The Effect of Polycyclic Hydrocarbons on the Synthesis of DNA in Lymphoid Organs, Bone Marrow and Regenerating Rat Liver

Many carcinogens inhibit DNA synthesis and cell cycle. Urethane acts on regenerating rat liver[1,2], lymphoid organs[3], skin[4] and lung[5]; 7,12-dimethylbenz(a)anthracene (DMBA) is effective in vivo on regenerating and growing rat liver[6–8], small intestine epithelium[8] and other rapidly dividing cells[9,10], and in vitro[11,12]; 3-methylcholanthrene (MCA) acts on lung[5]. It is not known if a relationship exists between inhibition of DNA synthesis and carcinogenic power of the substances employed, even though a certain kind of correlation has been suggested[7,8,13]. At present too little data is available on this point: aromatic polycyclic hydrocarbons, including compounds of similar structure but having very different carcinogenic indexes when tested on the skin, may be chosen for this purpose. We have studied the effect of DMBA, benz(a)-anthracene (BA), dibenz(a, h)anthracene (1,2,5,6-DBA), dibenz(a, c) anthracene (1,2,3,4-DBA), 3,4-Benzo(a)pyrene (BP), benzo(e)pyrene (1,2-BP) and perylene (P) on spleen, thymus, femur bone marrow and regenerating rat liver. DMBA, BP and 1,2,5,6-DBA are high oncogenic substances (in decreasing order), BA shows very weak oncogenic power, and 1,2,3,4-DBA, 1,2-BP and P are considered non-oncogenic. DMBA is known to be carcinogen for lymphoid organs and regenerating liver[14,15]; the oncogenic index of other hydrocarbons on these organs is actually unknown.

Materials and methods. Wistar outbred male rats were used: young adult (180–200 g) in the case of the experiments on lymphoid organs and bone marrow, and adult (280–300 g) in the case of the experiments on regenerating liver. Compounds were i.p. injected in sterile olive oil (0.19 μmole of compound per g of body weight, corresponding to 50.8 μg/g of DMBA, in 0.25 ml/100 g body wt. of oil). This is the maximal dose which can be employed without production of ascite later on[16]. In some cases a solution-suspension was obtained by homogenization and by warming. Partial hepatectomy was performed according to[17]; ^{3}H-thymidine (18.4 Ci/mmole, 25 μCi/100 g body wt.) was i.m. injected 30 min before killing. As regards spleen, thymus and bone marrow, 5 animals were killed at 8, 24, 48 h and 4, 8, 12 days after injection and processed separately. Concerning the regenerating liver, hydrocarbons were injected 2 h after hepatectomy and animals (in groups of 5) killed 22 h later and processed separately. A group of 18 animals was used as control: 6 untreated and 12 oil-treated (8 and 24 h after the injection), in the case of lymphoid organs and bone marrow, no significant difference having been found. In the case of hepatectomyzed rats, controls (20 animals) were oil-injected. Mortality was only observed with perylene which did not allow data beyond 24 h after injection to be obtained. The DNA was determined in tissue homogenates according to[18] and its labelling was measured by a liquid scintillation spectrometer.

Results. A constant depression is observed, not correlated to the oncogenic power of the substances used (Table I). The maximal effect is exerted by 1,2,3,4-DBA and DMBA. The strong inhibition by DMBA is more lasting in lymphoid organs than in bone marrow; 1,2-BP and BA affect thymus and spleen more than bone marrow; more marked the depression of DNA synthesis, clearer the increase in the DNA synthesis which takes place later on. Inflammatory and infective events randomly distributed in immunosuppressed animals, may explain the noticeable variability in our experiments. In Table II the effect on the DNA labelling in regenerating rat liver is reported. A strong inhibition is exerted by DMBA and BP, while 1,2-BP is less effective and the effect of BA is not significant. In this case a correlation seems to exist between the inhibition of DNA synthesis and the carcinogenic index. However 1,2,3,4-DBA is by far more active than 1,2,5,6-DBA.

Discussion. A correlation between oncogenic power and inhibition of DNA synthesis has not been shown. Therefore the depression of DNA synthesis does not seem to be a necessary and specific step for the action of chemical carcinogens. Probably the decreased thymidine incorporation merely reflects a toxic action of the compounds used. It should be considered, however, that the indexes of carcinogenic or initiating power in the organs examined are not available. In absence of measurement of thymidine uptake into the cytoplasmic pool, the altered thymidine incorporation into DNA could not necessarily reflect the DNA synthesis; the probable presence of unscheduled DNA synthesis should be taken into account. The inhibition of DNA synthesis cannot be attributed to covalent binding of hydrocarbons to DNA (even though the only available datum concerning the binding of BP and DMBA to rat spleen DNA[19] may be in agreement with this possibility): in fact the binding seems to be correlated to the oncogenic potency of the substances tested[20]. Moreover the amount of compound linked covalently to DNA is very small also in the case of powerful carcinogens, and it can hardly explain the massive effect on DNA synthesis. The inhibition of DNA synthesis may be exerted through weak interactions and intercalations, not correlated to the oncogenic power. However, the

