Metabolism of Benzo(a)pyrene with Isolated Hepatocytes and the Formation and Degradation of DNA-binding Derivatives*

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The metabolism of 

Benzo(a)pyrene with isolated hepatocytes from 3-methylcholanthrene-treated rats was examined with the aid of high pressure liquid chromatography. Covalent binding of [3H]benzo(a)pyrene metabolites to the intracellular DNA was investigated. The effects of phenylhydroxylamine, salicylamide, trichloropropene oxide, and diethylmaleate, individually or combined, on the metabolism and covalent DNA binding of benzo(a)pyrene were determined. The results indicate that the initial organic-soluble metabolites were arene oxides, phenols, quinones, and dihydrodiols and that these were subsequently converted to relatively polar, organic-soluble nonconjugated and sulfate-conjugated metabolites and to aqueous-soluble nonconjugated and glucuronide- and glutathione-conjugated metabolites. α-Naphthoflavone inhibited the formation of benzo(a)pyrene metabolites that covalently bound to hepatocyte DNA, while the binding was stimulated by salicylamide, trichloropropene oxide, or diethylmaleate.

Our observations indicate that benzo(a)pyrene-oxide hydration and glutathione conjugation, and glucuronide and sulfate conjugation of hydroxylated benzo(a)pyrene metabolites operate in concert to detoxify electrophilic DNA-binding benzo(a)pyrene metabolites in isolated hepatocytes. The degree of covalent binding of benzo(a)pyrene to the nuclear DNA of isolated hepatocytes seems thus to be correlated with the production of electrophilic benzo(a)pyrene metabolites and the rate of their disposal by epoxide hydratase and conjugation reactions.

It now appears well established that carcinogenic polycyclic aromatic hydrocarbons such as benzo(a)pyrene (1-3), are not inherently harmful, but are activated during their biotransformation to electrophilic metabolites that are toxic (4, 5), are mutagenic (6-10), and covalently bind to tissue macromolecules (1-3). Although the covalent interaction of these metabolites with cellular macromolecules may involve other nucleophiles in addition to DNA (11), there are good correlations between carcinogenic potency and DNA binding (12-15).

Benzo(a)pyrene is metabolized by the cytochrome P-450-linked monoxygenase to reactive intermediates, which either spontaneously rearrange to phenols, are converted by epoxide hydratase to dihydrodiols (2, 16, 17), or are conjugated with glutathione (2, 18, 19). Benzo(a)pyrene quinones probably arise by spontaneous oxidation of phenols or diols (20, 21). Certain benzo(a)pyrene metabolites, such as dihydrodiols and phenols, may "recycle" through the monooxygenase or monooxygenase-hydratase systems to yield reactive or deactivated relatively polar products, respectively (22, 23). Various benzo(a)pyrene intermediates that form conjugates with glucuronic acid (24) and sulfate (25) have also been found.

It is believed that the reactive metabolites are the initially formed, or recycled, products of the cytochrome P-450-linked monooxygenase and that subsequent conjugations with glucuronic acid, sulfate, or glutathione are true detoxication reactions (25). The most potent of the metabolites in regard to cell transformation and covalent binding to DNA appear to be benzo(a)pyrene 7,8-dihydrodiol-9,10-oxide and benzo(a)pyrene 4,5-oxide (6, 7, 10, 27). However, benzo(a)pyrene phenols are also mutagenic (27-29) and capable of being metabolically activated to DNA-binding products (30-32).

Studies on the activation of benzo(a)pyrene to DNA-binding metabolites have mainly been performed in experimental systems containing liver microsomes and pure DNA or isolated cell nuclei (30, 33-35). These models do, however, lack the conjugative detoxication mechanisms and are therefore not suited for investigating the "balance" between toxifying and detoxifying reactions which may in fact be decisive for the toxic effect produced. As an experimental model for such studies we have chosen isolated hepatocytes which actively catalyze the cytochrome P-450- and epoxide hydratase-linked, as well as the conjugative, reactions (36, 37) and which, when isolated from 3-methylcholanthrene-treated rats, have been found to convert benzo(a)pyrene to product(s) that bind to endogenous DNA (38, 39).

