Binding of Dibenzo(a,e)fluoranthene, a Carcinogenic, Polycyclic Hydrocarbon without K-Region, to Nucleic Acids in a Subcellular Microsomal System

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ABSTRACT

Dibenzo(a,e)fluoranthene (DBF), a highly carcinogenic polycyclic hydrocarbon without an apparent K-region, binds covalently to DNA, transfer RNA, and polynucleotides when incubated with hepatic microsomal fractions under standard conditions. Optimal binding conditions for [3H]DBF were established.

Methylcholanthrene-pretreated mouse liver microsomes induced a higher level of binding of [3H]DBF to DNA than did similarly induced rat liver microsomes. 7,8-Benzoflavone strongly inhibited the binding of this polycyclic aromatic hydrocarbon to DNA, while cyclohexene oxide and trichloropropene oxide had an enhancing effect when used in the presence of rat liver microsomes. An unexpected inhibitory effect was observed with cyclohexene oxide in mouse liver microsome-enriched medium.

[3H]DBF bound twice as much to denatured as to native DNA. Incubation of [3H]DBF in the presence of liver microsomes and polynucleotides (polyadenylate, polyuridylicate, polyguanylate, and polyninosinate) indicated that binding occurs mainly with guanine. Binding of [3H]DBF to DNA of various origins was found to be directly proportional to the amount of GC pairs. Preliminary results indicate a covalent bond between DBF and nucleic acids.

INTRODUCTION

Lacassagne et al. (23) demonstrated the powerful carcinogenicity of DBF in mice. This PAH belongs to the family of benzo- and dibenzofluoranthenes, carcinogenic pollutants as common as BP. They are found in large quantities in tars, e.g., cigarettes (40); exhausts of urban areas and combustion engines; road dusts as well as soil and sediments close to sea coasts, lake fronts and river banks; oil tars; creosote oil; and smoked foods (16).

The carcinogenicity of a few benzo- and dibenzofluoranthenes on mouse skin was studied by Wynder et al. (45), and the electronic properties and sarcomagenicity of several benzo- and dibenzofluoranthenes were analyzed by Lacassagne et al. (24).

For a long time DBF was confused chemically with dibenzo(a,e)pyrene (28) (Chart 1). Subsequently, both hydrocarbons were unambiguously prepared by total synthesis (7, 29, 42). Tested again under similar conditions, they both showed high sarcomagenicity (26).

A prominent difference in electronic structure distinguishes the 2 PAH's. To be carcinogenic all dibenzo- and dibenzo(e)pyrenes must apparently have a K-region (22, 23, 27). Dibenzo(a,e)pyrene, which is completely devoid of such a region, is totally inactive (25). However, DBF, which has no apparent K-region, is a strong carcinogen.

The hypothesis that K-region epoxides represent the ultimate carcinogenic metabolites (4, 37) is now being questioned (3, 8, 15, 20, 31, 38) despite the fact that the K-region epoxides are highly mutagenic toward bacteria (2) and display a potent transforming activity toward cells in culture (14).

The strongest evidence against this theory is the finding that all K-region oxides obtained by synthesis and tested in mice by several authors have low or no carcinogenic activity (5, 30, 32). On the other hand, the recent data on mutagenicity, binding to nucleic acid, and carcinogenicity of different metabolites of BP point to the products of secondary oxidative metabolism, such as vicinal epoxide dihydrodiols of non-K-region rings as the most probable ultimate carcinogenic metabolites (8, 19, 38).

These observations led us to compare the metabolism of 2 structurally different PAH's, one without any apparent K-region (DBF) and the other containing a typical K-region (i.e., BP). Hopefully, such a comparison will contribute to a clearer definition of the molecular structures of PAH-generating carcinogenic metabolites.

A series of DBF derivatives was synthesized and tested for their carcinogenic activities on X VIInc/Z mice. In addition the metabolism of DBF was investigated. When incubated in the presence of a NADPH-dependent microsomal system (11), DBF is transformed into some 20 metabolites that are currently being identified.

Many studies have dealt with the identification of the interaction between PAH and cellular components; they provide strong evidence for covalent binding of PAH to...
were prepared from XVIlnc/Z mice. This highly hybridized

MATERIALS AND METHODS

Preparation of Microsomes. Mouse liver microsomes were prepared from XVIlnc/Z mice. This highly hybridized strain belongs to the category of mouse strains responsive to aromatic hydrocarbon induction (33). Induction of AHH was brought about by i.p. injection of MC, 1 mg/100 g, 48 hr before killing. It is generally admitted that MC is a good inducer of AHH involved in the production of ultimate PAH metabolites (33).

