An Investigation into the Binding of the Carcinogen 15,16-Dihydro-11-methylcyclopenta[a]phenanthren-17-one to DNA in Vitro

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SUMMARY

After metabolic activation the carcinogen 15,16-dihydro-11-[3H]methylcyclopenta[a]phenanthren-17-one binds to DNA in vitro, and this binding is prevented by 7,8-benzoflavone. Radioactivity cannot be removed from the DNA with organic solvents or by chromatography on Sephadex G-50, even after heat denaturation of the DNA. Enzymatic hydrolysis yields radioactive fractions, which elute from a column of Sephadex LH-20 immediately after the natural nucleosides.

At least two species of reactive metabolites are involved in this binding, those with a half-life of a few hr and others with greater stability. After extraction from the aqueous incubation mixture, they could be detected in discrete polar fractions from separations of the complex metabolite mixture by high-pressure liquid chromatography. Their ability to bind to DNA decreased with time at ambient temperature, and they were rapidly deactivated by acid.

7,8-Benzoflavone acted by suppressing the formation of polar metabolites derived from enzymatic oxidation of the aromatic double bonds. The inhibitor had no effect on the enzymes hydroxylating saturated carbon; hence it is unlikely that metabolism of the methyl group is important in conversion of this carcinogen to its proximate form, although the presence of the 11-methyl group is essential for carcinogenic activity in this series.

INTRODUCTION

Previously, it was shown (4) that after metabolic activation the carcinogen 15,16-dihydro-11-methylcyclopenta[a]phenanthren-17-one (Compound II) binds to DNA in vitro and that this binding can be largely prevented by inclusion of the aryl hydrocarbon hydroxylase inhibitor 7,8-benzoflavone in the incubation mixture. The flavone also prolonged the latent period for tumor production when painted together with this carcinogen onto the skin of mice. The apparent significance of this was, however, brought to question when it was found that the parent, unsubstituted ketone (I), which does not evoke skin tumors, also bound to DNA after in vitro metabolism. For a more detailed investigation of this, a further study has been made of the microsomal metabolism and binding of these compounds to DNA in vitro, in the expectation that this would help us understand the mechanism by which this carcinogen exerts its biological action (Chart 1).

MATERIALS AND METHODS

The synthesis of the tritium-labeled ketones I and II has been described (4, 5). 7,8-Benzoflavone was from the Aldrich Chemical Co., Milwaukee, Wis. Calf thymus DNA type I (M.W. 20 million), type II (M.W. 1 million to 1.3 million), and salmon sperm DNA type III were from the Sigma Chemical Co., St. Louis, Mo., as were DNase type I, phosphodiesterase from Croatalus adamanteus venom type II, alkaline phosphatase type III, and NADPH.

Incubation Method and Extent of Binding to DNA. Male Sprague-Dawley rats (75 to 150 g) were given i.p. injections of 20-methylcholanthrene (10 mg/kg), fasted overnight, and killed by stunning followed by cervical dislocation. Livers were removed and immediately homogenized in ice-cold 0.25 M sucrose (2 ml/g of liver). After centrifugation at 9,000 × g for 15 min, and at 16,000 × g for 30 min, the microsomes were aggregated at 90,000 × g for 40 min and finally suspended in ice-cold 0.1 M Tris buffer (pH 7.2). Occasionally, the 9,000 × g supernatant was stored in 2-ml screw-cap plastic vials at −80° until required and then thawed rapidly; the microsomes were then prepared as described above. This microsomal suspension (0.6 ml) was incubated with NADPH (15 mg) in 0.1 M Tris buffer (10 to 20 ml) in 250-ml conical flasks open to the atmosphere at 37°. Substrate (500 μg), for or without 7,8-benzoflavone (1,500 μg), dissolved in methanol or dimethyl sulfoxide (0.1 ml), was added at zero time, and the flask was shaken gently during the subsequent incubation.

