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N-methylation reduces the DNA-binding activity of 7H-dibenzo[c,g]carbazole ~300-fold in mouse liver but only ~2-fold in skin: possible correlation with carcinogenic activity

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_N_-methyl-dibenzo[c,g]carbazole (MeDBC) lacks the potent hepatocarcinogenic activity in mice characteristic for 7H-dibenzo[c,g]carbazole (DBC), while both compounds are local carcinogens, leading to papilloma and carcinoma formation in skin after topical application. Because DNA binding is considered an essential step in the initiation of chemical carcinogenesis, the DNA adduction by MeDBC was compared with that by DBC in mouse liver and skin via a ^{32}P-postlabeling technique. Both compounds elicited chromatographically similar adducts in liver; however, the extent of total DNA binding of DBC was 343- and 265-fold greater than that of MeDBC 24 h after topical and i.p. administration, respectively, of a 37 μmol/kg dose. In skin, the adduct pattern elicited by either compound after topical application was different from that seen in liver, and three of four adducts derived from MeDBC were chromatographically distinct from those produced by DBC. Quantitative analysis revealed that total adduction in skin by DBC was 2.3-fold higher than by MeDBC. When the adduct levels were compared between liver and skin, topically applied MeDBC bound preferentially to skin versus liver DNA by a factor of 10, while the opposite was true for DBC. These data are in agreement with the carcinogenicity reported for DBC and MeDBC and support the hypothesis that the extent of covalent DNA modification by these compounds is associated with their biological activity. We conclude that an unsubstituted nitrogen is essential for the genotoxic activity of DBC in liver but not skin. The results also demonstrate the potential of the ^{32}P-postlabeling assay in predicting the organotropism of closely related carcinogens.

Introduction

Compared with polycyclic aromatic hydrocarbons (PAH*), the biological activity of *N*-heterocyclic polynuclear compounds has not been thoroughly studied, although these compounds are important environmental pollutants, and some have been shown to be carcinogenic (1–3). Among these compounds, 7H-dibenzo[c,g]carbazole (DBC) is found in cigarette and wood smoke (4,5), synfuel products (6), sediments of industrially polluted rivers (7), and is formed during the production of carbon black (8). Unlike PAHs, DBC is unusual in that it is both a potent local and systemic carcinogen (9–16). Kirby and Peacock (15) and Kirby (16) demonstrated that in contrast to DBC, *N*-methyl-dibenzo[c,g]carbazole (MeDBC) (Figure 1) and *N*-ethyl-dibenzo[c,g]carbazole do not possess any hepatocarcinogenic activity; however, like DBC, they are carcinogenic in mouse skin. Thus, the heterocyclic nitrogen strongly affects the biological activity of DBC. This hypothesis is supported by observations that the homocyclic analog of DBC, dibenz[a,j]anthracene, is a weak carcinogen in skin and is inactive in liver (2), and both dinaphtho(2,1,1′,2′)furan and dinaphtho(2,1,1′,2′)thiophene, the *O*- and *S*- isosters of DBC, respectively, lack carcinogenic activity altogether (17).

It is believed that DNA damage is a critical event in the initiation of chemical carcinogenesis because of the good correlation between the covalent binding in DNA by a compound and its carcinogenic potency (18,19). Schurdak and Randerath (20) and Schurdak *et al.* (21) have shown that after a single s.c., i.p. or topical administration of DBC, there is extensive and preferential binding to liver DNA compared with other tissues, and suggested that this may be related to the hepatocarcinogenicity of the compound. Recently, Schurdak *et al.* (21) have identified the phenolic metabolite, 3-hydroxy-DBC, as a likely proximate metabolite of DBC leading to liver DNA adduction. The reason for MeDBC being a skin but not liver carcinogen may lie in the ability of the compound to more effectively bind covalently to skin versus liver DNA. In this paper, we have tested this hypothesis by using a ^{32}P-postlabeling technique (22–24) to compare the *in vivo* DNA binding between MeDBC and DBC in mouse liver and skin. The results showed that, after both topical and i.p. administration, DNA binding in liver by MeDBC was negligible. After topical application, both MeDBC and DBC bound significantly to skin DNA.

