

A Method for *In Vitro* Culture of Rat Zymbal Gland: Use In Mechanistic Studies of Benzene Carcinogenesis in Combination with ^{32}P -Postlabeling

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Zymbal glands were excised bilaterally from the ear ducts of female Sprague-Dawley rats (three/group), minced into approximately four fragments per gland, and transferred into a microtiter plate containing 1.5 mL per well of Waymouth's tissue culture medium supplemented with fetal calf serum, hydrocortisone, insulin, and gentamicin. After addition of a test compound or solvent vehicle, plates were incubated for 6, 24, 48, or 96 hr at 37°C in a humidified atmosphere of 5% CO₂ in air. Tissue in culture for 6 hr was histologically indistinguishable from the freshly excised tissue, while that in culture for 24, 48, and 96 hr showed a progressive deterioration often with necrosis and/or squamous metaplasia. More pronounced deterioration was noted in samples treated with 750 or 1500 µg/mL of benzene. Using a nuclease P₁-enhanced ^{32}P -postlabeling assay, aromatic DNA adducts were detected in cultured Zymbal glands exposed for 48 hr to benzene and its derivatives, as well as to 7,12-dimethylbenzanthracene (DMBA) and 2-acetylaminofluorene (AAF). Benzene produced very low levels of adducts (0.5 adducts per 10⁹ nucleotides), whereas its congeners produced relatively high levels of adducts (50-2000 lesions per 10⁹ nucleotides), which decreased in the order benzoquinone > hydroquinone > phenol > benzenetriol > catechol. Each adduct profile overall was characteristic for the compound studied, suggesting the formation of compound-specific electrophiles. AAF and DMBA adducts were identical to those formed *in vivo* in animals. Our results show that the Zymbal glands are capable of metabolizing different carcinogens to DNA-reactive intermediates, a process that may be causally associated with tumor formation *in vivo* in this organ.

Introduction

Rat Zymbal glands, located at the base of the external auditory canal, have a distinctive pink-yellow color, weigh about 7 to 8 mg, and have a diameter of 3 to 5 mm. These glands, also present in other rodents and insectivores, but not in humans, have a typical sebaceous morphology, which is characterized by rounded sacs of plump, fat-containing cells (1). These cells subsequently break down, releasing fat droplets into the external ear canal (1). Maltoni et al. (2) and the National Toxicology Program (3) have reported a dose-dependent formation of solid tumors in Zymbal glands of rats following administration of repeated high doses of benzene. The mechanism by which

benzene induces this cancer is unknown, but it has been suggested from *in vitro* and *in vivo* metabolism studies that benzene is enzymatically activated to reactive intermediates that can bind to DNA (4), thereby forming covalent adducts, which, in turn, may causally be associated with mutation and the initiation of carcinogenesis (5).

The liver is generally considered to be the primary organ for benzene metabolism, but the incidence of liver tumors (hepatomas) has been reported to be three times lower than that of Zymbal gland carcinomas in rats after daily administration of 500 mg/kg benzene for 104 weeks (2). This difference in the susceptibility to carcinogenesis may, in part, derive from the unique metabolic capabilities of Zymbal gland tissue.

In order to facilitate the study of Zymbal gland metabolism, we have devised a culture system to maintain fragments of Zymbal glands for several hours after excision from rats. The capability of the cultured glands to metabolize benzene and its derivatives [catechol,

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1,2,4-benzenetriol (BT), phenol, hydroquinone (HQ), and benzoquinone (BQ)], as well as 7,12-dimethylbenzanthracene (DMBA) and 2-acetylaminofluorene (AAF), was then assessed by measuring covalent binding of these compounds to DNA using a highly sensitive ^{32}P -post-labeling assay (6,7). DNA adducts were readily detectable with all compounds tested, except benzene, which produced relatively low levels of adducts. DMBA- and AAF-DNA adduct profiles were similar to those formed *in vivo* in the epidermis and Zymbal gland, respectively, of treated animals.

Materials and Methods

Benzene was purchased from American Burdick and Jackson, a subsidiary of American Hospital Supply Corporation (Muskegon, MI). Catechol, BT, HQ, and BQ were obtained from Aldrich Chemical Company. Phenol (Mallinckrodt, Cat. No. 0025-2.5) was distilled prior to use. DMBA, AAF, and calf thymus DNA were from Sigma Chemical Company. Waymouth's culture medium and calf serum were from Gibco Laboratories. Carrier-free γ - ^{32}P -ATP (about 4000 Ci/mmol) was synthesized from $^{32}\text{P}_i$ (8) or purchased from ICN Radiochemicals (Cat. No. 35020). Polyethyleneimine (PEI)-cellulose plates were prepared (9) in the laboratory. The sources of all other materials needed for ^{32}P -postlabeling analysis of adducts have been documented previously (6-8).