Binding to DNA was assayed by adding aliquot portions of the incubation mixture to solutions of calf thymus DNA (M.W. 20 million) containing 1 mg of DNA in 1 ml of 0.1 M Tris buffer (pH 7.2), 10−3 M in sodium chloride. After incubation at 37° for 20 min, 2 volumes of 0.1 M Tris-saturated phenol were added, the mixture was shaken vigorously, and the DNA was precipitated from the aqueous layer by the addition of ethoxyethanol. The DNA was collected on glass fiber circles with suction and washed thoroughly with aqueous ethoxyethanol, with ethanol, and finally with ether. After dissolution in 10−3 M sodium chloride solution, the binding index, expressed as μmoles of substrate per mole of DNA phosphorus, was estimated as previously described.
(4). In order to find the conditions for optimum binding to DNA, amounts of the incubation mixture containing from 5 to 200 \( \mu \)g of substrate were added to 1-mg portions of DNA diluted with buffer so that the final volume was 9 ml in each case. Binding indices showed a linear increase up to 50 \( \mu \)g of substrate and then remained constant. This ratio was therefore generally used.

Binding indices were up to 10 times higher than those obtained with microsomes for uninduced rats (4), but they varied severalfold with different batches of microsomes, as previously found. For this reason the same, freshly prepared microsomal preparation was used throughout each comparative experiment. Indices obtained with the carcinogen (II) as substrate were less (about one-half to two-thirds) than those with the noncarcinogen (I), as found before. Also inclusion of 7,8-benzoflavone in the incubations always reduced these values to 5 to 10% of those obtained in its absence, with either ketone as substrate. Essentially no binding occurred when incubations were carried out with boiled microsomes; on the other hand, use of heat-denatured DNA increased binding approximately 2-fold.

Since the binding indices found previously (about 50 \( \mu \)moles/mole phosphorus for DNA with a molecular weight of 20 million) would have resulted from binding of the substrate at the end groups only, binding was compared with that using calf thymus DNA with a molecular weight of 1 million to 1.3 million and "high-molecular-weight" salmon testes DNA. The results in Table 1 demonstrate that this is not true.

Nature of the Binding of Ketones I and II to DNA. Ketone I (150 \( \mu \)g) was incubated together with DNA (3 mg), microsomes, and NADPH in the presence of air for 45 min as already described. The DNA was recovered by phenol extraction and dissolved in \( 10^{-3} \) M sodium chloride (4 ml); the binding index was 128. This solution was chromatographed on a column of Sephadex G-50 (28-\( \times \)1.5-cm diameter) with Tris buffer (pH 7.2). UV-absorbing material was eluted as a single broad peak which contained 89% of the DNA and 90.8% of the radioactivity applied to the column. The fractions comprising this peak were concentrated to 2.7 ml, and the DNA was denatured by heating in a boiling water bath for 7 min, followed by rapid cooling in ice. Elution from the same column gave a large, sharp peak containing 83% of the tritium and 92% of the DNA, followed immediately by a small peak (17 and 8%, respectively). Total recoveries were 86.4% tritium and 95% DNA.

In other experiments Substrates I and II were separately incubated with DNA (50 mg) and the microsomal system. Binding indices measured on the recovered DNA (about 90% recovery in each case) were 126 (for I) and 252 (for II, different batch of microsomes). The samples were hydrolyzed with DNase (0.6 mg, 1300 Kunitz units) in 0.01 M Tris-0.01 M MgCl\(_2\), pH 7.0 (44 ml), at 37° for 4 hr. The solutions were made alkaline by adding an equal volume of 0.1 M Tris (pH 9.0), phosphodiesterase (3.05 mg, 0.5 unit), and alkaline phosphatase (23 \( \mu \)l, 12.5 units), and incubation was continued for 64 hr. After concentration and centrifugation to remove a small quantity of insoluble material, methanol was added to 30% v/v concentration, and the solution was placed on a column of Sephadex LH-20 (85-\( \times \)1.5-cm diameter) packed in 30% v/v aqueous methanol. Elution was made as a single peak (for I) or as 2 peaks (for II, as shown) between 225 and 300 ml. Elution volumes for a number of synthetic cyclopenta[a]phenanthrenes are as follows: 15,16-dihydrocyclopenta[a]phenanthrene-17-one (I), 680 ml; 6,7-dihydroxy-6,7,15,16-tetrahydrocyclopenta[a]phenanthrene-17-one, 380 ml; 15,16-dihydro-16-hydroxy cyclopenta[a]phenanthrene-17-one, 640 ml; 15,16-dihydro-6-hydroxy cyclopenta[a]phenanthrene-17-one, 805 ml.