Rat liver microsomes were prepared from our strain of Wistar rats which were given injections of MC, 1 mg/100 g, 48 hr before killing. Relatively weak doses of MC were used to avoid the excess fatty degeneration of the hepatocytes affected by the toxic MC metabolites that often hampers microsome preparations. For the same reason we avoided fasting the mice before killing.

Mouse and rat liver microsomes were prepared according to the techniques described by Garner et al. (10) and Ames et al. (1). The microsomes were suspended in Tris-sucrose buffer at a concentration of 12 mg protein per ml, and 1-ml aliquots were stored in liquid nitrogen. Protein concentration was determined by the biuret reaction (12) with bovine serum albumin as standard. Each stage of the procedure was checked microscopically.

Chemicals and Radioisotopes. DBF was synthesized according to the technique described by Lavit-Lamy et al. (28, 29). The hydrocarbon was again purified chromatographically on 60 Merck thin-layer Kieselgel (Merck AG, Darmstadt, West Germany) in a cyclohexane/dioxane (80/20) as solvent and the second using cyclohexane/dioxane (80/20).

The labeled compound (910 mCi/mol) was then placed in acetone at a concentration of 250 µg/ml and stored in the cold and dark.

DNA samples from different animal and bacterial species were purchased from Sigma Chemical Co. (St. Louis, Mo.). They were placed in 0.01 M NaCl at a concentration of 3 mg/ml. DNA solutions were denatured by heating at 105° for 4 min followed by immediate cooling to 0°.

Polyribonucleotides (Sigma) were prepared as an aqueous solution at a concentration of 15 mM and estimated spectrophotometrically, after alkaline hydrolysis with 0.3 M KOH for 18 hr at 37°. MRE 600 Escherichia coli tRNA (Boehringer-Mannheim GmbH, Munich, West Germany) in aqueous solution was used at a concentration of 3 mg/ml.

Determination of Microsome-mediated Binding of [3H]DBF to Nucleic Acids. The assay system used for microsome-mediated binding of [3H]DBF to nucleic acids was based on the system originally described by Gelboin (28). Each tube contained, in a total volume of 1 ml, 50 µmol of Tris-Cl (pH 7.5), 0.36 µmol of NADPH, 3 µmol of MgCl2, 0.1 ml of the microsomal preparation (containing 1.2 mg proteins), and 15 µl of an acetone solution of [3H]DBF at 250 µg/ml added just before incubation, i.e., 3.75 µg [3H]DBF. Quantities of nucleic acid and other ingredients are indicated in the chart and table legends. Reaction mixtures, prepared at 0°, were incubated at 37° and slightly stirred throughout incubation. To each tube was added 0.1 ml 10% sodium dodecyl sulfate, and the assay was left for 5 min at room temperature before addition of 1 ml of water-saturated phenol. The tubes were periodically shaken with a Vortex at 20° during 20 min. Samples were centrifuged in a low-speed centrifuge to separate the aqueous and phenolic phases completely. The aqueous phase was removed by a Pasteur pipet and extracted 4 times with 1 ml ether, 4 times with 1 ml ethyl acetate, and again once with 1 ml ether. Excess residual ether was eliminated by a light air current. A 0.5-ml sample was taken from each tube, and the DNA fraction was precipitated by addition of either 0.5 ml 10% TCA or 1 ml ethanol and 50 /¿l 3.75 µg [3H]DBF. Quantities of nucleic acid and other ingredients are indicated in the chart and table legends. Reaction mixtures, prepared at 0°, were incubated at 37° and slightly stirred throughout incubation. To each tube was added 0.1 ml 10% sodium dodecyl sulfate, and the assay was left for 5 min at room temperature before addition of 1 ml of water-saturated phenol. The tubes were periodically shaken with a Vortex at 20° during 20 min. Samples were centrifuged in a low-speed centrifuge to separate the aqueous and phenolic phases completely. The aqueous phase was removed by a Pasteur pipet and extracted 4 times with 1 ml ether, 4 times with 1 ml ethyl acetate, and again once with 1 ml ether. Excess residual ether was eliminated by a light air current. A 0.5-ml sample was taken from each tube, and the DNA fraction was precipitated by addition of either 0.5 ml 10% TCA or 1 ml ethanol and 50 µl 20% potassium acetate. The assays were left at room temperature for 30 min filtered on a Whatman GF/C glass fiber filter. The TCA precipitate was rinsed twice with 3 ml 5% TCA. Both types of precipitate were washed twice with 3 ml ethanol and twice with 3 ml ethyl acetate. The filters were dried under an IR lamp and covered with 10 µl scintillation liquid containing 4 g PPO and 0.1 g dimethyl-POPOP per liter toluene, after which 0.1 ml M Hyamine hydroxide in methanol was added. The mixture was then left for 2 hr at room temperature, and radioactivity was determined in an Intertechnique scintillation counter (Intertechnique, 78350 Plaisir, France).