Materials and methods

Chemicals

DBC and dimethylsulfoxide (DMSO) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Trioctanoin was from Sigma Chemical Co. (St Louis, MO). The sources of the materials for adduct analysis have been previously reported (22,23). Polyethyleneimine–cellulose thin layers were prepared in the laboratory (22) to ensure reproducible adduct resolution. MeDBC was prepared by *N*-alkylation of DBC using the method of Stevens and Tucker (25). The compound was characterized by u.v., fluorescence, nuclear magnetic resonance and mass spectrometry. H.p.l.c. utilized a methanol–water gradient (76–100%) at a flow rate of 1 ml/min at room temperature in a Varian 8500 h.p.l.c. unit fitted with a 25 × 4.6 mm, 10-μm particle size Whatman Partisil-10 ODS-2 reversed-phase column. The effluent was monitored by u.v. absorption at 268 nm and indicated >99.9% purity of the MeDBC preparation (D.B.Stong, R.T.Christian, K.Jayasimuhulu, R.M.Wilson and D.Warshawsky, in preparation).

Animals and treatment

For topical treatments, a 6-cm^2 area of the backs of 8–10-week-old female CD-1 mice (Charles River Breeding Co., Wilmington, MA) was shaved with an electric clipper (Oster, Milwaukee, WI). Groups of three mice each received 37 μmol/kg of either MeDBC or DBC applied to the shaved area in 0.2 ml acetone.

*Abbreviations: PAH, polycyclic aromatic hydrocarbon; DBC, 7H-dibenzo[c,g]carbazole; MeDBC, *N*-methyl-dibenzo[c,g]carbazole; DMSO; dimethylsulfoxide; IF, intensification factor; <RAL>, relative adduct labeling under ATP-deficient conditions; RAL, relative adduct labeling under conditions of ATP excess; D, direction or directional; 3-OH-DBC, 3-hydroxy-dibenzo[c,g]carbazole.

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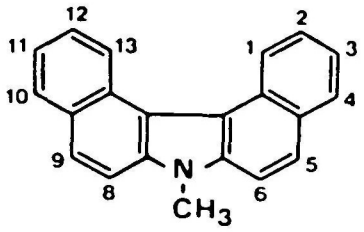

Fig. 1. The structure of *N*-methyl-dibenzo[c,g]carbazole.

For i.p. injections, groups of three mice each were given 37 μmol/kg of either MeDBC or DBC in 0.2 ml trioctanoin:DMSO (6.7:1). Control mice received vehicle alone. Mice were killed by cervical dislocation 24 h after treatment. To obtain DBC adducts for cochromatographic analysis, two groups of four mice each received 44 μmol/kg DBC topically and were killed 0.5 and 6 h after treatment. Liver and dorsal skin were taken from all animals and kept at −80°C until DNA isolation.

DNA isolation and adduct analysis

DNA was isolated by solvent extraction and enzymatic digestion of protein and RNA (26). DNA adducts were assayed by a ^{32}P-postlabeling technique (22−24). Nucleotide digests were labeled in the presence of a limiting amount of [γ-^{32}P]ATP (1.7 μM) relative to DNA-P (400 μM) (24) to increase the sensitivity of adduct detection compared with the standard procedure, which employs an excess of [γ-^{32}P]ATP over DNA-P (22,23). Intensification factors (IFs) (24) for DBC adducts in liver and skin have been reported (20,21). To determine the IFs for MeDBC adducts in skin, DNA from mice treated topically with 37 μmol/kg MeDBC for 24 h was labeled in parallel under both the standard and adduct intensification conditions. The IFs for individual adducts were calculated as the ratio of the relative adduct labeling obtained under intensified (= <RAL>) to that obtained under standard conditions (= RAL). MeDBC adduct levels in liver were determined using the IFs established for DBC liver adducts, since in this organ, the adducts derived from both compounds were chromatographically identical (see Results).

Normal nucleotides were analyzed as described (22). For adduct purification, 2.3 M sodium phosphate, pH 5.8 (solvent I), replaced the 1.1 M lithium chloride direction (D)1 solvent (22), and a magnet transfer technique was used during D3 (27). Chromatography in 2.7 M ammonium formate (D2) was omitted. Conditions for two-dimensional adduct separation in D3 and D4 were 2.8 M lithium formate, 6 M urea, pH 3.5 (solvent II) and 0.72 M lithium chloride, 0.35 M Tris−HCl, 7.7 M urea, pH 8.0 (solvent III), respectively. A final development in 1 M sodium phosphate, pH 6.8 (solvent IV) was included to reduce background (21).