Rate of Binding of Ketones I and II to DNA In Vitro. The substrates were incubated separately with DNA as already described with the microsomal system. Aliquot portions were withdrawn at 5-min intervals, DNA recovered, and

<table>
<thead>
<tr>
<th>Type of DNA</th>
<th>Binding index (( \mu )moles of substrate/mole DNA phosphorus)</th>
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<tr>
<td>Calf thymus (20 million)</td>
<td>200 ± 12, 148 ± 6</td>
</tr>
<tr>
<td>Calf thymus (1-1.3 million)</td>
<td>191 ± 23, 127 ± 5</td>
</tr>
<tr>
<td>Salmon sperm</td>
<td>341 ± 48, 204 ± 11</td>
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binding indices were measured. Covalent binding reached a maximum in 30 to 45 min for I but 45 to 60 min for II.

In order to determine whether this difference reflected the rate of formation of activated metabolites or the rate of their reaction with DNA, the latter rate was measured. Substrates I and II were incubated as usual for 45 min; microsomes were removed by centrifugation at 90,000 x g for 45 min at +4°, and DNA was added to the supernatant at 37° at zero time. Duplicate aliquots were withdrawn at 1-min intervals, DNA was recovered, and binding indices were measured. Binding was at a maximum for I within the 1st min, but for the carcinogen (II) it reached a maximum only after 10 min (Chart 3).

Stability of the Reactive Metabolites of I and II. Ketones I and II were incubated separately by the standard procedure for 30 min. Microsomes were removed, and the incubations were continued at 37° for periods of up to 22 hr. Duplicate aliquots were withdrawn at intervals, they were incubated for 15 min at 37° with DNA, the DNA was recovered, and binding indices were determined. Under these conditions binding reached a maximum in 2 to 3 hr and then fell to about three-fourth of this value during the next 10 hr. Thereafter it remained fairly constant for a further 10 hr (Chart 4).

The effect of pH on the stability of the activated metabolites(s) from the carcinogen (II) was tested as follows. The ketone was incubated as usual for 45 min, after which the microsomes were removed. The incubation solution was divided into 2 equal parts; one was brought to pH 1 with N HCl, and both were incubated further at 37° for 10 min. The pH of the acidified sample was brought back to 7.2 by careful addition of N NaOH, and a volume of buffer equal to the total volume of acid and alkali used was added to the untreated sample. Triplicate aliquots of both samples were incubated with DNA as usual to give the following binding indices: unacidified, 343 ± 23 μmoles II per mole DNA phosphorus; acidified, 94 ± 3 μmoles II per mole DNA phosphorus.

Stability of the Activated Metabolites to Extraction with Organic Solvents. Standard incubations were carried out for 30 to 45 min and terminated by cooling in ice; the solutions were extracted 5 times with equal volumes of ethyl acetate that had been washed with saturated aqueous sodium sulfate, evaporated under reduced pressure below 40°, and redissolved in a small volume of redistilled methanol. Recovery of radioactivity was usually better than 80%. Aliquot portions of the methanol solutions containing radioactivity equivalent to 50 μg of substrate were added to DNA, and binding indices were obtained. These were generally somewhat less than those obtained from the original aqueous incubation solution. When kept at room temperature in stoppered glass tubes in the absence of light, the methanolic solutions lost their ability to react with DNA. The rate of loss seemed to vary in an unpredictable manner with preparations from either I or II, taking from 3 to 7 days or more for the binding index to fall to 5% of its original value (see Chart 7d).