[1] K. M. HWANG, S. A. MURPHREE and A. C. SARTORELLI, Cancer Res. *33*, 2149 (1973).
[2] G. PRODI, P. ROCCHI, A. M. FERRERI and S. GRILLI, Z. Krebsforsch. *82*, 277 (1974).
[3] G. PRODI, P. ROCCHI and S. GRILLI, Z. Krebsforsch., in press (1975).
[4] T. J. SLAGA, G. T. BOWDEN, B. G. SHAPAS and R. K. BOUTWELL, Cancer Res. *33*, 769 (1973).
[5] M. B. SHIMKIN, T. SASAKI, M. MCDONOUGH, R. BASERGA, D. THATCHER and R. WIEDER, Cancer Res. *29*, 994 (1969).
[6] S. K. JUHN and G. PRODI, Experientia *21*, 473 (1965).
[7] H. MARQUARDT and F. S. PHILIPS, Cancer Res. *30*, 2000 (1970).
[8] H. MARQUARDT, F. S. PHILIPS and A. BENDICH, Cancer Res. *32*, 1810 (1972).
[9] E. V. JENSEN, E. FORD and C. HUGGINS, Proc. natn. Acad. Sci., USA *50*, 454 (1963).
[10] F. S. PHILIPS, S. S. STERNBERG, A. P. CRONIN and P. M. VIDAL, Proc. Am. Ass. Cancer Res. *10*, 68 (1969).
[11] L. J. ALFRED, Nature, Lond. *208*, 1339 (1965).
[12] L. J. ALFRED and J. A. DI PAOLO, Cancer Res. *28*, 60 (1968).
[13] H. MARQUARDT, A. BENDICH, F. S. PHILIPS and D. HOFFMANN, Chemico-Biol. Interactions *3*, 1 (1971).
[14] A. W. POUND, New Zealand med. J. *67*, 88 (1968).
[15] H. MARQUARDT, S. S. STERNBERG and F. S. PHILIPS, Chemico-Biol. Interactions *2*, 401 (1970).
[16] G. PRODI, C. FINZI and A. T. DI MARCO, Arch. Sci. Biol. *53*, 256 (1969).
[17] G. M. HIGGINS and R. M. ANDERSON, Arch. Path. *12*, 186 (1931).
[18] H. N. MUNRO and A. FLECK, Analyst *91*, 78 (1966).
[19] G. PRODI, P. ROCCHI and S. GRILLI, Cancer Res. *30*, 1020 (1970).
[20] P. BROOKES and P. D. LAWLEY, Nature, Lond. *202*, 781 (1964).
[21] J. STJERNSWÄRD, J. natn. Cancer Inst. *35*, 885 (1965).
[22] J. STJERNSWÄRD, J. natn. Cancer Inst. *36*, 1189 (1966).
[23] A. T. DI MARCO, C. FRANCESCHI, L. XERRI and G. PRODI, Cancer Res. *31*, 1446 (1971).

Table I. DNA labelling time-course in thymus, spleen and bone marrow after injection of polycyclic hydrocarbons