We have previously reported some characteristics of benzo(a)pyrene metabolism in isolated hepatocytes, including a rough estimate of the pattern of metabolites, using thin layer chromatography for separation (40). In the present study, we have used HPLC for a detailed analysis of the benzo(a)pyrene metabolite pattern in the hepatocytes and in
the surrounding medium; DNA-binding as a measure of the accumulation of electrophilic metabolites capable of entering the nucleus; and selective inhibitors of various metabolic pathways in order to evaluate the contribution of different reactions to the activation or inactivation of benzo(a)pyrene.

**Materials and Methods**

Chemicals - [7,10-^4^C]Benzo(a)pyrene (95 to 50 mCi/mmol) and [4^-H]Benzo(a)pyrene (5.5 to 25 Ci/mmol) were bought from the Radiochemical Centre (Amersham, Buckinghamshire, England). TCPO was obtained from Aldrich-Europe (Beerse, Belgium), α-naphthoflavone from Koch-Light Laboratories, Ltd. (Colnbrook, Bucks, England), salicylaldehyde from Merck (Darmstadt, Germany), and collagenase (EC 3.4.24.3) from Boehringer Mannheim GmbH (Mannheim, Germany). Diethylmaleate, protease type V, RNase (EC 3.1.4.22) type 1-A, β-glucuronidase (EC 3.2.1.31) type B-10, and aryl sulfotase (EC 3.1.6.1) type V were from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were of analytical grade.

**Animals and Treatment** - Male Sprague-Dawley rats (200 to 250 g) were allowed food and water ad libitum. Control rats received 0.5 ml of corn oil intraperitoneally once daily for 3 days, while pretreated rats were given 20 mg of 3-methylcholanthrene/kg body weight (0.5% corn oil) intraperitoneally once daily for 3 days. Rats were killed 36 h after the last injection.

**Isolation of Hepatocytes** - Rat hepatocytes were isolated by essentially the perfusion method of Berry and Friend (41) as modified by Høegberg et al. (37, 42) with some further modifications. Perfusion with Hank's buffer containing 2% bovine serum albumin and 0.5 mM ethylene glycol bis(P-aminoethyl ether)N,N'-tetraacetic acid was continued for 7 min, after which the liver was perfused for a further 6 min with Hank's buffer containing collagenase (1 mg/ml) and 4 mM CaCl_2. The average yield of hepatocytes was 25% (30 X 10^6/g of liver) (43), of which less than 10% allowed exogenous NADH to penetrate their plasma membrane. NADH leakage was measured photometrically (37). A trypan blue exclusion of more than 95% was generally observed.

**Incubation of Hepatocytes with Benzo(a)pyrene** - Freshly isolated hepatocytes were incubated with benzo(a)pyrene (80 μM) in Krebs-Henseleit buffer containing 2% bovine serum albumin at 37°C, using 25-ml Erlenmeyer flasks shaking gently under an atmosphere of 85% O_2 and 15% CO_2. For analysis of benzo(a)pyrene metabolism, 1.5 X 10^6 hepatocytes/ml were incubated with [1^4^C]benzo(a)pyrene (10 mCi/mmol; added in acetone, 25 μl/m of incubate). For analysis of the covalent binding of benzo(a)pyrene to endogenous DNA, 5 X 10^6 hepatocytes/ml were incubated with [3^-H]benzo(a)pyrene (3.7 μCi/mmol). At zero time α-naphthoflavone (0.5 mM), salicylamide (2 mM), TCPO (2 mM), or diethylmaleate (0.02% w/v) (final concentrations) were added. After a 5-min preincubation with or without one of these agents, benzo(a)pyrene was added and the incubation was continued for another 30 min. For analysis of benzo(a)pyrene metabolism, 1 ml of incubate was sampled into an equal volume of a solution in corn oil containing 0.5% 0, and 6.5% CO_2. For analysis of benzo(a)pyrene metabolism, 1 ml of incubate was sampled into an equal volume of a solution in corn oil containing 0.5% 0, and 6.5% CO_2.

**Bound to DNA** - The DNA isolated from hepatocytes was exhaustively treated with RNase (1.7 X 10^3 Fishman units) or aryl sulfatase (11.7 units) and 25 μl for HPLC on a Waters Associates ALC/202 high pressure liquid chromatograph equipped with a μBondapack-C_18 analytical column and eluted with a choice of two alcohol/water gradients. These HPLC systems were based on those described by Selkirk et al. (44, 45) and Holder et al. (46).

