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STUDIES IN THE METABOLISM OF CARCINOGENIC POLYCYCLIC HETEROAROMATIC COMPOUNDS

I. THE HEPATIC MICROSOMAL METABOLISM OF 7-METHYLBENZ[*c*]ACRIDINE

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Abstract—An enzyme assay for the metabolism of the carcinogenic aza-aromatic polycyclic compound 7-methylbenz[*c*]acridine has been developed using a modification of a radiochemical assay described for the polycyclic aromatic hydrocarbon benzo[*a*]pyrene by DePierre *et al.* [J. W. DePierre, M. S. Moron, K. A. M. Johannesen and L. Ernster, *Analyt. Biochem.* **63**, 470 (1975)] and Van Cantfort *et al.* [J. Van Cantfort, J. DeGraeve and J. E. Gielen, *Biochem. biophys. Res. Commun.* **79**, 505 (1977)]. When the activities of control microsomes and microsomes of phenobarbital-, 3-methylcholanthrene- and 7-methylbenz[*c*]acridine-pretreated animals were compared, strong similarities were displayed toward oxidation of benzo[*a*]pyrene and 7-methylbenz[*c*]acridine. These similarities were seen in turnover numbers, Michaelis constants, and inducibility of both enzyme systems. 7-Methylbenz[*c*]acridine afforded a type I difference spectrum with 3-methylcholanthrene-pretreated microsomes. It is suggested that 7-methylbenz[*c*]acridine is oxidized by the same or a similar set of enzymes which is responsible for benzo[*a*]pyrene metabolism.

Amongst the environmental air pollutants which are known or thought to be carcinogenic are alkylbenzacridines, dibenz [*a*, *h*]-, and [*a*, *j*]acridine [1, 2]. The dibenzacridines are constituents of tobacco smoke formed by pyrolysis of nicotine [3], and these, together with benz[*c*]acridine and many alkylbenzacridines, are listed as known or suspected carcinogens [2, 4, 5]. Amongst other known carcinogenic environmental pollutants are the polycyclic aromatic hydrocarbons (PAH)† [4]. Much evidence has been obtained that these PAH are metabolized to reactive intermediates which are responsible for the manifested biological properties of the parent hydrocarbon. The major bioactivation pathways for PAH involve the MFO system and epoxide hydrase [6] and form diolepoxides which are thought to be ultimate carcinogens. These have been shown to be formed *in vitro* [7], to react nonenzymically with DNA [8, 9], and to be mutagenic [10] and tumorigenic [9, 11].

Apart from some structure–activity relationship work [12, 13] on polynuclear aza heteroaromatic compounds, little work has been done with these substances. The "bay region" theory, which correlates the electrophilicity of postulated ultimate carcinogens with the tumorigenicity of the parent PAH [14], has been extended to alkylsubstituted benz[*a*]- and benz[*c*]acridines [15] using quantum mechanical calculations. 7-MBAC, one of the more tumorigenic benzacridines, fitted well with these theoretical calculations. In the present work, 7-MBAC was selected for study from the polycyclic aza-aromatic compounds with a view to identifying metabolic activation pathways that may be characteristic of this class of carcinogen. This paper reports a radiochemical assay for total 7-MBAC metabolism and its comparison with total BP metabolism. The assay is based on that first described by DePierre *et al.* [16] and later modified by Van Cantfort *et al.* [17] for AHH (BP metabolism).

MATERIALS AND METHODS

Materials

[G-^{3}H]Benzo[*a*]pyrene (BP) was purchased from the Radiochemical Centre, Amersham, U.K., was purified as described previously [17] and was used at a specific radioactivity of 11 mCi/mmole. [G-^{3}H]-7-Methylbenz[*c*]acridine was obtained from Dr. M. A. Long, U.N.S.W., Kensington, N.S.W., Australia, and was purified by preparative thin-layer chromatography (t.l.c.); it was used at a specific activity of 11 mCi/mmole. It was shown to be more than 97.8 per cent pure by t.l.c. and 98.6 per cent pure by reverse isotope dilution analysis. No radioactive impurities could be detected by t.l.c. Thin-layer chromatography was conducted on silica gel using 2% acetone in chloroform for development. Biochemicals were purchased from the Sigma Chemical Co., St. Louis, MO. 7-MBAC was synthesized by the published method [18]. ACS was purchased from Amersham Australia Pty. Ltd. and used without dilution.

