacrificed with 3-methylcholanthrene (20 mg/kg in peanut oil) and fasted overnight.

The incubation mixture was the following: 4 mg calf thymus DNA, 4 mg NADPH (Sigma), 100 μmoles sodium phosphate buffer, pH 7.4, 200 μmoles EDTA, 0.4 ml microsomal suspension (5 mg protein) and 40 μg [^3H]BP in 0.2 ml ethanol in a total volume of 5.8 ml. The incubations were performed in air at 37° for 15 min and stopped by adding 0.2 ml 3% sodium lauryl sulfate. The mixture was homogenized 2 min and concentrated CsCl (P.A. Merck, Darmstadt) was added to give 22 ml of a solution of density 1.702 g/cm^3 as determined from refractive index measurements [16]. The solution was centrifuged at 25,000 rpm and 25° in a Spinco no. 30 fixed-angle rotor for 64 hr. Incubation was run in duplicate and centrifuged separately. This step allows the separation of

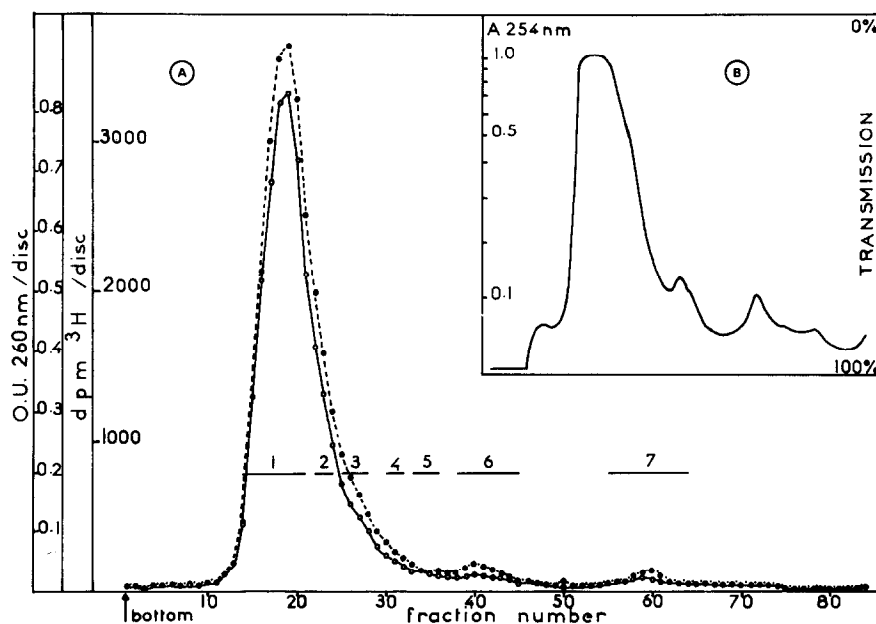


Fig. 1. Fractionation of calf thymus DNA which has bound [^3H]BP metabolite *in vitro*, in a Ag^+ - Cs_2SO_4 preparative density gradient at pH 9.2. A) (○—○—○) Optical unit/disc; (●—●—●) dpm ^3H /disc. B) (—) Transmission at 254 nm recorded on LKB apparatus (see Methods). Even though it seems difficult to localize the satellite DNA's on the absorbance pattern, they are quite evident on the transmission pattern.

DNA from ribosomal RNA and microsomal proteins. It was shown [17] that the protein contamination is less than 0.2%. The two gradients were collected and fractions of each gradient corresponding to DNA were pooled and dialyzed overnight against 1 M NaCl. The two DNA samples were precipitated with 2 vol of cold ethanol, rinsed once with 70% ethanol and twice with 100% ethanol.