For cochromatography (21) of MeDBC- and DBC-derived adducts, aliquots of ^{32}P-labeled DNA digests were mixed at the indicated ratios and then analyzed by t.l.c. The mixtures analyzed were: liver DNAs, DBC (0.5 h):MeDBC (24 h) (1:4); skin DNAs, DBC (6 h):MeDBC (24 h) (2.2:1). The ratios were chosen in consideration of the different adduct levels. For 2-D t.l.c., solvent II was used to separate the adducts in D3. Resolution in D4 was achieved under various chromatographic conditions employing three solvents: solvent III; 0.5 M Tris−HCl, 0.5 M boric acid, 1.3 M sodium chloride, 10 mM EDTA, 8 M urea, pH 8.0 (solvent V); or 1 M sodium phosphate, 4 M urea, pH 8.0 (solvent VI). Autoradiography was performed as described (21, 22).

Results

The DNA adduct patterns produced by MeDBC and DBC in liver after topical or i.p. administration of a 37 μmol/kg dose are shown in Figure 2. While the patterns were qualitatively similar for the two compounds, resembling the DNA fingerprint seen previously with DBC (20,21), inspection of the maps indicated large quantitative differences. (Note that film exposures in Figure 2 for the DBC samples were 40 min, while those for MeDBC were 48 h.) Cochromatographic analysis using solvent II in combination with solvents III, V or VI failed to distinguish the MeDBC-derived adducts from the DBC-derived adducts (data not shown). Quantitative data for both compounds have been presented in Table I. The total level of liver DNA adduction by DBC was 343- and 265-fold greater than that by MeDBC after topical and i.p. administration respectively. Notably, the route

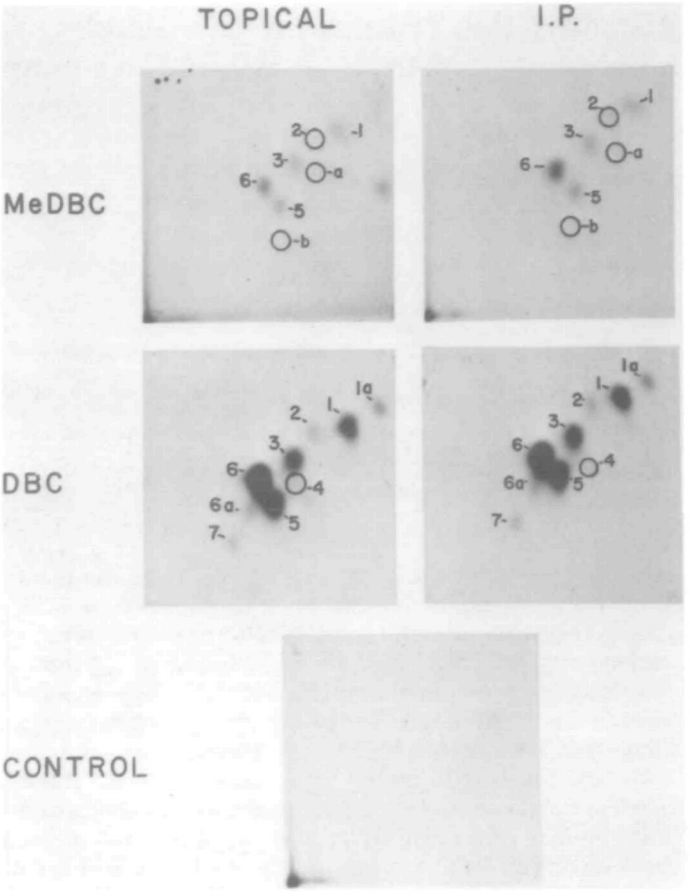

Fig. 2. DNA adduct patterns in mouse liver 24 h after topical or i.p. administration of 37 μmol/kg of MeDBC or DBC. ^{32}P-postlabeling was performed under adduct intensification conditions, and 178 μCi of labeled nucleotides were spotted for D1 purification (24). Separation of the adducts in D3 was with solvent II, and in D4 with solvent III. Du Pont Cronex 4 film was exposed as follows: MeDBC and control, 48 h, DBC, 40 min. Adduct numbers in the MeDBC pattern correspond to liver adduct numbers. Adducts requiring longer film exposure for their detection have been circled. Spots a and b are uncharacterized spots not seen in control or DBC-treated DNA and may be minor MeDBC adducts.

of administration did not significantly affect the level of adduction by either compound.