**System A** was a linear gradient of changing solvent composition, changing from 45% water, 55% methanol:ethanol (2:1) to 20% water, 80% methanol:ethanol (2:1). **System B** was also a linear gradient, from 50% water, 50% methanol:ethanol (1:1) to 25% water, 75% methanol:ethanol (1:1). For system B was used benzo(a)pyrene 4,5-oxide. It failed to separate benzo(a)pyrene 4,5- and 7,8-dihydridodiols, but this was achieved with System A (Fig. 1). Both gradients were run for 1 h with a solvent flow rate of 1 ml/min. At the end of 1 h with either system the solvent was changed to 100% methanol:ethanol mixture in order to elute metabolites. The effluent stream was monitored for absorbance at 254 nm and collected as fractions (100 X 0.7 ml) for scintillation counting. Fractions were counted in 10 ml of Aquasol containing 1% acetic acid using a Beckman LS-150 scintillation counter. Counting efficiency for all fractions was 94%. Better than 98% of the radioactive material applied to the HPLC column was recovered while the overall recovery of radioactive material added to the hepatocyte incubations was more than 85%. All estimations of benzo(a)pyrene metabolites have been corrected to 100% recovery for each experiment. The added butylated hydroxytoluene was extracted into the ethyl acetate and eluted on the HPLC with benzo(a)pyrene, after the benzo(a)pyrene metabolites were identified. Throughout this report the identifications of radioactive benzo(a)pyrene metabolites are based on their co-chromatography with authentic compounds. In the light of other studies using similar HPLC conditions (46, 47), it is possible that the metabolites 4,5-dihydridiol and 7,8-dihydridiol, that any benzo(a)pyrene 4,5- or 11,12-quinoine metabolites eluted with the identified quinones, and that the metabolite identified as 3-hydroxybenzo(a)pyrene included some 1- and 7-hydroxybenzo(a)pyrene.

**DNA Extraction and Assay of [4^-H]Benzo(a)pyrene Covalently Bound to DNA** - The DNA isolated from hepatocytes was exhaustively purified of protein and RNA. The centrifugal pellet of hepatocytes from 5 ml of incubation mixture was solubilized for 30 min at room temperature with 5 ml of 1% sodium dodecyl sulfate containing 10 mM EDTA, using slow inversion-rotation. This was then digested for 60 min at 37°C with 5 ml of 0.2 mTris buffer, pH 7.5, containing 10 mM EDTA and 2.5 mg of protease. Next, the sample was extracted for 30 min at 2°C with 10 ml of phenol/chloroform (1:1), the emulsion was broken by centrifugation at 10,000 X g for 10 min, and the organic phase was aspirated off. The aqueous phase was twice extracted with 5 ml of ether, then solid NaCl was added to 1 M concentration and 20 ml of cold (°4°C) ethanol were slowly added. At this stage the sample was stored overnight at °4°C. The ethanol-precipitated nucleic acids and glycerogen were redissolved in 3 ml of 0.1 M NaCl pH 7.5 solution and filtered (1 ml solution was a precipitate, 3 ml). The sample was then added to stand for 24 h at °2°C. Ribosomal RNA was precipitated by centrifugation at 79,000 X g for 30 min. The supernatant was collected and 9 ml of cold ethanol (°4°C) were slowly added. The precipitate was redissolved in 3 ml of 0.1 M NaCl pH 7.5. The sample was then added to a final concentration of 100 and 10 mM, respectively. After digestion of the redissolved precipitate for 30 min at °37°C with RNase (50 μg/ml, preheated for 10 min at °85°C in order to inactivate DNase), the sample was dialyzed against 0.1% BSC for 24 h, with one change.

1 The abbreviations used are: TCPO, trichloroperoxide; HPLC, high pressure liquid chromatography; GSH, reduced glutathione; SSC, standard saline citrate (0.15 m NaCl, 0.015 m sodium citrate, pH 7.6).
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was added to each fraction and the precipitated DNA was collected. Then, 0.5 ml of 10% trichloroacetic acid was added to each fraction, and their absorbances were determined at 260 nm. The precipitated DNA was dissolved in 0.5 ml of 1% acetic acid, and then radioactivity was determined in 0.25 ml of 10% trichloroacetic acid. The counting efficiency for all samples was 39%, assuming 12 ng of DNA/10^6 hepatocytes (48). The spectral properties of the DNA isolated as above (A_{260}/A_{280} = 1.89; means for 20 samples) indicate that it was of high purity (49).