2.2. Separation of satellite DNA's on Cs_2SO_4 - Ag^+ gradients

The separation of satellite DNA's was performed according to Filipisky et al. [13]. The two samples of DNA were dissolved overnight at 4° , the first one in 0.005 M $\text{Na}_2\text{B}_4\text{O}_7$ pH 9.2, the second one in 0.005 M $\text{Na}_2\text{B}_4\text{O}_7$ pH 7.9, and dialyzed against 200 vol of the same buffer three times. The DNA (28–30 A_{260} /gradient) was mixed with AgNO_3 in order to obtain $\text{Ag}^+/\text{DNA-P}$ molar ratio equal to 0.35. The density of the solution was then adjusted to 1.48 g/cm^3 with Cs_2SO_4 (Suprapur Merck) and to 22 ml with the corresponding buffer, pH 9.2 or 7.9. The solutions were

centrifuged in a Spinco L 2 preparative ultracentrifuge in a no. 30 fixed-angle rotor for 65 hr at 25° at 25,000 rpm. Gradients were developed from the bottom. Transmission was recorded at 254 nm on a LKB Uvicord apparatus. Fractions of 14 drops were collected.

2.3. Specific activity determination of different DNA's in gradients pH 9.2 and 7.9

Absorbance at 260 nm was determined after dilution of an aliquot of each fraction. Another aliquot was laid on glass fibre paper discs (Whatman 2.5 cm diameter), dried, precipitated for 15 min in 10% TCA at 0° , 5 min in 5% TCA, washed 5 min in 95% ethanol, 5 min in 100% ethanol, then in ether and dried. This step permits the elimination of free or loosely bound [^3H]BP. Each filter was counted individually with 5 ml of PPO-POPOP scintillant in an Intertech-nique liquid scintillation spectrometer. The standardization was made using [^3H]leucine. The efficiency of the counting on filters was 0.348.

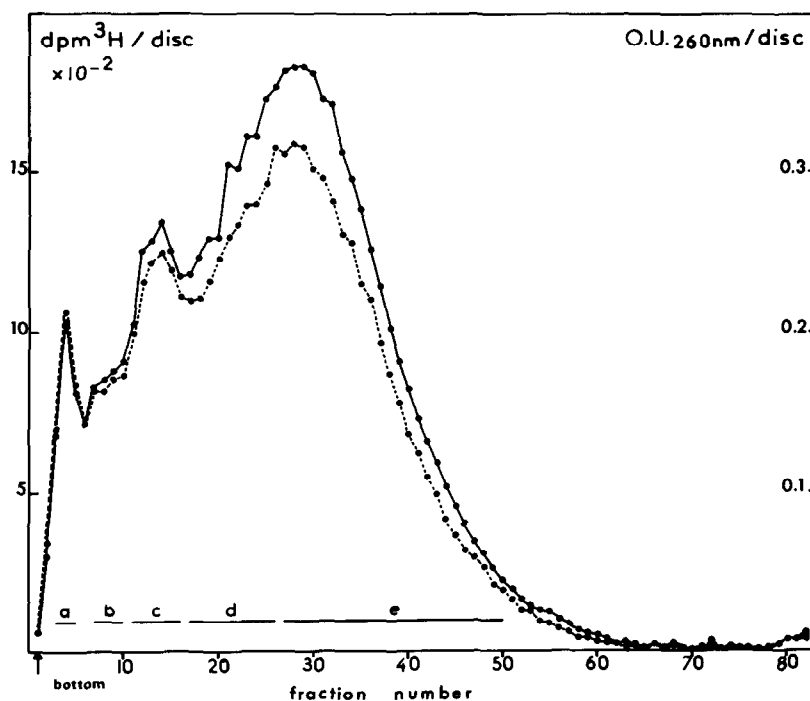


Fig. 2. Fractionation of calf thymus DNA which has bound [^3H]BP metabolite *in vitro*, in a $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ preparative density gradient at pH 7.9. (○—○—○) Optical unit/disc; (●---●---●) dpm ^3H /disc.

The specific radioactivity of DNA which has bound [^3H]BP is expressed as the ratio of dpm ^3H per optical unit at 260 nm of each fraction.