No significant differences were observed between those 2 precipitation techniques. DNA recovery was measured after each test and found to be almost always total.

For tRNA and polyribonucleotide recovery, an aliquot of...
the aqueous phase (0.5 ml) was precipitated with 5% TCA. The precipitate was centrifuged and hydrolyzed in 1 ml 0.6 M NaOH at 37° for 18 hr. The pH of the solution was adjusted to 7 with 1.2 ml 0.5 M HCl, and its absorbance was measured at the maximum absorption wavelength corresponding to the base of the polyribonucleotide under study.

RESULTS

Optimal Experimental Conditions for [3H]DBF Binding to DNA

Determination of Incubation Time. We measured the amount of [3H]DBF binding to salmon sperm DNA (0.9 mg/assay) in an NADPH-dependent microsomal system as a function of incubation time. Chart 2 shows that [3H]DBF simultaneously binds to the DNA and to the endogenous microsomal RNA's. Binding gradually increased during the first 30 min of incubation for both native and denatured DNA, as well as for endogenous RNA, before reaching a plateau. The amount of DBF bound to denatured DNA was higher than that bound to native DNA.

[3H]DBF Binding as a Function of DNA Concentration. Maximum binding of [3H]DBF was achieved at 0.3 mg DNA per assay (Chart 3). For higher concentrations the binding slowed down for both native and denatured DNA. All the DNA was recovered in 5% TCA without noticeable loss of soluble material.

[3H]DBF Binding as a Function of the Quantity of Microsomes. Maximum binding to DNA was attained when the concentration of microsomal proteins was 1.2 mg/assay; binding of [3H]DBF to microsomal RNA gradually rose to a limiting value of about 2.4 mg protein per assay (Chart 4). Therefore we always used 1.2 mg microsomal protein per assay, a quantity that allowed maximum binding to DNA but limited binding to microsomal RNA.

[3H]DBF Binding as a Function of Its Concentration.

[3H]DBF binding experiments were carried out with native and denatured DNA (0.3 mg/assay) to determine the minimum amount of [3H]DBF necessary to saturate the system. This value was found to be 3 µg/assay (Chart 5). This is certainly an overestimation since [3H]DBF was present in the reaction mixture as a very fine suspension and was therefore not completely dissolved. Several control tests were carried out by adding an NADPH enzyme-regenerating system (composed of glucose 6-phosphate and glucose-6-phosphate dehydrogenase) to the microsomal system. No significant differences were observed as compared to our optimal standard conditions.

[3H]DBF Binding in the Presence of Specific Inhibitors.
The influence of certain specific inhibitors of monoxygenases and epoxide hydrolases on \([^{3}H]DBF\) binding to DNA was investigated.

7,8-Benzo-flavone, known to inhibit monoxygenases (44), inhibited \([^{3}H]DBF\) binding to DNA in both mouse and rat MC-pretreated liver microsomal systems (Table 1).

We confirmed that TCPO, an epoxide hydratase inhibitor that acts by reducing epoxide hydrolysis (34), increased \([^{3}H]DBF\) binding to DNA. This is true at weak TCPO concentrations. In mouse liver microsome experiments, the maximum effect was obtained at a concentration of 10 \(\mu\)M, and in the rat liver microsome experiments, it was obtained at a concentration of 20 \(\mu\)M. These results agree with those of Pietropaolo and Weinstein (35), who obtained a similar effect for BP binding to \(E.\ coli\) tRNA. However, when TCPO concentrations were higher than 0.1 mM, we observed a strong inhibitory effect with mouse liver microsomes but not with rat microsomal system.

Similar experiments were carried out with CHO as epoxide hydratase inhibitor.

As expected, CHO increased \([^{3}H]DBF\) binding to DNA in experiments with rat liver microsomes. Contrariwise, in the presence of mouse liver microsomes, CHO inhibited the binding by 20% at concentrations between 10 \(\mu\)M and 0.1 mM; for higher concentrations this effect decreased (Table 1). The discrepancy of these results cannot yet be explained.