RESULTS

High Pressure Liquid Chromatography of Metabolites—Fig. 1 shows the HPLC elution patterns of ethyl acetate-soluble radioactivity obtained for incubations of [14C]benzo(a)pyrene with hepatocytes isolated from 3-methylcholanthrene-treated rats. The elution patterns of authentic benzo(a)pyrene derivatives are included for comparison. The first eluting fraction of organic-soluble metabolites is designated as "Fraction I" and probably contains polyhydroxylated benzo(a)pyrene derivatives (22) and sulfate conjugates (25). The water-soluble benzo(a)pyrene metabolites were not separated and were measured as their combined radioactivity.

Benzo(a)pyrene Metabolism and DNA Binding as Function of Time—Time courses of production of [14C]benzo(a)pyrene metabolites with suspensions of hepatocytes isolated from 3-methylcholanthrene-treated rats are shown in Fig. 2. The sequences of changes in metabolite concentrations were similar with either (a) 1.5 × 10^6 or (b) 5 × 10^6 cells/ml of incubation mixture, but occurred more rapidly at the higher cell concentration. The concentration of 5 × 10^6 hepatocytes/ml was selected for studies of the covalent binding of [3H]benzo(a)pyrene to hepatocyte DNA, while 1.5 × 10^6 cells/ml were used when studying changes in [14C]benzo(a)pyrene metabolite profiles. The general pattern of changes as shown was typical for all the experiments: that is, an increase during the early phase of incubation of organic-soluble benzo(a)pyrene metabolites which later declined as they were recycled or conjugated, while the Fraction I metabolites and total aqueous-soluble metabolites continued to increase. Benzo(a)pyrene quinones remained at a fairly constant level throughout the incubation. It is probable that amounts of benzo(a)pyrene phenols and dihydrodiols much higher than those shown were present with 5 × 10^6 cells/ml at times earlier than 1 min. We reported earlier (40) a much higher dihydrodiol to phenol ratio with isolated rat hepatocytes, but the thin layer chromatography system that was used would not have distinguished between benzo(a)pyrene dihydrodiols and the more polar Fraction I metabolites.

A variation in the rate of accumulation and decline of phenols and dihydrodiols with different cell preparations was observed, presumably due to differences in metabolic activity, and this range of behavior is illustrated in Table I. The total organic-soluble metabolites include the percentage (10 to 20%) of organic-soluble 14C that did not co-chromatograph with either the Fraction I metabolites or any of the authentic benzo(a)pyrene derivatives that eluted before benzo(a)pyrene itself.

The data in Table I typify the metabolite pattern both during the initial phase of incubation (5 min), when the concentration of benzo(a)pyrene was not rate-limiting, and at a time (30 min) when more than 94% of the benzo(a)pyrene had...
been metabolized. The major initial (5 min) organic-soluble metabolites were the Fraction I metabolites, 3-hydroxybenzo(a)pyrene and benzo(a)pyrene 9,10- and 7,8-dihydrodiols. Towards the end phase of metabolism the Fraction I metabolites constituted the only appreciable organic-soluble products under these conditions.

When isolated hepatocytes from 3-methylcholanthrene-treated rats were incubated with [3H]benzo(a)pyrene, tritiation of the cellular DNA steadily increased during the first 25 min of reaction as reported earlier (39). Tritiation of DNA was prevented by addition of the benzo(a)pyrene hydroxylase inhibitor α-naphthoflavone to the incubations. Since benzo(a)pyrene itself does not covalently bind to DNA, but many of its metabolites do (30-32), we interpret this labeling, which resisted removal by organic solvents, as being due to benzo(a)pyrene metabolites bound covalently with endogenous DNA. When increasing concentrations of calf thymus DNA were added to hepatocytes immediately after stopping the benzo(a)pyrene incubation with sodium dodecyl sulfate/EDTA solution, the resulting decrease in bound radioactivity per μg of DNA indicated that no further 3H binding occurred during the isolation of hepatocyte DNA. Pezzuto et al. (33) showed by using 3H2O with liver microsomes and isolated liver nuclei, under conditions where [3H]benzo(a)pyrene metabolites covalently bound to the nuclear DNA, that tritiation of the DNA was not due to isotope exchange.