In skin, topical application of MeDBC resulted in four adducts, while DBC produced six adducts (Figure 3). The DBC-derived adduct fingerprint in skin DNA has been previously shown to be qualitatively distinct from that in liver DNA (21). In the present experiments, the MeDBC-derived adducts numbered 2, 3 and 6a appeared similar to the same numbered DBC-derived adducts, but the major labeled adduct 1 was unique to the MeDBC fingerprint. Cochromatography in solvents II + V revealed, however, that both adducts 2 and 3 elicited by either compound were separable, while this was not the case for adduct 6a (Figure 4).

Because after topical application, MeDBC- and DBC-derived skin adducts were different, IFs for the former were determined as described in Materials and methods and are given in Table II. Interestingly, adduct 6a derived from MeDBC had the same IF as adduct 6a produced by DBC (21). This observation further supported the chemical identity of adduct 6a suggested by cochromatography (Figure 4). The adduct levels elicited by MeDBC and DBC in skin are presented in Table III. Examination of the data revealed that total adduction by DBC was 2.3-fold

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Table I. DNA adduct levels in mouse liver, determined by the adduct intensification version of the ^{32}P-postlabeling assay and expressed as RAL × 10^7, 24 h after topical or i.p. administration of 37 μmol/kg MeDBC or DBC[a]

	Topical		I.p.	
Adduct	MeDBC	DBC	MeDBC	DBC
1	0.22 ± 0.04[b](34)[c]	39.9 ± 8.9 (18)	0.20 ± 0.09 (22)	45.7 ± 2.9 (19)
2	0.16 ± 0.05 (25)	18.3 ± 5.5 (8)	0.17 ± 0.07 (19)	24.3 ± 11.5 (10)
3	0.08 ± 0.02 (12)	26.1 ± 2.6 (12)	0.18 ± 0.04 (20)	30.3 ± 8.6 (13)
4	ND[d]	12.4 ± 7.6 (6)	ND	8.2 ± 2.7 (3)
5	0.07 ± 0.01 (11)	39.9 ± 7.1 (18)	0.09 ± 0.02 (10)	41.5 ± 16.9 (18)
6	0.12 ± 0.03 (18)	75.4 ± 10.9 (34)	0.18 ± 0.03 (20)	74.6 ± 16.0 (32)
7	ND	10.7 ± 1.2 (5)	0.07 ± 0.02 (8)	11.4 ± 2.8 (5)
Total	0.65 ± 0.07	222.7 ± 18.6	0.89 ± 0.13	236.0 ± 27.8

[a]The RAL values were calculated by dividing <RAL> values obtained under adduct intensification conditions by the IFs previously established for DBC adducts in liver (20). Because IFs of individual adducts are different (20), the RAL values shown here do not correlate proportionally with the spot intensities in Figure 2. Spots a, b, 1a and 6a were not quantitated.
[b]Standard deviation of the mean from at least three analyses.
[c]Percentage of the total level.
[d]ND = not detected.