For the organ culture experiments, Zymbal glands were aseptically excised from both the ear ducts of female Sprague-Dawley rats (4 months of age, weighing approximately 280 g), minced into four to six fragments per gland, and placed into Waymouth's MB752/1 tissue culture medium supplemented with 10% fetal calf serum (heat inactivated at 57°C for 30 min), 350 $\mu\text{g}/\text{mL}$ glutamine, 5 $\mu\text{g}/\text{mL}$ hydrocortisone, 5 $\mu\text{g}/\text{mL}$ insulin, and 0.1% gentamicin (10). The gland fragments from three rats were transferred into a 24-well microtiter plate (1.5 mL of the above medium per well), to which was added a test chemical or solvent vehicle (1%, v/v). DMBA and AAF were dissolved in DMSO, BT in ethanol, and others in water. Final concentrations in the incubation media were: DMBA, 5 $\mu\text{g}/\text{mL}$; AAF, 40 $\mu\text{g}/\text{mL}$; benzene, 7500 $\mu\text{g}/\text{mL}$ (solubility in water is 1800 $\mu\text{g}/\text{mL}$); others 750 and/or 1500 $\mu\text{g}/\text{mL}$. The plates were incubated for 6, 24, 48, or 96 hr at 37°C in a humidified atmosphere at 5% CO_2 in air. For the histopathological examination, freshly excised Zymbal glands, as well as the treated tissues, from each of the time points were fixed in neutral formalin, embedded in paraffin, cut at 6 μm thick, and stained with hematoxylin and eosin. For the adduct study, tissues were collected at 12 hr (DMBA) or 48 hr (others) after treatment. *In vivo*-modified AAF-DNA was prepared from the Zymbal glands of rats at 24 hr after daily oral gavage doses of 40 mg/kg AAF in ethanol/DMSO/olive oil (1:2:14, v/v, 3 mL/kg) for 5 days; control animals received solvent vehicle alone.

DNA was isolated from the Zymbal glands by treatment with ribonucleases (A, T₁) and proteinase K, fol-

lowed by a solvent extraction as described previously (11), except that the volumes of reagents were scaled down by 3-fold. An average yield of DNA from pooled Zymbal glands in several extractions corresponded to 17 (± 6) μg DNA per rat. The presence of adducts in DNA was analyzed using a nuclease P₁-enhanced postlabeling assay (7,8). Briefly, DNA (10-15 μg) was enzymatically digested to 3'-deoxyribomononucleotides (12), which were then treated with nuclease P₁, which dephosphorylates normal nucleotides but not aromatic adducted nucleotides (8). The latter were ^{32}P -labeled using γ - ^{32}P -ATP and polynucleotide kinase and resolved by PEI-cellulose TLC according to the following two methods.

In method A, which utilizes the techniques previously described (13-15), the adducted ^{32}P -nucleotides of AAF, benzene, and its derivatives were purified on a PEI-cellulose thin-layer plate (Fig. 1) in direction D1 in 2.3 M sodium phosphate, pH 5.77 (15), *in situ* magnet transferred to a second 20 x 12.5-cm size PEI-cellulose sheet (15), and resolved by a three-directional development (15) of the sheet in the following solvents: D3, 2.5 M lithium formate, 4.7 M urea, pH 3.5, to 20 cm; D4, 0.4 M sodium phosphate, 0.25 M Tris-HCl, 4.25 M urea, pH 8.0, to 12.5 cm; D5, 1.7 M sodium phosphate, pH 6.0, to 12.5 cm. DMBA adducts were chromatographed the same way as above except the composition of D3 and D4 solvents were D3, 3.2 M lithium formate, 6.0 M urea, pH 3.5; D4, 6.4 M sodium phosphate, 0.4 M Tris-HCl, 6.8 M urea, pH 8.0.

In method B, which was specially designed to permit the recovery of additional adducts more polar than those isolated in method A, the labeled solution was applied to

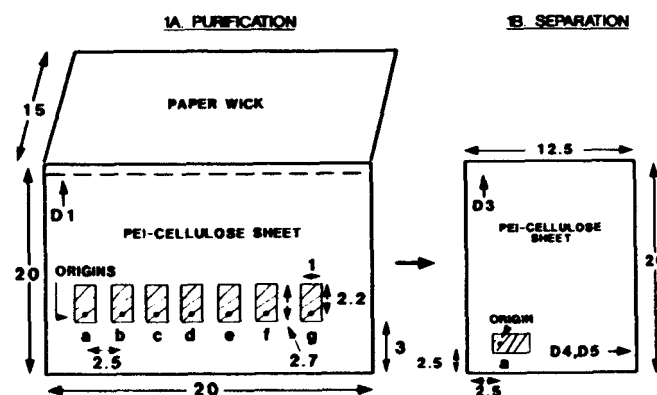


FIGURE 1. Scheme of PEI-cellulose TLC for purification and separation of adducted ^{32}P -nucleotides (method A). This scheme is essentially the same as the one previously described by Lu et al. (15) with a few minor modifications. ^{32}P -Labeled DNA digests were applied to the origins (a to g) of a PEI-cellulose sheet that had been attached to a Whatman paper No. 1 wick by stapling (A). The sheet was then developed in direction D1 to remove leftover γ - ^{32}P -ATP and ^{32}P -labeled contaminants. Each origin area (shaded area) containing purified aromatic adducts was cut out and attached to a second PEI-cellulose sheet (B) with the aid of button-type magnets for contact-transfer of adducts (15). The sheet was then developed along D3, D4, and D5 with solvents given in the text to transfer and resolve adducts. Measurements are in centimeters.