\(\text{[}^{3}H\text{]DBF Binding to Different Polyribonucleotide and Poly-}
\text{deoxyribonucleotide Chains}\)

The affinity for \([^{3}H]DBF\) of various purines and pyrimidine in the DNA-binding reaction was investigated by incubating different single-sequence polyribonucleotide chains with the PAH under optimal experimental conditions. Experiments with polyuridylate were inconclusive because of extreme sensitivity of this polyribonucleotide to endogenous nucleases, which hydrolyze it very fast into products not precipitable by 5% TCA. Polyadenylate, polycytidylate, and polynosinate, despite repeated experiments, did not react with \([^{3}H]DBF\), nor did double-stranded polynucleotides (double-stranded copolymer of polyadenylate and polyuridylicate; double-stranded copolymer of polycytidylate and polynosinate). On the other hand, poly(G) showed strong affinity to \([^{3}H]DBF\) (Chart 6). The extent of \([^{3}H]DBF\) binding increased progressively as a function of poly(G) amount per assay up to 0.3 mg. For higher concentrations the curve leveled off to a plateau, indicating that a limiting quantity of bound \([^{3}H]DBF\) had been reached. This binding [566 pmol/mg poly(G)] proved approximately twice as high than for denatured DNA (265 pmol/mg) and 3.7 times that for native salmon sperm DNA (150 pmol/mg).

These results strongly suggest that guanine is the main site of \([^{3}H]DBF\) binding in the different nucleic acids. To verify this hypothesis, we carried out \([^{3}H]DBF\) binding experiments using native or denatured DNA or tRNA of

![Chart 5. \([^{3}H]DBF\) binding as a function of its concentration. Determinations were made with 0.3 mg native and denatured salmon sperm DNA. Incubation lasted for 40 min. ●, denatured DNA; ○, native DNA.]

![Chart 6. \([^{3}H]DBF\) binding to poly(G) as a function of polymer concentration. Incubation time for each sample was 40 min.]

### Table 1

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Concentration (M)</th>
<th>([^{3}H]DBF) binding to DNA (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>7,8-Benzoflavone</td>
<td>10(^{-4})</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>2\times10(^{-4})</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>5\times10(^{-4})</td>
<td>17</td>
</tr>
<tr>
<td>TCPO</td>
<td>10(^{-4})</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>2\times10(^{-4})</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>5\times10(^{-4})</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>10(^{-3})</td>
<td>123</td>
</tr>
<tr>
<td>CHO</td>
<td>10(^{-4})</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>2\times10(^{-4})</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>5\times10(^{-4})</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>10(^{-3})</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>5\times10(^{-4})</td>
<td>130</td>
</tr>
</tbody>
</table>

In these experiments the DNA concentration was 0.6 mg/assay and the incubation time was 40 min. Inhibitors dissolved in acetone were added at the final concentration indicated. The assay conditions were the same as indicated in “Materials and Methods.”
various origins with proportions of GC pairs ranging from 31 to 70%. We carefully checked that all of the DNA was recovered. The results of these experiments show that the extent of binding is dependent on the proportion of GC pairs in the native as well as in the denatured DNA’s (Table 2). In addition, current experiments to establish the chemical nature of the DBF-nucleic acid bond showed a strong resistance to hydrolysis. This bond is resistant to heating at 95° for 45 min, at both pH 7.0, and 12.2, nor does TCA affect such binding (see “Materials and Methods”). These preliminary results suggest the covalent nature of the DBF-nucleic acid bond.

DISCUSSION

DBF binds to DNA, tRNA, and polynucleotides when incubated with MG-pretreated rat or mouse liver microsomes and the cofactors necessary for microsomal mixed-function oxidase activity. The binding reached an optimal value after 30 min incubation when the medium contained 1.2 mg of microsomal proteins, 0.3 mg of DNA, and 3 μg of [3H]DBF. Denatured DNA binds more [3H]DBF than native DNA.

As expected, 7,8-benzoflavone, a specific inhibitor of AHH, strongly reduced [3H]DBF binding to DNA in both mouse and rat microsomal systems. This suggests that the metabolites responsible for binding could be an epoxide, as seems to be the case with BP. This conclusion still must be confirmed since this compound can either inhibit or activate monooxygenases (39). With the epoxide hydroxylase inhibitors, the observed effects are more complex. TCPO, when used at low concentrations (10 μM to 0.1 mM), enhances [3H]DBF binding to DNA. At higher concentrations we observed an inhibition with mouse microsomes (40% inhibition for 1 mM TCPO).