**Release of Benzo(a)pyrene Metabolites from Hepatocytes**

As previously reported there is a release of organic-soluble benzo(a)pyrene metabolites from the hepatocytes into the medium (40). By using HPLC, these metabolites could now be identified (Table II). Of the metabolites formed during the initial (5 min) phase of metabolism, 69% of the benzo(a)pyrene...
9,10-dihydrodiol was found outside the cells, while the majority of 9-hydroxybenzo(a)pyrene, 3-hydroxybenzo(a)pyrene, and the Fraction I metabolites remained inside the hepatocytes. The 4,5- and 7,8-dihydrodiols, and the benzo(a)pyrene quinones were distributed approximately equally inside and outside the cells. An almost identical distribution pattern was observed after incubating benzo(a)pyrene with the hepatocytes for 30 min in the presence of salicylamide, which allowed accumulation of the phenols and dihydrodiols by inhibiting their conjugation (see later).

Effects of α-Naphthoflavone, Salicylamide, TCPO, or Diethylmaleate on Benzo(a)pyrene Metabolism—The effects of selective enzyme inhibitors on benzo(a)pyrene metabolism in hepatocytes isolated from 3-methylcholanthrene-treated rats were investigated. The results of these experiments are illustrated in Fig. 3 and Table III. For each inhibitor the results of one experiment are shown, together with results of the appropriate control incubation (hepatocytes from the same preparation but with inhibitor absent). The experiments were repeated three times, with virtually identical results. The proportion of leaking hepatocytes was determined and was not significantly altered by benzo(a)pyrene and TCPO or diethylmaleate.

α-Naphthoflavone inhibits the 3-methylcholanthrene-induced microsomal monooxygenase, probably at cytochrome P-448 (50). Accordingly, α-naphthoflavone decreased the amounts of all the organic- and aqueous-soluble metabolites formed from benzo(a)pyrene with isolated hepatocytes of 3-methylcholanthrene-treated rats (Fig. 3 and Table III). We have shown in Fig. 3 the results of α-naphthoflavone inhibition at both 5 and 30 min since the effect was more pronounced at the earlier time, when there were still relatively large quantities of benzo(a)pyrene phenols and dihydrodiols in the absence of α-naphthoflavone.

Salicylamide inhibits glucuronidase and sulfate conjugation (51, 52) and in our experiments 2 mM salicylamide accordingly lowered the amounts of the Fraction I metabolites and the water-soluble metabolites, whereas the amounts of phenols, quinones, and dihydrodiols were increased (Table III and Fig. 3). The total metabolism of benzo(a)pyrene was not affected by addition of salicylamide.

β-Glucuronidase and aryl sulfatase hydrolysis of the metabolites obtained after incubation of benzo(a)pyrene with hepatocytes for 30 min confirmed the effects of salicylamide (Table IV). β-Glucuronidase and aryl sulfatase hydrolyzed 48 and 20%, respectively, of the water-soluble metabolites into organic-soluble benzo(a)pyrene metabolites such as dihydrodiols, quinones, and phenols. The total amount of Fraction I metabolites was not affected by β-glucuronidase treatment, whereas with aryl sulfatase the amount was decreased, indicating that the Fraction I consisted partly of sulfate conjugates. Free quinones were probably formed during spontaneous oxidation of the benzo(a)pyrene phenols or from hydrolyzed dihydroxybenzo(a)pyrene glucuronides (20, 21) and sulfates. The data in Table IV are from one experiment and were supported by the results of a second investigation (not shown).

TCPO inhibits epoxide hydratase, thereby leading to increased amounts of arene oxides and their spontaneous isomerization products, phenols, and to decreased dihydrodiols (53). This effect of TCPO was also seen in the metabolism of benzo(a)pyrene with isolated hepatocytes (Fig. 3, Table III). It has to be noted that TCPO added to the hepatocyte suspensions also reduced the intracellular level of GSH by approximately 98%. With TCPO the amount of Fraction I metabolites was decreased. Of the possible arene oxides, only the stable K region oxide, benzo(a)pyrene 4,5-oxide, was identified. TCPO slightly inhibited total benzo(a)pyrene metabolism over a period of 30 min and it increased the ratio of organic- to aqueous-soluble metabolites. The presence of salicylamide, diethylmaleate, or TCPO caused an increase in a minor unidentified metabolite, which eluted from the HPLC in Fractions 29 to 32 between the benzo(a)pyrene 9,10-dihydrodiol and 4,5-dihydrodiol; IV, benzo(a)pyrene 7,8-dihydrodiol; V, benzo(a)pyrene 1,6-quinone; VI, benzo(a)pyrene 3,6-quinone; VII, benzo(a)pyrene 6,12-quinone; VIII, benzo(a)pyrene 4,5-oxide; IX, 9-hydroxybenzo(a)pyrene; X, 3-hydroxybenzo(a)pyrene. For α-naphthoflavone, the results for metabolism of benzo(a)pyrene during 5 min, following 5 min of preincubation, are also given (inset). Metabolites were analyzed by HPLC and scintillation counting as described under "Materials and Methods."
TABLE III