Separation of Metabolites by High-Pressure Liquid Chromatography. A Varian 4200 liquid chromatograph system used in this study had the following characteristics: column, 50 x 0.8 cm inside diameter, Micro-Pack CH-10; solvents, methanol-water gradients, 85% water-15% methanol changing to 100% methanol at 1% per min; flow rate, 200 ml/hr; detection, continuous UV monitoring at 254 nm. Aliquot portions (100 μl) of the methanolic solutions described above containing 100 to 500 μg of metabolites were injected onto the column, and the gradient was started. Fractions were collected every minute (Chart 5) or as indicated (Charts 6 and 7). Aliquots (usually 100 μl) of each fraction were counted for radioactivity, and it was found that on this basis the UV trace gave a good indication of the metabolites present except when 7,8-benzoflavone was used, when additional (nonradioactive) UV-absorbing peaks derived from metabolism of this inhibitor were also observed (see Charts 6c and 7c). Chart 5 demonstrates the effect of varying the ratio of Substrate II to microsomes (a) from standard conditions (500 μg Substrate II: microsomes from 4 g of liver), (b) to 250 μg, and (c) to 50 μg of carcinogen (II). Charts 6 and 7 show (a) the metabolite pattern soon after incubation; (b) when the DNA-binding capacity had diminished to 5% of the original, and (c) the pattern of metabolites formed in the presence of 7,8-benzoflavone, for Substrates I and II, respectively.

Volumes equivalent to 5 μg of metabolites (calculated from the radioactivity) from each fraction [Fractions 1 to 24 (Chart 6a)] were added to DNA (0.5 mg), and binding indices were measured as usual. This was repeated with fractions from a similar chromatographic separation 75 hr later (Chart 6b). Only the following fractions bound to DNA (index > 25 μmoles (I) per mole DNA phosphorus) at 4 hr:

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Binding index</th>
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<tr>
<td>7</td>
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<tr>
<td>11</td>
<td>100</td>
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<tr>
<td>12</td>
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<tr>
<td>20</td>
<td>94</td>
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<td>21</td>
<td>40</td>
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(possibly overlap from Fraction 20)

DECEMBER 1976
RESULTS AND DISCUSSION

In our previous reports on the in vitro metabolism (4, 7) and binding (4) of the cyclopenta[a]phenanthren-17-ones I and II to DNA, livers from either uninduced rats or rats induced with 3 injections of 20-methylcholanthrene at 1 mg/kg were used as the source of microsomes. The major metabolites from both ketones were nonphenolic mono-ols, and binding was of the order of 50 μmoles/mole DNA phosphorus. In the present experiments the animals received over 3 times the total amount of methylcholanthrene used previously, as 1 injection (10 mg/kg), and increased metabolism to more polar compounds was observed. Presumably, this is more favorable for the induction of the aryl hydrocarbon hydroxylases involved in this metabolism than the smaller, divided dose used hitherto. In vitro DNA binding was up to 10 times that previously found; it therefore seemed probable that this further metabolism was involved.
Previously, we observed that ketones I and II bound to DNA after *in vitro* metabolic activation and that binding was decreased if the incubation was carried out in the presence of the aryl hydrocarbon hydroxylase inhibitor 7,8-benzoflavone. It was considered that this binding was the result of a covalent reaction because radioactivity (from the substrate) could not be removed from the DNA by extensive washing with organic solvents. It is now found that DNA treated in this way is eluted from columns of Sephadex G-50 both before and after heat denaturation without loss of radioactivity, thus confirming the original conclusion. After enzymatic hydrolysis into its constituent nucleosides and chromatography of the latter on columns of Sephadex LH-20 (2), DNA treated with the carcinogen (II) gave the elution pattern shown in Chart 2. Most of the radioactivity appeared as 2 peaks, following immediately after the natural nucleosides; small amounts of radioactivity were also associated with 2 other materials that were eluted later. DNA treated with the noncarcinogen (I) yielded a similar picture, with the difference that the main radioactive peak was unaccompanied by the smaller peak. The very small quantities of all these hydrolytic fractions have thus far precluded their identification.