Time after injection (days)		0	0.33	1	2	4	8	12	18
DMBA	Thymus	100 ± 12.4	27.3 ± 6[a]	33.1 ± 3.7[a]	48.2 ± 1.6[a]			63.4 ± 12.8[c]	100 ± 7.6[c]
	Spleen	100 ± 6.8	29.7 ± 4.5[a]	43.9 ± 9.3[a]	58.7 ± 13.5[a]	67.3 ± 22.2[c]		109.3 ± 18[c]	114.6 ± 20[c]
	Bone marrow	100 ± 7.8	55.6 ± 11[a]	65.4 ± 8.7[b]	159 ± 8[a]	164 ± 15[a]		86 ± 5.7[c]	91.1 ± 11[c]
BA	Thymus	100 ± 12.4		48 ± 12[a]	56.1 ± 12[a]				
	Spleen	100 ± 6.8		60.6 ± 22[c]	55.6 ± 11[a]				
	Bone marrow	100 ± 7.8		107 ± 23[c]	137 ± 20[c]				
1,2,5,6-DBA	Thymus	100 ± 12.4	55 ± 7.8[b]	63 ± 6.8[c]	56.1 ± 7[b]	110 ± 9[c]	167.2 ± 23[b]		
	Spleen	100 ± 6.8	76 ± 15[c]	47 ± 7.3[a]	94.7 ± 9[c]	125 ± 1.1[c]	166 ± 19[a]		
	Bone marrow	100 ± 7.8	80.6 ± 4[c]	66.2 ± 4.7[b]	124 ± 6.6[c]	94 ± 7[c]	167 ± 18[a]		
1,2,3,4-DBA	Thymus	100 ± 12.4		17.3 ± 3.3[a]	49.6 ± 12.6[a]		173.8 ± 9[a]	175.6 ± 20[b]	
	Spleen	100 ± 6.8		16.6 ± 1.4[a]	96.6 ± 26[c]		130 ± 16[c]	155 ± 20[a]	
	Bone marrow	100 ± 7.8		23.5 ± 3.7[a]	76.4 ± 4[c]		110 ± 15[c]	170 ± 9[a]	
BP	Thymus	100 ± 12.4	74.4 ± 13[c]	83.6 ± 4[c]	79.9 ± 5[c]	130 ± 7[c]	134.6 ± 26[c]		
	Spleen	100 ± 6.8	114 ± 29[c]	94 ± 14[c]	90.9 ± 11[c]	122 ± 2.5[c]	135 ± 20[c]		
	Bone marrow	100 ± 7.8	101 ± 6.9[c]	100 ± 14[c]	114 ± 10.4[c]	116 ± 5.8[c]	129 ± 8.4[c]		
1,2-BP	Thymus	100 ± 12.8		32.2 ± 5[a]	35.5 ± 6[a]				
	Spleen	100 ± 6.8		49.7 ± 4[a]	69 ± 4[a]				
	Bone marrow	100 ± 7.8		78.8 ± 5.6[b]	125 ± 14[c]				
P	Thymus	100 ± 12.4		63 ± 21[c]					
	Spleen	100 ± 6.8		51.7 ± 4[a]					
	Bone marrow	100 ± 7.8		80.4 ± 22[c]					

Specific activities '(as dpm/mg) are reported as the percent of those of controls (time 0). DNA specific activities of controls are: thymus 6,400 ± 800; spleen 22,000 ± 1,500; bone marrow 29,600 ± 2,300. Each value represents mean of 5 animals ± SE; the Student's t-test is so referring: [a] $p < 0.01$; [b] $0.01 < p < 0.05$; [c] $p > 0.05$.

Table II. DNA labelling of regenerating rat liver (as both dpm/mg $\times 10^{-3}$ and the percent of controls) 24 h after hepatectomy and 22 h after the i.p. injection of oil (controls) or polycyclic hydrocarbons (treated rats)

Compound	DMBA	BA	1,2,5,6-DBA	1,2,3,4-DBA	BP	1,2-BP	Controls
dpm/mg	70 ± 14	238.2 ± 34.5	183.2 ± 38.2	92.4 ± 9.8	122.5 ± 13.9	160.75 ± 22.74	265.3 ± 24.6
%	30 ± 6.2[a]	89.9 ± 12.9[c]	69.1 ± 14.1[c]	34.8 ± 0.4[a]	46.2 ± 5.2[a]	60.6 ± 8.5[b]	100 ± 9.3

For statistical evaluation see Table I.

inhibition of DNA synthesis can affect the carcinogenic power through an immunosuppressive effect leading to a decreased immunosurveillance[21-23].

Riassunto. È esaminato l'effetto di sette idrocarburi policiclici, a diverso potere oncogeno, sulla sintesi del DNA nel timo, milza, midollo e fegato rigenerante di ratto. Il DMBA è inibente in tutti i tessuti esaminati, come pure il 1,2,3,4-DBA. Il BP ha un notevole effetto solo sul fegato rigenerante mentre il 1,2,3,4-DBA é più efficace dell'1,2,5,6-DBA in ogni caso. Non é stata stabilita una correlazione tra il potere oncogeno delle sostanze esaminate e la inibizione della sintesi del DNA.

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Cytoplasmic DNA of Hepatoma Tumor Cells Studied by ^{3}H-Actinomycin D Binding

In this study the cytoplasmic DNA content of mouse hepatoma tumor cells was compared with that of normal mouse liver cells. The method chosen was that of ^{3}H-actinomycin D (^{3}H-AD) incubation followed by radioautography. Since ^{3}H-AD binds specifically to DNA[1], it can be used to localize small amounts of DNA with a high degree of specificity in radioautographic preparations at the light microscope level[2-6].