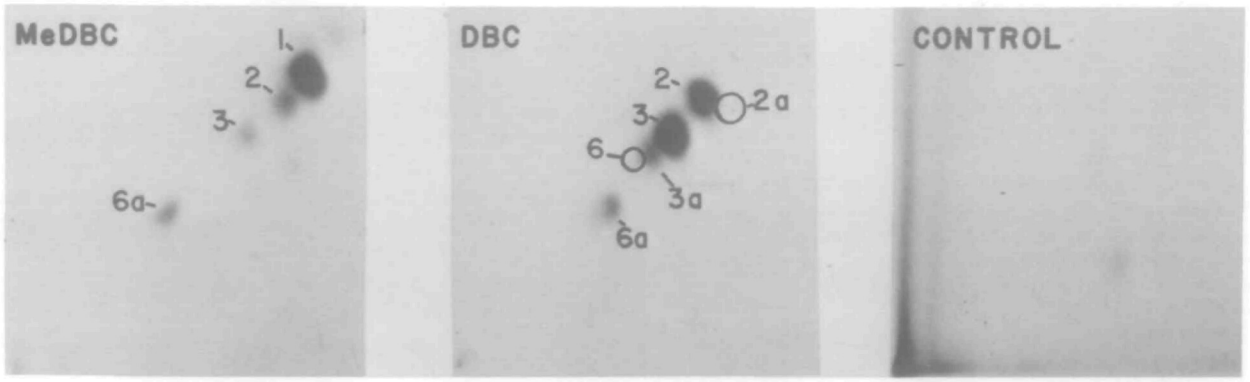

Fig. 3. DNA adduct patterns in skin 24 h after topical application of 37 μmol/kg of MeDBC or DBC. ^{32}P-postlabeling and adduct separation were as in Figure 2. Kodak XAR-5 film was exposed as follows: MeDBC and DBC, 2 h; control, 48 h. The numbers of the skin and liver adducts do not correspond to each other. Adduct 2a required a 4-h film exposure for its detection.

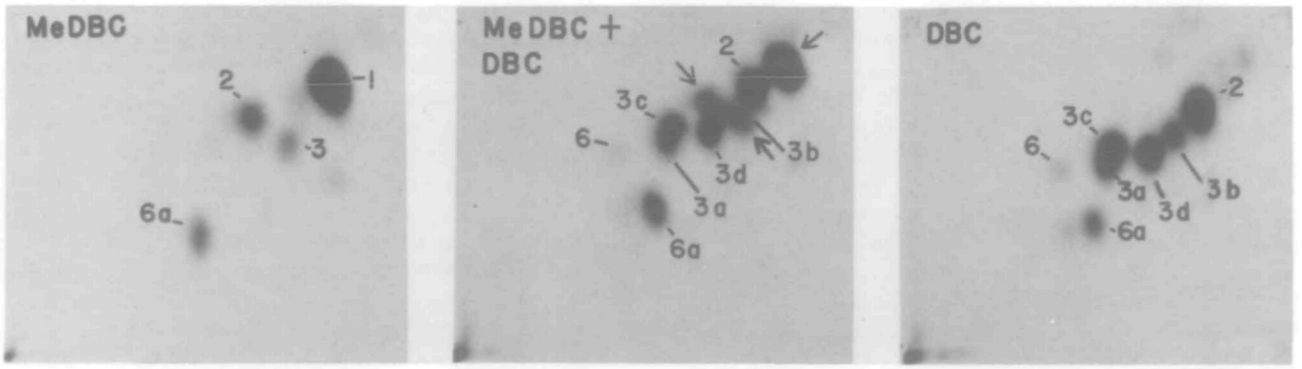

Fig. 4. Cochromatography of MeDBC and DBC adducts in skin after topical application. MeDBC-adducted DNA was taken 24 h after a dose of 37 μmol/kg, and DBC-adducted DNA was obtained 6 h after application of 44 μmol/kg. The amounts of labeled nucleotides spotted for D1 purification (24) were as follows: DBC alone, 211 μCi; MeDBC alone, 103 μCi; for cochromatography, 70 μCi MeDBC–DNA digest was mixed with 151 μCi DBC-DNA digest (ratio 1:2.2, MeDBC:DBC). Separation in D3 was with solvent II, and in D4 with solvent V. Du Pont Cronex 4 film was exposed for 24 h. The numbering systems in the outer panels correspond to those in Figure 3. DBC spot 3 (Figure 3) was resolved into three subfractions, designated 3b, 3c and 3d (21). In the middle panel, the arrows indicate the MeDBC-derived adducts (corresponding to 1, 2 and 3 in the left-hand panel) that did not comigrate with any DBC adduct. The numbers in the middle panel indicate the DBC-derived adducts. Adduct 6a was the only adduct that cochromatographed in the mixed sample.

higher than by MeDBC. Adduct 6a was formed to similar extents by both compounds. Comparison of quantitative data showed that, in contrast to topically applied DBC, which bound 14 times less to skin than to liver DNA, MeDBC adducted skin DNA 11 times more than it did liver DNA (Tables I and III).