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† Abbreviations: PAH, polycyclic aromatic hydrocarbons; 7-MBAC, 7-methylbenz[*c*]acridine; BP, benzo[*a*]pyrene; 3-MC, 3-methylcholanthrene; PB, phenobarbital; AHH, aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase; DMSO, dimethylsulfoxide; and MFO, mixed function oxidase.

Microsomes

Liver microsomes were prepared [19] from groups of six male Wistar rats (100–150 g) that had been either pretreated with corn oil (control) or with PB, 3-MC or 7-MBAC. All inducers were administered i.p.; doses were 70, 80 and 100 mg/kg PB on 3 consecutive days; 20 mg/kg 3-MC in corn oil daily for 2 days; and 20 mg/kg 7-MBAC in corn oil daily for 2 days. Animals were fasted for 24 hr after the last dose and then killed. When preparing microsomes for spectral work, the last dose was given 48 hr before killing. The protein content of the microsomes was determined by literature methods [20] using bovine serum albumin as standard in the protein assay.

Aryl hydrocarbon hydroxylase

Fluorescence assay. Incubation mixtures (3 ml) contained freshly prepared hepatic microsomal protein (0.5 mg unless otherwise stated), NADP (1.5 μmoles), glucose-6-phosphate (12.5 μmoles) magnesium chloride (9 μmoles), glucose-6-phosphate dehydrogenase (1 I.U.), potassium phosphate buffer, pH 7.4 (300 μmoles), and BP (195 nmoles). The reaction was started by addition of substrate dissolved in acetone (50 μl), and the mixture was shaken for 10 min at 37° in air under diffuse light. Acetone (2 ml) was added to stop the reaction. Hexane (4 ml) was then added, and after vortexing for 20 sec an aliquot (3.0 ml) of the organic phase was stored overnight at 4° [21]. Activity was then measured by the published method [22] and expressed as nmoles fluorescent phenols equivalent to 3-hydroxy-BP produced per min per mg protein. Blank values were obtained by addition of acetone (2.0 ml) to the incubation mixture before addition of substrate.

Radiochemical assay. Incubation mixtures (1 ml) contained microsomal protein (0.2 mg unless otherwise stated), glucose-6-phosphate (1.25 μmoles), magnesium chloride (4.5 μmoles), NADP (0.5 μmole), glucose-6-phosphate dehydrogenase (1 I.U.), and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer, pH 7.4 (10 μmoles). The reaction was started by addition of [^{3}H]BP (80 nmoles unless otherwise stated) containing 2×10^6 dpm in acetone (50 μl) and shaken for 6.5 min (unless otherwise stated) under air in diffuse light at 37°. The reaction was stopped by addition of sodium hydroxide (0.15 M) in 85% (v/v) DMSO (1 ml) and extracted with hexane (3 × 4 ml). The organic phases were discarded and the radioactivity in the aqueous phase (0.5 ml) was determined after addition of water (1.0 ml). Appropriate blank determinations, obtained by addition of NaOH–DMSO before incubation, were subtracted from the enzyme determinations. Radioactivity measurements were made with a Packard model 3255 liquid scintillation spectrometer using ACS (10 ml). Time linearity was investigated for 25 min; protein concentrations were up to 1.2 mg/ml and substrate concentrations to 120 μM. For Michaelis–Menten kinetics, bovine serum albumin (1 mg/ml) was added to the incubation mixtures.

7-MBAC metabolism

Enzyme activities were determined using [^{3}H]-7-MBAC in a method analogous to the radiochemical assay for AHH described above. The concentration of 7-MBAC was 40 μM unless otherwise stated.

Difference spectra

These were determined [23, 24] using fresh induced microsomes in tandem cuvettes on an Aminco–Bowman DW-2 spectrophotometer at BP and 7-MBAC concentrations of 0.4 μM. A protein concentration of 2.5 mg/ml was used.

RESULTS

In initial experiments the behaviour of [^{3}H]-7-MBAC was investigated under the radiochemical extraction assay conditions without addition of microsomes. Two hexane extractions resulted in about 1% of the 7-MBAC remaining in the alkaline DMSO phase, and this was decreased to 0.3% by the use of a third extraction. This remnant of radioactivity sets the limit to the sensitivity of the assay. In another experiment hepatic microsomes from 3-MC-pretreated rats were incubated with [^{3}H]-7-MBAC under conditions which resulted in about 30 per cent metabolism. The combined hexane phases from three extractions were evaporated at 25° under a stream of dry nitrogen, and the residue was exam-