Incubation for 30 min with hepatocytes isolated from 3-methylcholanthrene-treated rats was conducted as described under "Materials and Methods."

| Additions         | None | α-Naphthoflavone | Salicylamide | TCPO | Diethylmaleate | Salicylamide + TCPO | Diethylmaleate |
|-------------------|------|------------------|--------------|------|----------------|---------------------|---------------|
| Metabolites       |      |                  |              |      |                |                     |               |
| Total             | 76.7 | 16.3             | 75.2         | 71.7 | 73.2           | 85.3                | 69.2          |
| Organic           | 15.9 | 8.4              | 36.6         | 27.2 | 24.5           | 41.1                | 38.1          |
| Aqueous           | 60.8 | 7.9              | 38.4         | 44.5 | 48.7           | 24.2                | 31.1          |
| % aqueous metabolites of total | 79   | 48               | 51           | 62   | 67             | 37                  | 45            |

TABLE IV

Effect of β-glucuronidase or aryl sulfatase on pattern of benzo(a)pyrene metabolites with isolated hepatocytes

Incubation of benzo(a)pyrene for 30 min with hepatocytes isolated from 3-methylcholanthrene-treated rats was carried out as described under "Materials and Methods." After the incubation, the mixtures were reacted at pH 5.5 with either β-glucuronidase, aryl sulfatase plus saccharo-1,4-lactone, or with no enzyme (control) as detailed under "Materials and Methods." Abbreviations are as in Fig. 1.

| Metabolites       | Control | β-Glucuronidase | Aryl sulfatase |
|-------------------|---------|-----------------|----------------|
|                   | nmol/1.5 × 10^6 cells |               |               |
| Total             | 74.3    | 74.3            | 72.6           |
| Organic           | 29.6    | 50.6            | 37.1           |
| Aqueous           | 45.3    | 23.7            | 35.5           |
| % aqueous metabolites of total | 61     | 32              | 49             |
| Fraction I        | 19.9    | 91.3            | 9.7            |
| 9,10 D            | 0.8     | 1.9             | 4.1            |
| 4,5 D             | 0.2     | 0.8             | 0.3            |
| 7,8 D             | 0.1     | 0.7             | 0.4            |
| 1,6 Q             | 3.7     | 5.0             | 7.2            |
| 3,6 Q             | 1.9     | 7.4             | 9.2            |
| 6,12 Q3           | 0.4     | 0.9             | 0.6            |
| 9 OH              | 0.2     | 0.8             | 1.7            |
| 5 OH              | 0.6     | 10.1            | 9.9            |

* Benzo(a)pyrene 6,12-quinone was not separated from benzo(a)pyrene 4,5-oxide.

The effects on benzo(a)pyrene metabolism of combining two inhibitors were cumulative but not strictly additive (Fig. 3, Table III). The combinations were slightly more inhibitory to total benzo(a)pyrene metabolism than were the individual inhibitors, but this increased inhibition was manifested solely in the amount of aqueous-soluble metabolites, whereas the organic-soluble metabolites were enhanced (Table III). Dual inhibitor combinations inhibited the production of aqueous-soluble metabolites more effectively than did single inhibitors.

Results are presented as mean values for three experiments with standard error.

Fig. 4. Effects of α-naphthoflavone (ANF), salicylamide (SA), TCPO, and diethylmaleate (DEM) on covalent binding of [3H]benzo(a)pyrene metabolites to intracellular DNA. [3H]-Benzo(a)pyrene was incubated for 30 min with hepatocytes (5 × 10^6 cells/ml) isolated from 3-methylcholanthrene-treated rats. DNA was isolated from the hepatocytes and analyzed for covalently bound radioactivity as described under "Materials and Methods." Results are presented as mean values for three experiments with standard error.
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enhancement of ['H]benzo(a)pyrene/DNA binding, about 4-fold.