Discussion

In the present study we utilized a ^{32}P-postlabeling assay to compare the DNA adduction by MeDBC and DBC in mouse liver and skin. The aim of this work was (i) to ascertain whether the

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Table II. Intensification factors for MeDBC-derived adducts in mouse skin (Figure 3) after topical application of 37 μmol/kg[a]

Adduct	<RAL>	RAL	IF[b]
1	150.0 ± 39.4[c]	3.50 ± 0.80	42.9
2	39.4 ± 7.2	1.14 ± 0.30	34.6
3	15.6 ± 3.6	0.28 ± 0.04	55.7
6a	22.5 ± 4.3	2.25 ± 0.64	10.0

[a]<RAL> was obtained by labeling under ATP-deficient conditions employing 1.7 μM [γ-^{32}P]ATP and 400 μM DNA-P. RAL was derived by labeling under standard conditions of 120 μM [γ-^{32}P]ATP and 100 μM DNA-P.
[b]IF = intensification factor = <RAL>/RAL.
[c]Standard deviation of the mean from at least three analyses.

Table III. DNA adduct levels in skin, determined under intensification conditions and expressed as RAL × 10^7 values, 24 h after topical application of 37 μmol/kg MeDBC or DBC

Adduct[a]	MeDBC	DBC
1	3.50 ± 0.90[b](49)[c]	ND[d]
2	1.14 ± 0.03 (16)	4.65 ± 0.66 (29)
2a	ND	0.80 ± 0.20 (5)
3	0.28 ± 0.10 (4)	5.34 ± 1.25 (33)
3a	ND	1.67 ± 0.43 (10)
6	ND	0.78 ± 0.19 (5)
6a	2.25 ± 0.40 (31)	2.91 ± 0.30 (18)
Total	7.17 ± 0.99	16.15 ± 1.53

[a]With the likely exception of 6a, MeDBC and DBC adducts of the same number were not identical (see Results).
[b]Standard deviation of the mean from at least three analyses.
[c]Percentage of the total level.
[d]ND = not detected.

genotoxic activity of MeDBC and DBC correlated with the reported carcinogenic activity in these organs, and (ii) to obtain information concerning the importance of the unsubstituted nitrogen in the genotoxicity of DBC. Advantages of the ^{32}P-postlabeling assay are that it enables one to detect and estimate DNA adducts down to 1 adduct in 10^9-10^{10} nucleotides, and it does not require the costly synthesis of radiolabeled compounds. Our results showed that in liver, total adduction by DBC was over two orders of magnitude greater than that by MeDBC (Table I), and thus, DBC was a much more potent hepatic genotoxicant. These data were in accord with the reports that while DBC is a potent hepatocarcinogen (9,10, 13–16), MeDBC is devoid of such activity (15), and suggest that the lack of hepatocarcinogenicity of MeDBC may, in part, stem from insufficient DNA damage elicited by this compound.

The lack of binding by MeDBC in liver also implicates the unsubstituted nitrogen in the generation of electrophiles from DBC in liver. Studies on the metabolism of DBC indicated that oxidation by liver microsomes *in vitro* results in a highly reactive, short-lived metabolite which may be *N*-hydroxy-DBC (28,29), in addition to numerous phenols (30). When Perin *et al.* (28) investigated the metabolism of MeDBC by liver microsomes, they found that the presence of the alkyl group did not alter the oxidation of the aromatic system, and no significant N-demethylation occurred. These workers concluded that the presence of the methyl group would only block reactions directly involving the nitrogen, i.e. N-oxidation. Our data support the hypothesis that, in addition to oxidation at C3 (21), this reaction may be involved in the activation of DBC to DNA-binding compounds *in vivo*. Thus, two essential features of the metabolic activation of DBC in liver *in vivo*, i.e. the presence of an unsubstituted nitrogen and oxidation of DBC to 3-hydroxydibenzo[c,g]carbazole (3-OH-DBC) (21), have been elucidated.