Table 1. Comparison of hepatic microsomal metabolism of 7-MBAC and BP*

	7-MBAC		BP			
Pretreatment	Radiochemical assay†	Induced / Control	Radiochemical assay†	Induced / Control	Fluorescence assay‡	Induced / Control
Corn oil	1.152 ± 0.093		0.530 ± 0.048		0.110 ± 0.001	
PB	3.21 ± 0.10	2.79	1.11 ± 0.10	2.09	0.260 ± 0.001	2.36
3-MC	5.15 ± 0.20	4.47	2.77 ± 0.30	5.23	0.238 ± 0.004	2.17
7-MBAC	2.09 ± 0.10	1.81	1.77 ± 0.11	3.34	0.250 ± 0.003	2.27

* Radiochemical assays were carried out using 0.2 mg microsomal protein/ml, incubation times of 6.5 min and 80 μM BP or 40 μM 7-MBAC after PB or 7-MBAC pretreatment. After 3-MC-pretreatment, 0.1 mg microsomal protein/ml and an incubation time of 3 min were used. The fluorescence assay was carried out using 65 μM BP, 0.5 mg microsomal protein/ml, and an incubation time of 10 min. Results are means ± S.D. five determinations on each of two batches of animals.

† Results are expressed as nmoles 7-MBAC or BP oxidized·(mg protein)$^{-1}$·min^{-1}.

‡ Results are expressed as nmoles fluorescent phenols equivalent to 3-hydroxy-BP·(mg protein)$^{-1}$·min^{-1}.