A similar effect has been reported by Dipple and Nebzydoski (9) for TCPO concentrations of 40 to 120 μM in a system of mouse cells in culture. These authors believe that under these experimental conditions TCPO prevents formation of an intermediate metabolite, such as a trans-dihydrodiol, for example, without which the ultimate metabolite responsible for DNA binding cannot be produced.

On the other hand, Selkirk et al. (36) have shown that TCPO does indeed inhibit dihydrodiol formation but also reduces BP metabolism down to 50% and significantly modifies the metabolite proportions. TCPO could therefore also inhibit monooxygenase activity and produce a reducing effect on [3H]DBF binding.

Similarly, CHO increased [3H]DBF binding to DNA in the presence of rat liver but reduced this binding in the case of mouse liver.

Despite well-known differences in AHH and epoxide hydrolase content between rat and mouse liver, we presently have insufficient data to interpret our observations with CHO.

The results obtained with DNA clearly showed a parallel between DBF binding and the GC pair content of the nucleic acids. With polyadenylate, polyinosinate, and polyctydylate no binding occurred, but with poly(G) efficient binding of [3H]DBF was observed. Thus, guanine may be considered as the main site for DBF binding to the different nucleic acids tested.

Analysis of the number of DBF molecules bound to the different polynucleotide chains gives a proportion of 1 molecule of DBF for 70,000 nucleotides in salmon sperm DNA and for 22,600 nucleotides in poly(G). These values agree fairly well with those reported in the literature for BP, i.e., 1 BP per 50,000 nucleotides (11, 17, 43). That no selective losses occurred during DNA purification can be reasonably assumed from the results obtained with various synthetic polynucleotides.

Since [3H]DBF binding to DNA and poly(G) was resistant to treatment by heat, alkali, or strong acids, one may assume that it is covalent.

The question arises as to what kind of DBF metabolites bind to DNA. DBF has no apparent K-region. Analysis of DBF oxidation with osmic acid enabled Jacquignon et al. (18) to show that this reaction takes place at the 5-5a double bond, which possesses the strongest calculated index in the molecule. This oxidation progresses extremely slowly. We may therefore consider the 5-5a bond as a sterically hindered pseudo-K-region, which would explain its low reactivity (at least 10 times weaker than a true K-region).

None of the DBF metabolites isolated migrated chromatographically with model molecules corresponding to pseudo-K-region diols. It is therefore reasonable to conclude that the DBF metabolites bound to DNA are not epoxides of the pseudo-K-region.

Thus the most probable reacting metabolites might be the epoxides or vicinal dihydrodiol-epoxides located on the A and/or D rings. This hypothesis agrees with current ideas about the nature of ultimate carcinogenic PAH metabolites, such as those of BP where, according to several groups of workers, a non-K-region vicinal dihydrodiol-epoxide seems to be responsible for in vivo binding of this PAH to cell DNA (3, 8, 13, 15, 20, 31, 38). The bay-region theory of polycyclic hydrocarbon carcinogenesis of Jerina et al. (19) would also predict that the ultimate carcinogen of DBF might be the 3,4-dihydrodiol-1,2-epoxide.

This interpretation should be taken with caution. In fact, under similar conditions, Thompson et al. (41) showed that

### Table 2

[3H]DBF binding to several DNA and tRNA samples of diverse origin

<table>
<thead>
<tr>
<th>Species of DNA</th>
<th>% GC</th>
<th>Native DNA</th>
<th>Denatured DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>31</td>
<td>5</td>
<td>6.5</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>39</td>
<td>7.3</td>
<td>11.3</td>
</tr>
<tr>
<td>Salmon sperm</td>
<td>41.2</td>
<td>8.1</td>
<td>13</td>
</tr>
<tr>
<td>Herring sperm</td>
<td>42.9</td>
<td>8.4</td>
<td>12.7</td>
</tr>
<tr>
<td>E. coli</td>
<td>50</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td><em>Micrococcus lysodeikticus</em></td>
<td>71</td>
<td>11.1</td>
<td>17.2</td>
</tr>
<tr>
<td>tRNA E. coli</td>
<td></td>
<td></td>
<td>7.4</td>
</tr>
</tbody>
</table>

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important differences exist in the chemical nature of metabolites of 7-methylbenzanthracene bound to DNA whether this PAH is incubated with liver microsomes or in a system closer to in vivo conditions such as cultured mouse fibroblasts. These differences probably originate in the very low or nonexistent production of vicinal dihydrodiol-epoxides in a 1-step in vitro incubation.

In order to identify the nature of the DBF metabolite bound to DNA, we are currently analyzing enzymatically hydrolyzed DNA.

REFERENCES


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