**DISCUSSION**

With suspensions of hepatocytes isolated from 3-methylcholanthrene-treated rats the pattern of organic-soluble benzo(a)pyrene metabolites produced after 5 min metabolism was similar to the pattern reported for rat liver microsomes (Table I; Refs. 46, 47, 54), with 3-hydroxybenzo(a)pyrene and benzo(a)pyrene 7,8- and 9,10-dihydrodiols constituting the major metabolites. But unique to initial benzo(a)pyrene metabolism with isolated hepatocytes was the major fraction of relatively polar Fraction I metabolites that eluted from the HPLC before benzo(a)pyrene 9,10-dihydrodiol. This fraction, which increased steadily during a 30-min incubation, was probably closely related to the very polar organic-soluble benzo(a)pyrene metabolites that accumulate with liver microsomes during prolonged incubations and are considered to be polyhydroxylated benzo(a)pyrene derivatives recycled through the monooxygenase (22, 23).

It is clear from our results that the pattern of organic-soluble benzo(a)pyrene metabolites changes with the duration of the reaction and that eventually all the added benzo(a)pyrene becomes metabolized to water-soluble and comparatively polar organic-soluble metabolites (cf. Fig. 2).

The observed effects of β-glucuronidase confirm that glucuronide conjugation was a major reaction of benzo(a)pyrene phenols, as has been found in vivo (55) and using liver microsomes (24). Aryl sulfatase treatment, in turn, revealed that the inhibitory effect of salicylamide on the formation of the most polar of the organic-soluble benzo(a)pyrene metabolites was due to inhibition of sulfate conjugation (cf. Fig. 3; Table IV). With rodent lung tissue, sulfate conjugates of 3-, 7-, and 9-hydroxybenzo(a)pyrene are formed (25, 56) and similar results were obtained with isolated hepatocytes (cf. Table IV) where aryl sulfatase treatment produced an increase in phenols identified as 3- and 9-hydroxybenzo(a)pyrene; the metabolite identified as 3-hydroxybenzo(a)pyrene may have included some 1- and 7-hydroxybenzo(a)pyrene (46, 47).

The organic-soluble metabolites were distributed between the hepatocytes and the extracellular medium (Table II). This distribution was asymmetric for some metabolites and was probably not simply related to the relative polarities of the metabolites since the most polar Fraction I metabolites were preferentially retained, whereas the less polar benzo(a)pyrene 9,10-dihydrodiol was preferentially excluded from the cells. The possible hepatic excretion of benzo(a)pyrene metabolites such as phenols and dihydrodiols, which can be further metabolized to active derivatives (6, 29, 32, 57), may be of importance in vivo. Thus it is tempting to speculate that lung and skin, for example, which form much lower quantities of the initial benzo(a)pyrene metabolites than does liver, will be able to reactivate primary metabolites that have been formed in the liver and transferred to the target tissues.

Hepatocytes isolated from 3-methylcholanthrene-treated rats were able to activate benzo(a)pyrene to metabolite(s) that bound to endogenous DNA, whereas no binding could be detected in hepatocytes from control rats. In the absence of any added inhibitors, the maximal binding was approximately 1 pmol of benzo(a)pyrene metabolites/50 μg of DNA (equivalent to 5 × 10⁶ hepatocytes) (48), which represented 0.001% of the metabolized benzo(a)pyrene. Since the binding of benzo(a)pyrene metabolites to DNA continued to increase for several minutes after the disappearance of nonmetabolized benzo(a)pyrene, it is likely that the binding also involved further activated or recycled metabolites. Although the "initial" benzo(a)pyrene 4,5-oxide and reactivated benzo(a)pyrene phenols also show marked DNA-binding capacity, the recycled metabolite benzo(a)pyrene 7,8-dihydrodiol-9,10-oxide, is currently considered one of the most potent DNA-binding benzo(a)pyrene derivatives (30–32, 58).

Isolated rat liver nuclei have been shown to metabolize benzo(a)pyrene as well as its microsomal metabolites to DNA-binding derivatives (33–35) and it has been suggested that the primary benzo(a)pyrene metabolites produced by the endoplasmic reticulum are further activated in the close vicinity of the nucleus, possibly in the nuclear envelope, before reacting with nuclear constituents (34).