the origin (OR) of a PEI-cellulose sheet (20 x 20 cm) (Fig. 2). It was then developed overnight (16–17 hr) in direction D1 in 1.7 M sodium phosphate, pH 6.0, onto a Whatman No. 1 paper wick stapled to the top of the sheet. The wet sheet was cut below the paper wick, which was discarded and soaked twice in 1 L of deionized water for 5 min each. After drying, it was developed in D3, the same direction as the preceding development, with water to 1 cm from the bottom edge, then with 1.9 M lithium formate, 3.8 M urea, pH 3.5, to the top of the sheet. The sheet was soaked in water as before, dried, and developed in D4, perpendicular to the preceding development, in water to 1 cm, followed by 0.36 M sodium phosphate, 0.23 M Tris-HCl, pH 8.0, to the top, and dried. ^{32}P -adducts were detected by screen-enhanced autoradiography (6,7). Adduct radioactivity was determined by excising the spots from the chromatograms and Cerenkov counting (6,7). An estimation of adduct levels, expressed as relative adduct labeling (RAL), was made from the values of adduct count rates, adjusted for background radioactivity, and the specific activity of $\gamma\text{-}^{32}\text{P}\text{-ATP}$ determined by measuring the incorporation of ^{32}P into a known amount of dAp (7,8).

Results

Histopathological Examination of Tissues

Microscopic examination of the Zymbal glands maintained in tissue culture for various time periods without

any test substance revealed that the glands at 6 hr (not shown) were histologically indistinguishable from those at 0 hr, i.e., the freshly removed tissue (Fig. 3A), while the ones at 24 (not shown), 48 (Fig. 3B), and 96 hr (Fig. 3C) exhibited a progressive deterioration often with necrosis and/or squamous metaplasia.

Analysis of DMBA and AAF Adducts

Using the nuclease P_1 -version of the ^{32}P -postlabeling assay (7,8), we detected DNA adducts in the Zymbal glands treated in culture with DMBA (5 $\mu\text{g}/\text{mL}$) and AAF (40 $\mu\text{g}/\text{g}$), both being used in the present study as positive controls (Fig. 4). DMBA produced two major (spots 1 and 8) and five minor adducts (Fig. 4B) that were not detected in control DNA (Fig. 4A). The adduct pattern, except for an additional product 8, was qualitatively similar to that seen previously (7,8,16,17) in the epidermal DNA of mice that had been treated by topical application with DMBA. Cerenkov counting revealed that adducts 1 and 8 together comprised 70% of total DNA modifications, which corresponded to 25 adducts in 10^9 DNA nucleotides. Schmeiser et al. (18) have recently characterized adduct 1 as the major *syn* dihydrodiol epoxide deoxyguanosine (dG) product, and adducts 3 and 4 as the major *anti* dihydrodiol epoxide derivatives of deoxyadenosine (dA) and dG, respectively.

AAF produced four adduct spots (Fig. 4C) that were chromatographically identical to those obtained *in vivo* in Zymbal gland DNA of rats dosed orally with AAF (Fig. 4E). Because some aromatic amine adducts are not recovered completely by the nuclease P_1 -enhanced ^{32}P -procedure (7,8), AAF adducts were also evaluated by an alternate procedure involving butanol extraction of DNA digests prior to ^{32}P -labeling (19). More radioactivity was incorporated into spot 1 after butanol extraction (Fig. 4G, 4H) when compared with that after exposure to nuclease P_1 (Fig. 4D, 4E), suggesting the loss of this adduct by enzymic 3'-dephosphorylation. The recovery of adduct 1 after nuclease P_1 was 4% of that after butanol extraction; adduct 2 was recovered similarly with and without the enzyme (data not shown). Adduct 1, which has been characterized previously as $\text{dpGp}\cdot\text{C8}\cdot(\text{N}^2\cdot\text{AAF})$ (18), composed 89% of total modifications, corresponding to 31 adducts per 10^9 DNA nucleotides. Modification of the *in vivo* sample was about 540 lesions per 10^9 nucleotides, with adduct 1 representing 97% of the total. Adduct 2 has previously been identified as $\text{dpGp}\cdot\text{N}^2\cdot(\text{C3}\cdot\text{AAF})$ (18).

Analysis of Adducts from Benzene and Its Derivatives

When DNA samples isolated from the Zymbal glands that had been treated in culture with various derivatives of benzene (1500 $\mu\text{g}/\text{mL}$), as well as benzene itself (~ 1800 $\mu\text{g}/\text{mL}$), were analyzed by the nuclease P_1 -amplified ^{32}P -postlabeling assay, the autoradiograms shown in Figure 5 were obtained. DNA adducts were readily detectable