The finding that the minute amounts of adducts elicited by MeDBC in liver were chromatographically identical to those formed by DBC (Figure 2) was intriguing, and we believe that they were formed from DBC in both instances. Because of the unusually extensive binding of DBC to liver DNA (Table I and ref. 20), combined with the sensitivity of the ^{32}P-postlabeling assay, minute amounts of DBC in the MeDBC-treated tissues would be detected as DNA adducts. Furthermore, since the hepatic DBC–DNA adduct fingerprint is distinct from those of numerous other carcinogens, including hepatocarcinogens (26,31,32), studied by the postlabeling assay (22,23,32,33; K.Randerath, unpublished results), it is highly unlikely that the chromatographic identity could have arisen by coincidence or by the presence of trace amounts of other PAHs. A source of small quantities of DBC in the MeDBC-treated tissues could be DBC contamination in the MeDBC preparation. However, h.p.l.c. and mass spectrometry analysis failed to show any DBC in this preparation. It is remotely possible that DBC may have been present at levels below the limit of detection, but sufficient to form DNA adducts. A more likely source of trace amounts of DBC is from *in vivo* N-demethylation, since dealkylation is a major metabolic pathway for alkylated nitrogens *in vivo* (34). Furthermore, it has recently been found that MeDBC synthesized in a manner which completely precludes any DBC contamination gives similar results to those presented in Figure 2 and Table I (F.Perin, F.Zajdela and K.Randerath, unpublished results). Additionally, *in vivo* demethylation has been reported for *N*-methyl carbazole (35), a substance related to MeDBC. We believe, therefore, that the MeDBC adducts observed in liver were formed from DBC resulting via *in vivo* demethylation of MeDBC. Although demethylation of MeDBC was not observed by Perin *et al.* in rat or mouse liver microsomes *in vitro* (28), metabolism *in vivo* is more complete than *in vitro* with respect to metabolic enzymes and the availability of cofactors, which may account for the differences between the *in vivo* and *in vitro* results.

In contrast to the liver, the adduct patterns noted for MeDBC and DBC in skin were distinct from each other (Figures 3 and 4). This observation suggests that either the two compounds were metabolically activated differently altogether, or that activation was similar, but the migration of the MeDBC-derived adducts varied from that of the DBC-derived adducts due to the presence of the methyl group. In either case, the fact that adducts were formed to an appreciable extent by MeDBC in skin (Table III) indicated that the unsubstituted nitrogen of DBC is not crucial for activation to DNA-binding compounds in this tissue. In accord with the findings (21) that DBC produces chromatographically distinct adduct patterns in liver and skin, and that 3-OH-DBC appears to be a proximate binding metabolite in liver but not skin, these results demonstrate that both MeDBC and DBC exhibited a marked tissue specificity of metabolic activation.

Comparison of the quantitative data further revealed differences between liver and skin (Tables I and III). Total adduction of skin DNA by MeDBC was 2.3-fold lower than that by DBC (Table III), yet was still 11 times greater than in liver. The 2.3-fold lower level of adduction by MeDBC versus DBC correlated with the 2-fold lower potency of MeDBC in producing mouse skin tumors compared with DBC (15). Likewise, low liver DNA adduction (Table I) paralleled the lack of hepatocarcinogenicity of MeDBC. The correlation between DNA binding and biological activity of MeDBC is further supported by data of Parks *et al.* (36). The

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adduct patterns for both MeDBC and DBC in human xeroderma pigmentosum cells, in the presence of human hepatoma cells as a source of metabolic activation, were similar to those in mouse skin, with a 1.3- to 2.5-fold lower level of adduction by MeDBC relative to DBC. This quantitative difference was associated with a 2-fold lower mutagenicity of MeDBC relative to DBC in the xeroderma cells. Thus, the relative extent of *in vivo* and *in vitro* DNA adduction by MeDBC and DBC predicted the carcinogenic activity of these compounds in skin and liver, and their mutagenicity in cultured cells. While other factors (e.g. replicative fixation of DNA damage leading to specific mutations (37,38) and promotion of initiated cells) appear essential for the development of neoplasms, the data presented here and elsewhere (18,19,31) certainly allow the conclusion that a lack or a low level of covalent DNA binding of a particular carcinogen in a particular tissue predicts that the compound lacks appreciable carcinogenic activity in that tissue.

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