2.4. Analytical neutral CsCl density gradient centrifugation

The rest of fractions corresponding to satellite DNA's (as seen on transmission curve) were pooled, dialyzed exhaustively against 3 M NaCl, and then against 0.01 M Tris-HCl, 0.001 M EDTA, pH 8. The solutions contained 3–5 μg of DNA, 1 μg of 2 C phage DNA ($d = 1.742 \text{ g/cm}^3$) in Tris-HCl-EDTA buffer. The density of the solutions was adjusted to approx. 1.711 g/cm^3 by adding solid CsCl (Suprapur Merck). The centrifugation was performed in a Model E analytical ultracentrifuge. Ultraviolet absorption photographs were taken after 20 hr centrifugation at 44,770 rpm at 25° and analyzed with a Joyce and Loeb microdensitometer. Buoyant densities were calculated using 2 C phage DNA as a density marker by the method of Vinograd [16].

3. Results

Figs. 1 and 2 show the banding pattern of total thymus DNA in preparative $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ density gradients at two different pH and the radioactivity of fractions, corresponding to bound BP metabolite(s).

The absorbance patterns are in good agreement with those shown by Filipisky et al. [13] except in the case of the pH 7.9 gradient concerning the position of satellite DNA with a buoyant density equal to 1.705 g/cm^3 . It is evident that the radioactivity is distributed in all fractions corresponding to DNA. We chose several regions corresponding to a preponderant satellite DNA, characterized by its buoyant density in CsCl and compared the specific activity of DNA's between those regions. Since we did not enrich the preparation, the satellite DNA's are contaminated by one another. Table 1 and 2 show that each satellite DNA in pH 9.2 and pH 7.9 $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradients has a specific radioactivity significantly higher than the main DNA. It means that the satellite DNA's bind BP metabolite more efficiently than the main DNA. The

Table 1

CsCl buoyant density and specific activity of [^3H]BP metabolite-bound DNA, in different regions of $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradient at pH 9.2.

DNA region	1	2	3	4	5	6	7
Buoyant density in CsCl	1.699	1.703	1.709	1.711	1.715	1.722	1.704
Specific activity	4358 \pm 79 n = 8	4881* \pm 27 n = 3	5141** \pm 46 n = 4	5294** \pm 177 n = 3	5071* \pm 252 n = 4	5970** \pm 129 n = 6	5464** \pm 174 n = 7

The DNA regions are defined on fig. 1. The buoyant density in neutral CsCl (see Methods) corresponds to the major component of the region. The specific radioactivity of DNA is expressed as the mean of dpm ^3H /optical unit at 260 nm of the different fractions of the given region. (n = number of fractions per region) \pm standard error. The statistical analysis was done using an analysis of variance followed by t-test multiple comparisons.

* at $P < 0.01$
 ** at $P < 0.001$ (significantly different from main band DNA (region 1)).

level of binding is not exactly the same in both gradients as the incubations were done separately.

We can correlate the specific radioactivity of DNA's with their buoyant density in CsCl. In the case of gradient at pH 9.2, we calculated the coefficient of correlation r and obtained r equal to 0.784 (d d l = 5, $P < 0.05$) and for the gradient at pH 7.9, r equals 0.953 (d d l = 3, $P < 0.02$). The slopes of the two regression lines are very similar. Our results suggest that specific activity of [^3H]BP metabolite-bound DNA's and buoyant densities may be positively correlated.

4. Discussion

The data presented here show that there is no preferential binding of benzo(a)pyrene metabolite(s) to one calf thymus satellite DNA *in vitro*. Our results are in accordance with those reported recently by Zeiger et al. [18] who have shown that *in vivo* 9,10-dimethyl 1,2-benzanthracene binds to both mouse satellite and main-band DNA to the same extent.

However, a more detailed analysis showed that, in our experimental conditions, all calf thymus satellite DNA's bind BP metabolite(s) to a higher extent than

Table 2

CsCl buoyant density and specific activity of [^3H]BP metabolite-bound DNA, in different regions of $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradient at pH 7.9.