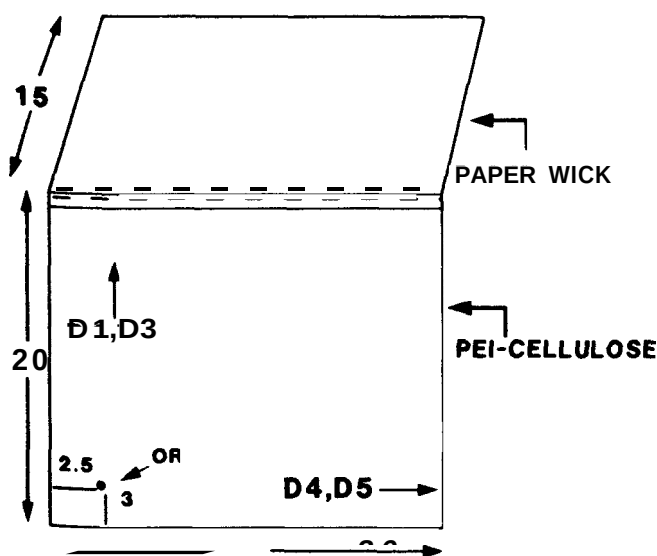
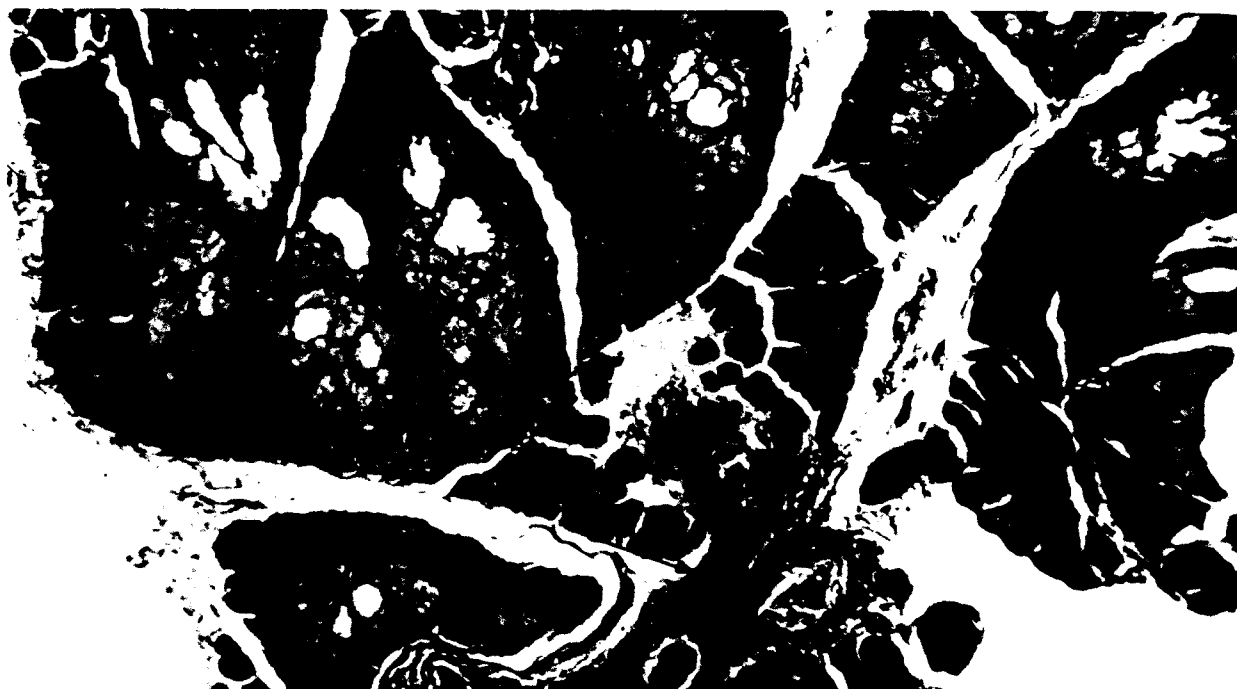
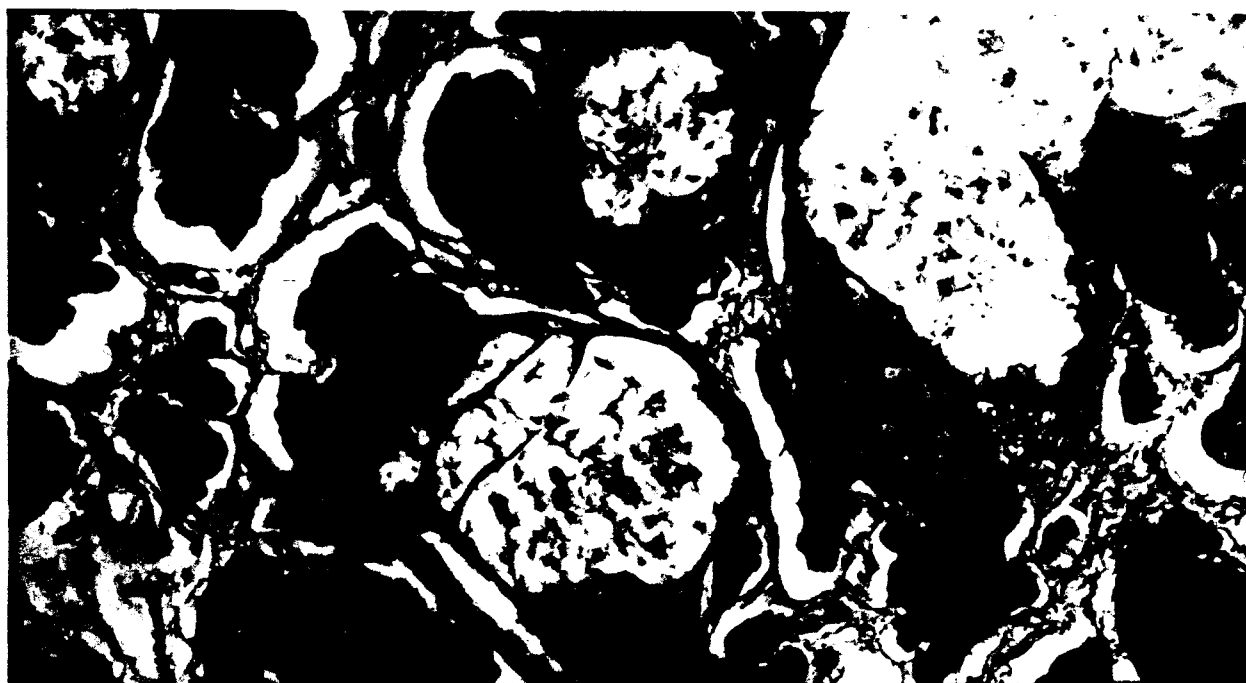