Although DNA binding with both ketones I and II was substantially higher than that observed previously (4), as before the binding indices varied severalfold with different batches of microsomes. It was therefore important to utilize the same microsomal preparations in comparative experiments. When the amount of metabolized substrate was varied with respect to the amount of DNA to which it was added, the binding index increased up to 50 μg substrate per mg DNA and thereafter remained constant; this optimum ratio of substrate to DNA was therefore used routinely. To exclude the possibility that binding was occurring at the end groups only, a comparison was made of the binding of both I and II to 2 specimens of calf thymus DNA with a molecular weight of 20 million and 1 million to 1.3 million daltons, respectively; salmon sperm DNA of unknown but high molecular weight was also included in this comparison (Table 1). Binding to both specimens of calf thymus DNA was almost the same, thus indicating that end groups are unimportant. Binding to salmon sperm DNA was substantially higher with both compounds. Since the base compositions of calf thymus and salmon sperm DNA are very similar (16), this suggests that these compounds bind only to a base or bases within certain discrete sequences.

The variation of binding indices with time when Substrates I and II were incubated together with DNA and the microsomal system demonstrated that maximum binding was reached in about 30 min for I and in about 45 min for II. This difference seemed to be accounted for by the different rates of reaction of the activated metabolites with DNA, because binding was almost immediate for I but took about 10 min to reach a maximum for II (see Chart 3). Initially, it was thought that the rapid reaction of these metabolites with DNA might have been brought about by intercalation, thus bringing the reactive center of the metabolite into close proximity with the receptor site in the DNA. However, the evidence is against this because binding to heat-denatured DNA was greater than with native DNA. Reactive metabo-

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**Chart 7. Separation of microsomal metabolites of the carcinogen (II).**

Conditions as in Chart 6. a, run 4 hr after the end of the incubation; b, run 7 days later; c, incubation in presence of 7,8-benzoflavone; d, fall in binding index with time.

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lites were conserved when they were extracted from the aqueous incubation mixture with ethyl acetate, the latter was evaporated, and the residue was dissolved in methanol. Binding indices for the methanolic solutions were somewhat less than those for the aqueous solutions from which they were derived, and they gradually lost their ability to react with DNA over several days at room temperature.

The reason for the severalfold difference in binding index with different batches of microsomes became evident when experiments varying the amount of substrates to the amount of microsomes were performed. Incubation of the carcinogen (II) as usual (500 μg II; microsomes from 4 g of liver) gave the profile of metabolites shown in Chart 5a; under these conditions the substrate is incompletely metabolized, appearing as the last peak at about 78 min. The triplet of peaks that eluted between 55 and 63 min consisted (in order of decreasing retardation) of the 16-hydroxy, 15-hydroxy (main peak), and 11-hydroxyethyl methyl derivatives of II. These compounds have been identified previously as in vitro metabolites after separation from the incubation mixture by thin-layer chromatography, when complete separation was achieved only after conversion to their acetates (7). In the present work these mono-ols were resolved adequately and were identified by their UV spectra and by the identity of their retention times with those of the pure, synthetic ketones. More polar metabolites were present in lesser amounts, but prominent among these were compounds running at 18 and 42 min. When the amount of substrate was halved (Chart 5b), virtually all of ketone II was metabolized and the amounts of the mono-ols were reduced, while the amounts of the more polar metabolites were all increased. At one-tenth the original concentration of II (Chart 5c), there was little evidence of either the mono-ols or the metabolite at 42 min. Nearly all the radioactivity was associated with polar compounds emerging from the column from 15 to 35 min, chief among which was the metabolite eluted at 18 min. The UV absorption of this material was identical with that of the major urinary metabolite of II, isolated in approximately 25% yield by ethyl acetate extraction of urine from rats that had received the carcinogen i.p. (6). This compound was shown (5) to possess the new dibenzoepin structure (III) and has now been found to have the same retention time (18 min) as the in vitro metabolite. Metabolites produced under these nonsaturating conditions also resembled the urinary metabolites in that unchanged II and mono-ols were almost absent, and the majority of the metabolites were polar. Moreover, recovery of radioactivity by ethyl acetate extraction of this incubation mixture was 43%, compared with 78% under the usual conditions (500 μg substrate). The figure for urine is about 50%. DNA-binding indices increased with decrease in the substrate: microsome ratio: 500 μg, 114; 250 μg, 298; 50 μg, 1523. It therefore seems probable that the variation in binding index seen with different batches of microsomes reflects the variation in enzyme content of the livers used (1). Although no attempt has been made to correlate aryl hydrocarbon hydroxylase levels with binding index, it appears that the latter might offer an alternative way of estimating this enzyme system.