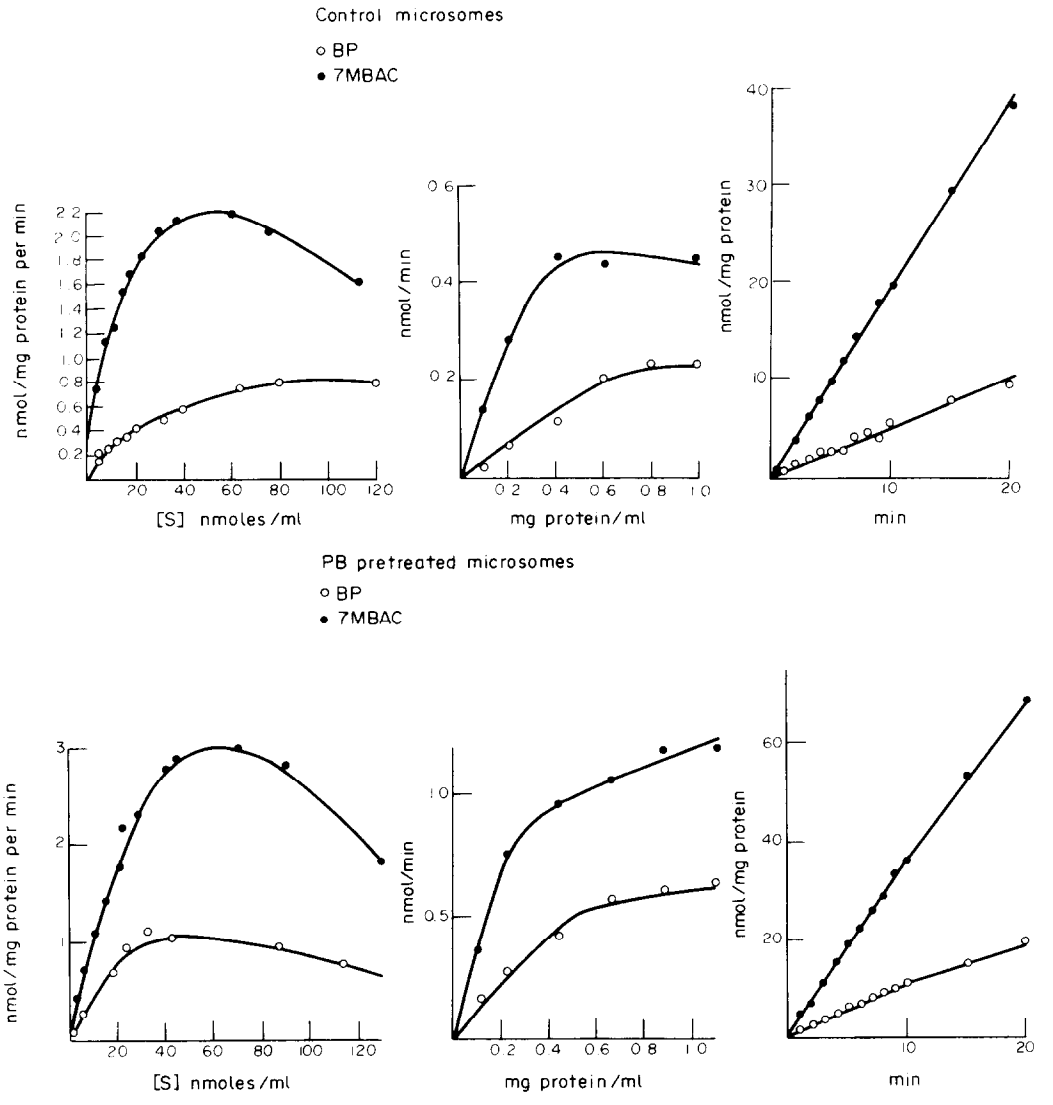

Fig. 1. Protein, time and substrate linearities for 7-MBAC and BP oxidation by rat hepatic microsomes from control and PB-pretreated animals. Metabolism was measured at 37° by the radiochemical extraction assay using 80 μM BP or 40 μM 7-MBAC for 6.5 min for protein linearity, 80 μM BP or 40 μM 7-MBAC and 0.2 mg protein/ml for time linearity, and 0.2 mg protein/ml for 6.5 min for substrate linearity.

ined by t.l.c. More than 96 per cent of the applied radioactivity cochromatographed with 7-MBAC, and no other radioactive peaks were detected.

The protein, time and substrate linearities of the enzyme with 7-MBAC and BP were examined by the radiochemical extraction assays. Results were obtained for microsomes from control and PB-pretreated rats (Fig. 1) and for microsomal preparations from 3-MC- and 7-MBAC-pretreated rats (Fig. 2). The reactions were linear for at least 10 min, to at least 0.2 mg protein/ml and to substrate concentrations of about 20–40 μM. For microsomes from 3-MC-pretreated animals and 7-MBAC as substrate, the reaction was only linear to lower substrate concentrations (≈10 μM). For all microsome preparations, high 7-MBAC concentrations caused inhibition of enzyme activity; this reached about 40 per cent inhibition at 120 μM 7-MBAC.

A comparison of the activities of microsomes with respect to 7-MBAC and BP metabolism was made using both the fluorescence and radiochemical assays for BP (Table 1). The two AHH assays measure, in part, different metabolites [25, 26]. The radiochemical method measures all the oxidized metabolites of BP that partition into the alkaline aqueous DMSO phase, while the fluorescence assay measures only fluorescent phenols. It is therefore expected that results obtained with the radiochemical assay method are higher. The ratios of induced to control activities for BP metabolism measured by both methods were the same for PB microsomes, but differed with 3-MC and 7-MBAC microsomes. As measured by the fluorescence assay, a 2-fold induction of AHH was seen for 3-MC microsomes, whereas a greater than 5-fold increase was evident with the radiochemical extraction assay. The induction ratios for 3-MC