FIGURE 2. Scheme of PEI-cellulose TLC for purification and separation of presumably less hydrophobic adducts derived from phenol and HQ (method B). ^{32}P -Labeled digests were applied at the origins (OR) of a PEI-cellulose thin-layer plate, which was developed along D1 to remove $^{32}\text{P}\text{-ATP}$ and partially resolve adducts. Further resolution of adducts was obtained by development along D3, D4, and D5 with solvents of composition given in the text. Measurements are in centimeters.



A



B

FIGURE 3. Light photomicrographs of rat Zymbal gland. (A) Freshly excised and fixed tissue. Focal fresh hemorrhage (arrow) in the supporting connective tissue and the normal architecture of the gland are seen. (B) Zymbal glands maintained in the culture medium without a test substance for 48 hr. Dissolution of glandular epithelium forming cystic spaces, which are partially filled with cell debris is seen. (C) Zymbal glands maintained in the culture medium without a test substance for 96 hr. Large cystic spaces with cell debris and squamous metaplasia (arrow) of the glandular epithelium are present.

Continued on next page



FIGURE 3. Continued.

C

following exposure to different derivatives of benzene, with unique adduct profiles for each compound. Catechol produced one main adduct (spot a) and several minor ones (spots b to m), some of the latter migrated in areas where the DNA derivatives of BT migrate. Phenol produced one major (spot 1) and seven minor adducts; HQ induced an adduct pattern of 3 major spots (spots 5 to 7) and many minor adducts. With BQ-DNA, one major (spot 6) and a few minor alterations were detected. The major adduct was found to be a guanine derivative, as a chromatographically identical product was formed when dGp was reacted *in vitro* with BQ and ^{32}P -labeled (data not shown). With benzene-DNA, no adducts were detected when the chromatogram was exposed to X-ray film for the same time period as that employed for the detection of metabolite-DNA adducts; however, upon five times longer film exposure, two faint adduct spots in the area where the major products of BQ and phenol migrate were seen when compared with the control map left in contact with the film for the same period. Adducts exhibiting similar mobilities on the ^{32}P maps were tentatively identified by overlapping the autoradiograms. Unequivocal evidence for the identity of adducts of interest would, however, require chromatography of a mixture of ^{32}P -labeled DNA digests in additional solvents and using different TLC systems (20). As shown in Figure 6, when ^{32}P -labeled digests were chromatographed according to

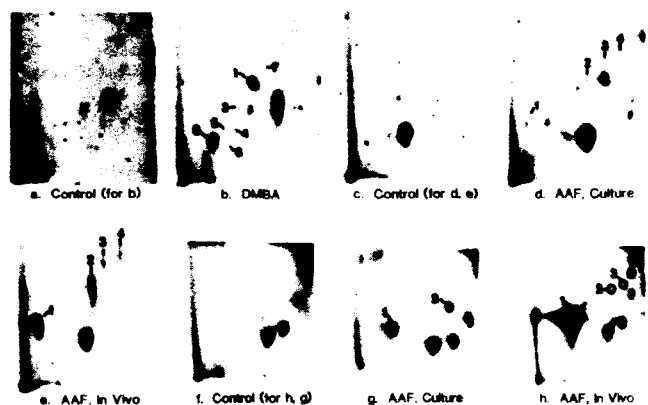


FIGURE 4. Autoradiograms of TLC maps of ^{32}P -labeled DMBA and AAF adducts. DNA specimens isolated from the Zymbal glands exposed in culture (a-d, f, g) or *in vivo* (e, h) to solvent vehicle (a, DMSO, c and f, ethanol/DMSO/olive oil) and the indicated carcinogens were digested to normal and adducted nucleotides. The latter were enriched, ^{32}P -labeled, contact-transferred, and resolved by TLC (15). Adducts of DMBA (a, b) and AAF (e-e) were enriched by nuclease P_1 treatment and resolved using solvents given in the text. AAF adducts were also enriched by butanol extraction (f-h) (19) and resolved according to the published conditions (14) with modifications entailing use of 2.3 M sodium phosphate, pH 5.77, as a D1 solvent and a magnet-transfer step after D1 development (15). Autoradiography was performed at -80°C for 2 hr (a, b), 5 hr (c, e), 15 hr (d), and 3 hr (f-h). Faint adduct spots requiring longer film exposure times (15-20 hr) have been circled.