Chart 5a illustrates the metabolic pattern produced by incubation of the noncarcinogen (I) under the normal conditions with a particularly active microsomal preparation; the initial DNA-binding index of the methanol solution was 810. Some substrate remained unmetabolized, but there was little of the 15-ol which is a major metabolite when less active microsomal preparations are used (binding index of the order of 200). The UV-absorbing Peaks 7 and 20 had disappeared when the metabolic profile was examined 24 hr later and Peaks 11 and 12 disappeared slowly, after 4 days giving the profile shown in Chart 6b; the DNA-binding index was then only 3% of the original value. The loss of both the UV absorption and the capacity of Peaks 7, 11, 12, and 20 to bind covalently to DNA was associated with the appearance of new UV-absorbing peaks (3A, 8). The effect of including the aryl hydrocarbon hydroxylase inhibitor, 7,8-benzoflavone on the metabolic profile is shown in Chart 6c; this experiment was carried out with the same microsomal preparation as in Chart 6a.

Chart 7 shows a series of experiments with the carcinogen (II). The original methanolic solution had a binding index of 221 and gave the profile seen in Chart 7a, with DNA binding largely confined to Fraction 19. The binding index fell to 5% of its original value in 7 days (Chart 7d) with loss of UV absorption in this fraction (Chart 7b) and appearance of a new UV peak (Fraction 14). As with the unsubstituted ketone (I), inclusion of 7,8-benzoflavone in the incubation suppressed formation of the more polar metabolites but left the mono-ols unchanged. Since further metabolism of this compound can involve only attack on the C=C double bonds of the aryl rings, it is clear that the inhibitor is very selective, inhibiting the aryl hydroxylase but not the enzyme system that hydroxylates saturated carbon atoms. Also since this inhibitor suppressed skin tumor production with II (4), metabolism of the methyl group cannot be important in the activation of this carcinogen to its proximate form, although the 11-methyl group is essential for carcinogenic activity in this series (3). The proximate carcinogen must arise through enzymatic attack on the aromatic rings to yield a species that can react spontaneously with the cellular target critical for the initiation of cancer. In view of the known involvement of aryl oxides in the oxidation of aromatic structures (14, 18), it seems probable that the DNA-binding metabolites described above are this type. Their general instability, and in particular their sensitivity to acid, is in line with this view.

In the case of the noncarcinogen (I) the reactive Fractions 7 and 20 (Chart 6a) seem to account for the less stable metabolites with the half-life of a few hr, while Peaks 11 and 12 represent the more stable DNA-binding compounds. From their elution times the latter could be di- or triol epoxides of the type suggested as the proximate form of benzo[a]pyrene (15). In the case of the carcinogen (II), only the latter type was visualized [Fraction 19 (Chart 7a)] although Chart 4 shows that the less stable DNA-binding metabolites were present initially. In addition both ketones must also yield even less stable intermediates which are the progenitors of the stable, polar metabolites.

Further progress in elucidating the nature of the proximate form of the carcinogen (II) depends on the application of a biological test that can distinguish between these reac-
tive intermediates. While uncertainty still seems to surround the usefulness of direct in vitro tests for carcinogenicity (cell transformation), certain in vitro mutagenicity assays have been found to be useful in detecting carcinogens (10), and one of these in which back mutation of *Salmonella typhimurium* TA100 to histidine nonrequirement is used has recently been used to study the metabolites of benz[a]anthracene and benzo[a]pyrene (9). The carcinogen (II) has now been shown to be strongly mutagenic towards this organism while the 3-methyl isomer, a noncarcinogen, is not (10). It is therefore hoped that this test system will enable us to decide which metabolite possesses the biological activity, as a preliminary to establishing its chemical nature. Work along these lines is in progress.

REFERENCES

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An Investigation into the Binding of the Carcinogen 15,16-Dihydro-11-methylcyclopenta[a]phenanthren-17-one to DNA in Vitro

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