Materials and methods. The tumor used in this study was a mouse hepatoma (Jax Code BW7756), obtained from the Jackson Laboratory, Bar Harbor, Maine and maintained by serial transplant in C57L/J mice. Livers from normal C57L/J mice served as controls All tissues were fixed for 2 h in glutaraldehyde[7], post-fixed for 1 h in osmium tetroxide[8], embedded in Araldite[9], and sectioned at 0.5 μm Control sections from both the hepatoma and the normal liver were incubated in DNase or RNase[10]. All tissues then were incubated in ^{3}H-AD (Schwarz Bioresearch Inc, Orangeburg, N.Y., specific activity 8.4 Ci/m*M*) using methods described in the literature[2-4,6]. Three experimental methods differing in the sequence of fixation and ^{3}H-AD incubation were used. These methods, as well as the methods of light microscope radioautography and Toluidine Blue staining are thoroughly described elsewhere[6].

Silver grains, indicating the sites where ^{3}H-AD had bound to DNA, were counted per unit area (123.4 μm^2) of cytoplasm, using a calibrated grid placed in the ocular. Grain counts were carried out over 20 randomly selected areas of cytoplasm per animal. The mean grain concentrations thus obtained were converted to a standard value: grain counts per 100 μm^2 cytoplasmic area. Standard errors of the means were calculated for all data.

Background counts were made on 'cold' control sections, which were dipped, exposed, developed, fixed and stained in a manner identical to the experimental slides, but which were not subject at any point to incubation in the radioactive compound[11]. Background values were then subtracted from the experimental grain count data.

Results and discussion. The results presented in the Table confirm that in this system ^{3}H-AD binds specifically to DNA. No significant differences were observed between RNase treated sections and non-treated sections, indicating that the presence or absence of RNA has no effect on ^{3}H-AD binding. On the other hand, after DNase extraction, no ^{3}H-AD binding occurred.

The Table shows that ^{3}H-AD binding is more than 2 times greater in the cytoplasm of hepatoma cells (an average of 27 grains per 100 μm^2) than it is in the cytoplasm of normal liver cells (an average of 12 grains per 100 μm^2). This mode of presenting the data compensates for differences in cell size. The Table also shows that the results obtained by all 3 experimental procedures are almost identical. This uniformity suggests that we had obtained maximal binding to DNA in all 3 experimental methods used.

Mitochondrial DNA (m-DNA) is the best documented class of cytoplasmic DNA. Differential H^3-AD binding to the m-DNA of tumor or an increase in amount of m-DNA in the hepatoma could account for the results reported here, especially since the m-DNA of tumors is significantly different from the m-DNA of normal cells.

The m-DNA molecules from normal tissues are circular monomers of double stranded DNA with a remarkably consistent contour length of 5 μm^2 [12,13]. In contrast,

Mean grain concentration per 100 μm^2 cytoplasmic area, representing the amount of ^{3}H-actinomycin D binding to cytoplasmic DNA in normal hepatic cells and hepatoma tumor in 18 mice

	Liver	Hepatoma
Experiment 1	12.41 ± 0.20	27.03 ± 0.18
Experiment 2	12.31 ± 0.19	27.00 ± 0.16
Experiment 3	12.35 ± 0.19	27.00 ± 0.16
Experiment 1 RNase	12.41 ± 0.18	26.97 ± 0.18
Experiment 1 DNase	0.10 ± 0.04	0.11 ± 0.04

Each value represents the average of 120 areas.

[1] W. MULLER and D. M. CROTHERS, J. molec. Biol. *35*, 251 (1968).
[2] E. P. CAMARGO and W. PALUT, J. Cell Biol. *35*, 713 (1967).
[3] B. S. EBSTEIN, J. Cell Biol. *35*, 709 (1967).
[4] B. S. EBSTEIN, J. Cell Sci. *5*, 27 (1969).
[5] J. ACZEL and H. ENESCO, J. Cell Biol. *59*, 2a (1973).
[6] J. ACZEL and H. ENESCO, Experientia *31*, in press (1975).
[7] D. D. SABATINI, K. BENSCH and R. J. BARNETT, J. Cell Biol. *17*, 19 (1963).
[8] R. E. NUNN, *Electron Microscopy: Preparation of Biological Specimens* (Butterworths, London 1970), p. 7.
[9] A. M. GLAUERT, J. R. microsc. Soc. *80*, 269 (1962).
[10] A. D. DEITCH, in *Introduction to Quantitative Cytochemistry* (Ed. G. L. WIED; Academic Press, New York 1966), p. 237.
[11] J. STILLSTROM, J. appl. Radiat. Isotop. *14*, 113 (1963).
[12] M. NASS, Science *165*, 25 (1969).
[13] D. A. CLAYTON, C. A. SMITH, M. J. JORDAN, M. TEPLITZ and J. VINOGRAD, Nature, Lond. *220*, 976 (1968).