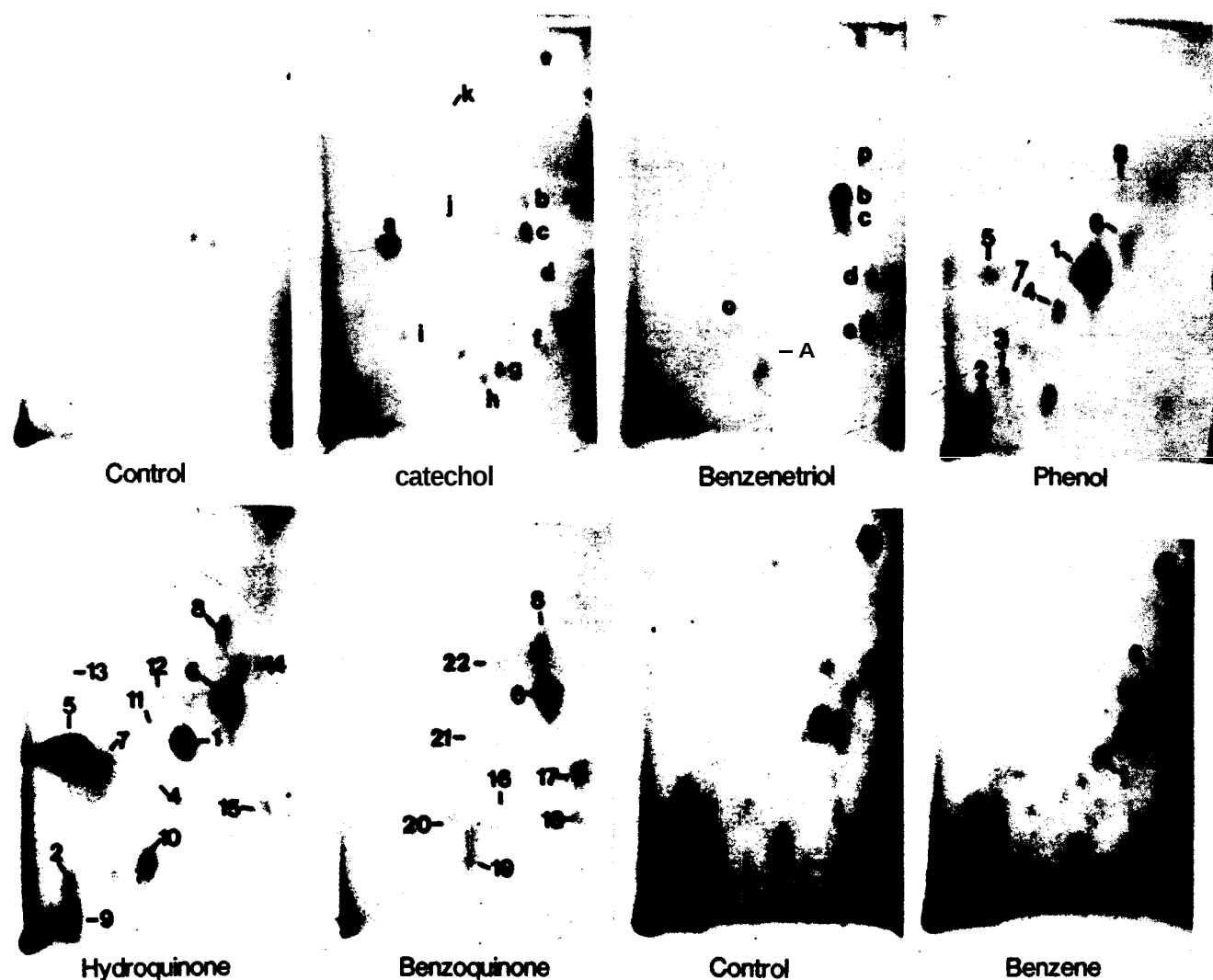


FIGURE 5. Autoradiograms of TLC maps of ^{32}P -labeled DNA adducts derived from benzene and its derivatives. DNA samples isolated from Zymbal glands exposed in culture for 48 hr to the solvent vehicle (control) and the indicated compounds were digested and ^{32}P -labeled. Labeled adducts in DNA digests were purified, contact-transferred, and resolved by two-dimensional TLC according to method A outlined in Fig. 1. D3 development was in the vertical direction, and D4 and D5 developments were in the horizontal direction. Autoradiographic conditions were: control (top row), catechol and BT, -80°C for 8 hr; phenol and HQ, -80°C for 2 hr; BQ, 23°C for 2 hr; control (bottom row) and benzene, -80°C for 36 hr. Note that the sensitivity of film detection is increased 3- to 4-fold at -80°C relative to 23°C . Chromatographically corresponding adducts on maps of catechol and BT, as well as those of phenol, HQ, BQ, and benzene, were identified with the same letters or numbers.

method B, which permits recovery of derivatives more polar than those detected above (Fig. 5), one additional adduct (no. 23) with phenol-DNA and two (nos. 23 and 24) with HQ-DNA were detected.

DNA modifications with different benzene derivatives ranged from 45 to 1980 adducts per 10^9 nucleotides and decreased in the order $\text{BQ} > \text{HQ} > \text{phenol} > \text{BT} > \text{catechol}$ (Table 1). Phenol and HQ induced dose-dependent adducts at two concentrations tested. More BQ adduct (no. 6) was formed when the HQ concentration was increased from 750 to $1500 \mu\text{g/mL}$. The adduct levels determined represent a minimum estimate, as the efficiency of labeling of various adducts and the extent of their resistance to 3'-dephosphorylation by nuclease P_1

are unknown, except for adduct 6 which was found completely resistant to nuclease P_1 -mediated 3'-dephosphorylation (21).

Discussion

In this report, we show for the first time that rat Zymbal glands can be maintained in culture for a period long enough to study early effects of genotoxins, e.g., DNA adduct formation. Employing the nuclease P_1 -enhanced ^{32}P -postlabeling assay, DNA adducts have been detected in Zymbal glands cultured in the presence of DMBA, AAF, benzene, and its derivatives.