DNA region	a	b	c	d	e
Buoyant density in CsCl	1.715	1.713	1.706	1.704	1.699
Specific activity	5133** \pm 2 n = 3	4857** \pm 45 n = 4	4723** \pm 32 n = 6	4441** \pm 51 n = 10	4189 \pm 26 n = 24

The DNA regions are defined on fig. 2. The buoyant density in neutral CsCl corresponds to the major component of the region. The specific radioactivity of DNA is expressed as the mean of dpm ^3H /optical unit at 260 nm of the different fractions of the given region. (n = number of fractions per region) \pm standard error. The statistical analysis was done using an analysis of variance followed by t-test multiple comparisons.

** Significantly different ($P < 0.001$) from main band DNA (region e).

do the main DNA. It is unlikely that this difference is due to a displacement of the BP metabolite from DNA by the formation of the Ag^+ -DNA complex since the BP metabolite has been shown to be covalently bound to DNA [5]. In addition, the localization of the DNA's in the Ag^+ - Cs_2SO_4 gradients, which is a function of the level of bound Ag^+ , varies with the pH conditions [13] and it can be seen that in the pH 7.9 gradient, the BP metabolite-bound DNA's having the highest specific activity are those which bind Ag^+ to the highest extent.

The most labelled satellite DNA has in the gradient at pH 9.2 a 22% and at pH 7.9 a 37% higher value than that of the main DNA. This difference may be explained by the correlation that we found between the level of labelled BP metabolite-bound DNA's and the DNA's buoyant density. The 22% increase corresponds to the region where the satellite DNA ($d = 1.715 \text{ g/cm}^3$) is predominant and the 37% increase to the region corresponding to the satellite DNA ($d = 1.723 \text{ g/cm}^3$).

As far as we can estimate the base composition of satellite DNA's from their buoyant densities [11], it is tempting to assume that the correlation between the level of bound BP metabolite and the DNA buoyant densities is due to the variation of G + C content in the different DNA species, since most carcinogens bind predominantly to the guanine residue ([19]: a review) and since some carcinogenic hydrocarbon K-region epoxides were shown to react more with poly G than with poly A [20].

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References

- [1] E.C. Miller, *Cancer Res.* 11 (1951) 100.
- [2] C. Heidelberger and M.G. Moldenhauer, *Cancer Res.* 16 (1956) 442.
- [3] P. Brookes and P.D. Lawley, *Nature* 202 (1964) 781.
- [4] P.L. Grover and P. Sims, *Biochem. J.* 110 (1968) 159.
- [5] H.V. Gelboin, *Cancer Res.* 29 (1969) 1272.
- [6] E. Boyland, *Brit. Med. Bull.* 20 (1964) 121.
- [7] P.L. Grover, A. Hewer and P. Sims, *Biochem. Pharmacol.* 21 (1972) 2713.
- [8] R.J. Britten and M. Waring, *Carnegie Inst. Wash. Year Book* 64 (1965) 316.
- [9] N. Sueoka, *J. Mol. Biol.* 3 (1961) 31.
- [10] S. Kit, *J. Mol. Biol.* 3 (1961) 711.
- [11] C.L. Schildkraut, J. Marmur and P. Doty, *J. Mol. Biol.* 4 (1962) 430.
- [12] E.H.L. Chun and J.W. Littlefield, *J. Mol. Biol.* 7 (1963) 245.
- [13] J. Filipisky, J.P. Thierry and G. Bernardi, *J. Mol. Biol.*, manuscript submitted for publication.
- [14] E.K.M. Kay, N.S. Simmons and A.L. Dounce, *J. Am. Chem. Soc.* 74 (1952) 1724.
- [15] M.G. Sevag, D.B. Lackman and J. Smolens, *J. Biol. Chem.* 124 (1938) 425.
- [16] J. Vinograd and J.E. Hearst, *Progr. Chem. Org. Natl. Prod.* 20 (1962) 395.
- [17] M. Meunier and J. Chauveau, *Int. J. Cancer* 6 (1970) 463.
- [18] R.S. Zeiger, R. Salomon, N. Kinoshita and A.C. Peacock, *Cancer Res.* 32 (1972) 643.
- [19] J.A. Miller, *Cancer Res.* 30 (1970) 559.
- [20] P.L. Grover and P. Sims, *Biochem. J.* 129 (1972) 41 P.