Salicylamide inhibits glucuronidase (51) and sulfate (52) conjugation of benzo(a)pyrene metabolites, and, as could be expected, addition of salicylamide to the cellular incubations decreased the level of aqueous-soluble metabolites, whereas the quantity of organic-soluble metabolites, such as phenols and dihydrodiols, was enhanced (cf. Fig. 3). Since the inhibitor also caused an increase in the binding of benzo(a)pyrene metabolites to DNA, these conjugation reactions obviously play a role in the detoxication of the initial benzo(a)pyrene metabolites. The increased DNA binding resulting from salicylamide inhibition of conjugation was thus probably related to an accumulation of benzo(a)pyrene phenols and dihydrodiols which could be further metabolized to electrophilic products (32, 34, 57).

Several benzo(a)pyrene oxides are conjugated with glutathione both spontaneously and enzymatically by glutathione transferases (18, 59, 60), and glutathione has been shown to decrease the binding of benzo(a)pyrene metabolites to nucleic acids of isolated liver cell nuclei (33, 61). In the intact hepatocytes, conjugation of benzo(a)pyrene metabolites with glutathione can be inhibited by addition of diethylmaleate, which decreases the intracellular GSH level (62) and is a glutathione transferase substrate (63). Diethylmaleate added to the cellular incubations lowered the formation of aqueous-soluble metabolites and elevated the amount of benzo(a)pyrene 4,5-oxide and benzo(a)pyrene phenols (cf. Fig. 3). The increased levels of benzo(a)pyrene 4,5-oxide and probably also of other "initial" benzo(a)pyrene oxides and benzo(a)pyrene phenols, were manifested in an enhanced binding to cellular DNA (cf. Figs. 3 and 4). Thus, a direct interaction of primary benzo(a)pyrene oxides with nuclear constituents has also to be considered when interpreting the observed results.

Interpretation of the action of TCPO in the present experiment is complicated since the agent had dual effects on isolated hepatocytes: it inhibited epoxide hydratase (53) and thereby the formation of dihydrodiols (cf. Fig. 3) and it also depleted GSH by reacting both spontaneously (53) and with glutathione transferase catalysis (64). Its effects on benzo(a)pyrene metabolism with the hepatocytes were, however, characteristic of its inhibition of epoxide hydratase and different from those of diethylmaleate (cf. Fig. 3). Thus, TCPO inhibited the formation of dihydrodiols and increased the levels of benzo(a)pyrene phenols and benzo(a)pyrene 4,5-oxide. The reduced amount of the most polar Fraction I metabolites and of aqueous-soluble metabolites indicates that some of these were further metabolites of benzo(a)pyrene dihydrodiols recycled through the cytochrome P-450-epoxide hydratase system, for example benzo(a)pyrene triols and tetrals (65). Any intermediate, recycled metabolites derived from benzo(a)pyrene phenols, such as phenol oxides, would presum-
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ably rearrange spontaneously to polyls if epoxide hydratase was inhibited and would be expected to accumulate as long as cytochrome P-450 remained active. The enhancement of DNA binding in presence of TCPO (cf. Fig. 4) again suggests that electrophilic products other than the benzo(a)pyrene 7,8-dihydrodiol-9,10-oxide contribute to the observed binding and indicates that initial benzo(a)pyrene oxides and activated phenols may bind to cellular DNA under these conditions. Activation of accumulated benzo(a)pyrene phenols to products that bind to DNA is in agreement with the observed mutagenic activity of phenols upon further oxidative metabolism (29).

In summary, the present study indicates that benzo(a)pyrene oxide hydrolase and glutathione conjugation, and glucuronide and sulfate conjugation of hydroxylated benzo(a)pyrene metabolites, operated in concert to detoxify electrophilic, DNA-binding metabolites of benzo(a)pyrene in isolated hepatocytes since TCPO, salicylamide, and diethylmaleate were more effective in combination than individually in stimulating DNA binding. Thus, the degree of covalent binding of benzo(a)pyrene to nuclear DNA of isolated hepatocytes is apparently regulated by the relative rate of production of electrophilic benzo(a)pyrene metabolites, by the cytochrome P-450-mediated monoxygenase, and the rate of their disposal by epoxide hydratase and the various conjugation reactions.

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