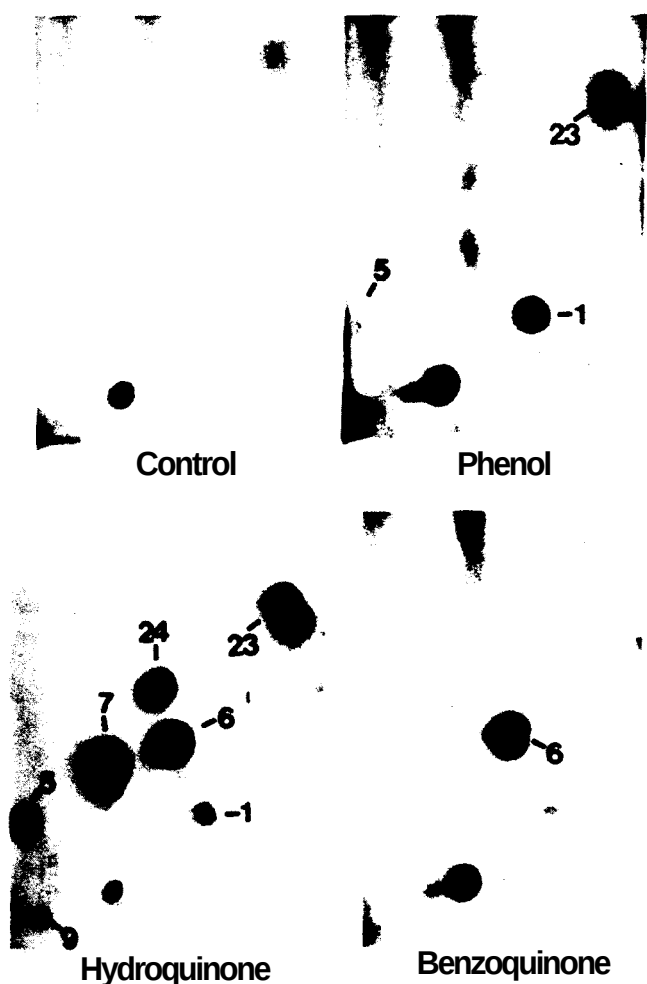


FIGURE 6. Autoradiograms of TLC maps of ^{32}P -labeled DNA adducts derived from phenol, HQ, and BQ. DNA specimens prepared as given in the legend of Fig. 5 were digested and ^{32}P -labeled. Labeled adducts in DNA digests were purified and resolved according to method B outlined in Fig. 2. D1 and D3 developments were in the vertical direction and D4 development in the horizontal direction. Autoradiographic conditions were similar to those given in the legend of Fig. 5.

Detection of DNA adducts with the indirect carcinogens, DMBA and AAF, indicates that Zymbal glands in culture are capable of metabolically activating these carcinogens to electrophilic intermediates that can bind to DNA. The activation pathways in the cultured Zymbal glands appear to be similar to those occurring *in vivo*, in as much as the adduct profiles (Fig. 4) were qualitatively the same as those seen *in vivo* in DMBA-treated mouse epidermal DNA (8,16-18) and in AAF-treated rat Zymbal glands in the present studies. DMBA, however, induced an additional product (no. 8) in cultured Zymbal gland, which has not been detected *in vivo* in mouse skin (8,16,17), suggesting the formation of some tissue-specific reactive intermediates. DMBA and AAF metabolism is mediated by a mixed-function oxidase system (22,23). Adduct detection in Zymbal glands is consistent with the observation by Pohl and Fouts (24), who have shown the

presence of cytochrome P-450-dependent aryl hydrocarbon hydroxylase activity in rat Zymbal gland homogenates.

The comparison of adduct patterns among different benzene metabolites studied (Fig. 5) enables the prediction of possible electrophiles formed from the individual compounds. Metabolism studies with liver and bone marrow (4,25,26) have shown that phenol can be oxidized to catechol, HQ, and biphenol by cytochrome P-450-dependent monooxygenase, peroxidase, or active oxygen released during the oxidative burst. Our findings that the major derivatives of phenol (Fig. 5, adducts 1 and 6) were different from those of catechol and overlapped with a fraction of HQ adducts suggest, but do not prove, that, in cultured Zymbal glands, conversion of phenol to catechol is negligible, while its conversion to HQ represents a minor pathway. This further suggests that phenol may be predominantly metabolized to biphenols, which, following oxidation to their semi-quinone and quinone entities, can bind to DNA.

The putative reactive metabolites derived from HQ are likely to be *p*-benzosemiquinone and/or BQ, which can be formed either by autooxidation of HQ at physiological pH in the presence of oxygen or by enzyme-mediated oxidation of HQ (4,25,26). In addition, HQ can be converted to BT, which can autooxidize to electrophiles such as α -hydroxy-*p*-benzosemiquinone and α -hydroxy-*p*-benzoquinone (4,25,26). Since only a minor fraction of HQ adducts (Fig. 5, adducts 6 and 8, composing < 20%) corresponded to BQ- and BT-derived modifications (Fig. 5, adducts 6, 8, and b), it appears that in Zymbal gland culture, BQ and BT are minor reactive intermediates formed from HQ, while the *p*-benzosemiquinone may predominate.

The DNA-reactive species derived from catechol may be either a mixture of *o*-benzosemiquinone and *o*-benzoquinone formed by enzyme mediated-oxidation or a mixture of semiquinone and quinone species of BT (4,25,26). About 20% of total modifications produced by catechol exhibited chromatographic properties similar to those derived from BT (Table 1), indicating that the conversion of catechol to BT is a minor pathway. Therefore, the alternate pathway leading to the formation of the semiquinone and quinone is probably the major one. The unique ^{32}P -fingerprints for each metabolite tested (Figs. 5 and 6) are consistent with the data of Rushmore et al. (27), who have observed different Sephadex LH-20 column profiles of dG adducts in ^3H -GMP-prelabeled DNA of rabbit bone marrow mitoplasts exposed *in vitro* to HQ, BQ, phenol, catechol, and BT.

Although phenol or other metabolites of benzene induced relatively high levels of adducts, benzene itself produced adducts at levels barely detectable by the ^{32}P -postlabeling assay. This could result from a) less efficient conversion of benzene to phenol in the Zymbal gland cultures; b) the high volatility of benzene, reducing the effective dose reaching Zymbal glands in the culture medium; and/or c) the metabolism of benzene predominantly to a ring-opened derivative (28), which may form polar adducts not detectable by postlabeling under the

Table 1. Levels of DNA adducts formed in Zymbal glands in culture treated with benzene and its metabolites. ^aas estimated by a nuclease P₁-enhanced postlabeling.¹

Compound	Concentration, μg/mL	RAL × 10 ⁹ for adduct spot ^b (No. of adducts per 10 ⁹ DNA nucleotides)						Total
		a	b	c	d	e	Others	
Catechol	1500	16.2	3.0 (7)	6.0 (13)	NC ^c	5.2 (11)	15.0 (33)	45.4
BT	1500	ND ^d	20.2 (38)	8.7 (17)	5.1 (10)	8.4 (16)	10.3 (20)	52.7
		1	5	6	7	23	24	Others
Phenol	750	30.7 (42)	ND	ND	ND	33.5 (46)	ND	9.4 (13)
	1500	75.1 (52)	ND	3.5 (2)	ND	52.9 (36)	ND	14.8 (10)
HQ	750	36.1 (3)	317 (29)	39.6 (4)	260 ^f (24)	254 (24)	90.2 (8)	83.5 (8)
	1500	53.3 (4)	269 (22)	228 (18)	202 (16)	215 (17)	121 (10)	162 (13)
BQ	1500	ND	ND	1370 (69)	ND	ND	ND	614 (31)
Benzene	~1800	NC		0.5				0.5

^aAdduct spots (Figs. 5 and 6) were cut from the chromatograms and counted by Cerenkov assay. RAL values were calculated from count rates using the specific activity of γ-³²P-ATP (7,8).

^bFor adduct assignments, see Figs. 5 and 6.

^cNot countable.

^dNumbers in parentheses indicate percentages of each adduct relative to total.

^eNot detected.

^fThe recovery of this adduct varied markedly. The value represents a maximum estimate.

experimental conditions described. Currently, none of these possibilities can be dismissed.

Reaction of BQ or HQ with dG has been shown by Jowa et al. (29) to generate (3'-OH)benzetheno(N1,N²)dG as the major derivative on the basis of HPLC and NMR analysis. In comparison with this data, adduct 6, which was detected as the major product of the interaction of BQ with DNA or dGp (Fig. 5), may be (3'-OH)-benzetheno-(N1,N²)deoxyguanosine 3',5'-[³²P]bisphosphate. The detection of multiple ³²P-postlabeled adducts with HQ-DNA is in accordance with the multiple HPLC peaks obtained from the hydrolysate of calf thymus DNA modified *in vitro* by ¹⁴C-HQ (29).

Analysis of AAF adducts here (Fig. 4) and those of 4-aminobiphenyl previously (7,8) indicate that the C8-guanine derivatives are susceptible to 3'-dephosphorylation by nuclease P₁, while the N²-guanine derivatives are not. Adducts of benzo[a]pyrene, safrole, and mitomycin C, which predominantly bind at the N² position of guanine, have also been shown to be resistant to the enzyme activity (7,8). Taken together, the data suggest that an adducted 3'-nucleotide with a carcinogen bound to a base at the exocyclic position, but not at the endocyclic position, is resistant to nuclease P₁-mediated dephosphorylation. When the attachment is simultaneously at the exocyclic and endocyclic positions, for example, a cyclic adduct, as in the case of the major adduct of BQ-DNA above, the adduct may not be susceptible to the enzyme activity (21). Such differential enzyme activity may be useful in adduct characterization.

Our results demonstrate the utility of postlabeling techniques to determine the capacity of tissues or cells to metabolize a mutagen/carcinogen of interest by measuring DNA adduct formation. Because of high sensitivity

and the need for only a few micrograms of DNA, the ³²P-postlabeling assay affords adduct measurement in a small number of cells, as evident from the present studies on Zymbal glands weighing only about 15mg per rat. The ³²P-postlabeling protocol, with further refinement to reduce background noise, may potentially be applicable to monitor the formation of DNA adducts in blood cells of humans exposed to benzene and thereby provide useful information for risk assessment and management.

By combining the highly sensitive postlabeling assay with the newly devised Zymbal culture system, we have begun to elucidate the metabolic pathways and macromolecular interactions in this target tissue. Insights gained from this work will subsequently be applied to the design and interpretation of analogous *in vivo* studies, with the ultimate goal of better understanding the mechanisms involved in the carcinogenic process.

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