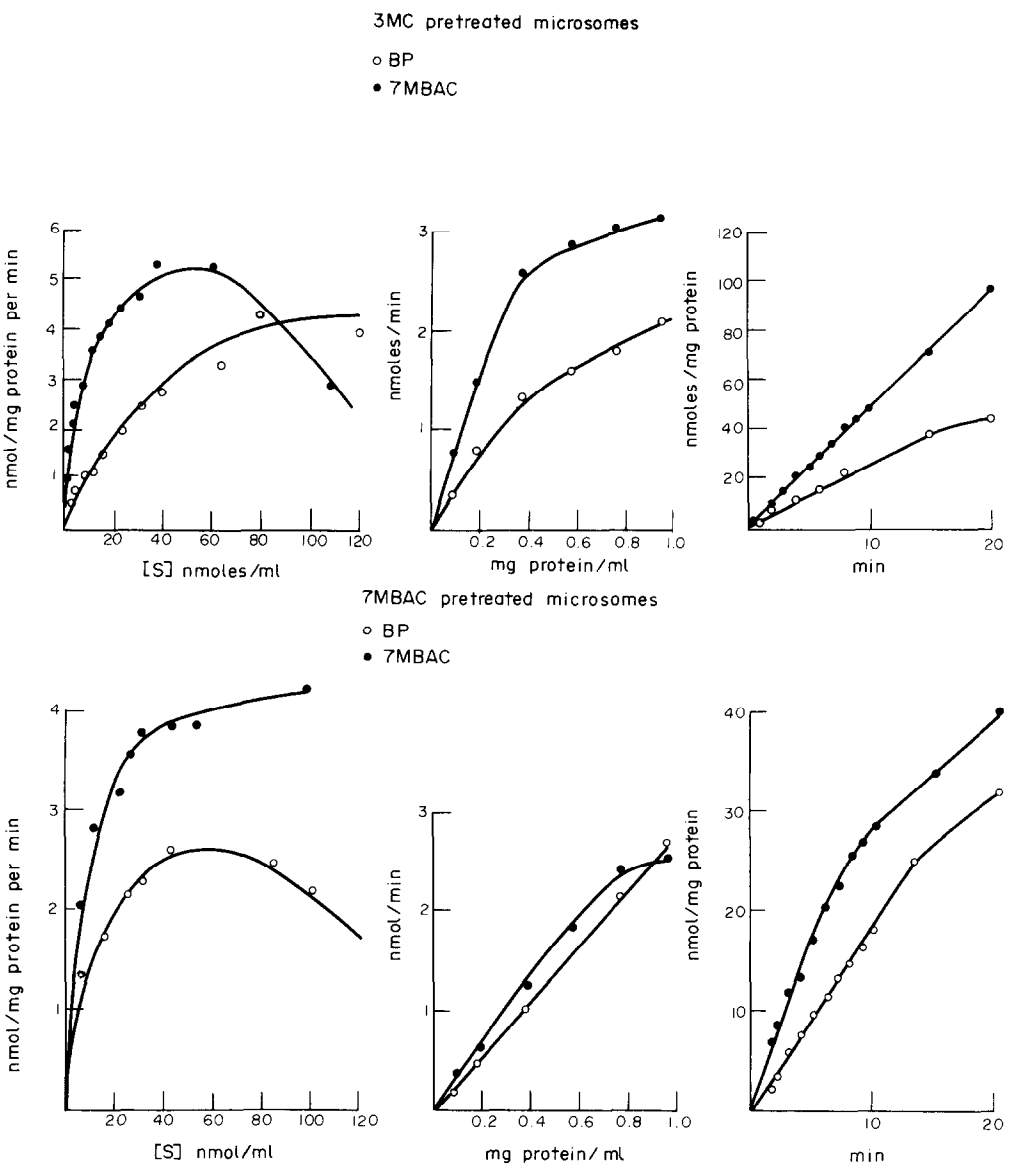

Fig. 2. Protein, time and substrate linearities for 7-MBAC and BP oxidation by rat hepatic microsomes from 3-MC- and 7-MBAC-pretreated animals. Details of conditions for incubations with 7-MBAC-pretreated microsomes are given in the legend to Fig. 1. The details for 3-MC-pretreated microsomes are similar except that incubation time was 3 min and the protein concentration used was 0.1 mg/ml.

microsomes which we obtained by the fluorescence assay varied from 2 to 5. The higher values are in better agreement with other published values [27, 28], and possible reasons for the differences, which also appear in the literature, have been discussed [29]. 7-MBAC and BP metabolisms measured by the extraction assay were similar for microsomes from 3-MC- and PB-pretreated rats as judged by induction ratios, but 7-MBAC appeared to be a somewhat better substrate than BP as measured by rates of metabolism (Table 1). The differences in substrate oxidation were not as great when metabolism was measured at lower protein and substrate concentrations used in K_m and V_{max} determinations. The same similarities between BP and 7-MBAC as substrates after 7-MBAC induction were not evident, possibly due to the presence of unlabeled 7-MBAC or metabolites derived from the inducing dose.*

Attempts to determine apparent Michaelis constants for 7-MBAC and BP by the radiochemical assay over a substrate concentration range of 5–30 μM gave poor results. When lower microsomal protein and substrate concentrations and bovine serum albumin [30, 31] were used in the incubation mixtures, satisfactory values were obtained (Table 2). The K_m values for BP agreed with those reported by Robie *et al.* [31] for microsomes from control and 3-MC-pretreated rats.

Difference spectra recorded for microsomes from

* G. M. Holder, C. M. Ireland, D. J. Wright and A. J. Ryan, unpublished results.

Table 2. Comparison of kinetic constants obtained for microsomal metabolism of BP and 7-MBAC*

Pretreatment	BP† Apparent K_m	BP† Apparent V_{max}	7-MBAC† Apparent K_m	7-MBAC† Apparent V_{max}
Corn oil	0.44	0.39	0.66	0.62
PB	1.04	1.28	3.24	2.66
3-MC	0.57	11.39	0.43	11.19
7-MBAC	0.23	1.34	0.39	1.55

* Metabolism was measured at 37° by the radiochemical extraction assay using from 0.2 to 2 μM BP or 7-MBAC, and incubating for 1.5 min (3-MC-pretreated microsomes) or 3.0 min (all other microsomal preparations) at a protein concentration of 0.02 mg/ml. The results were determined using the direct linear plot method of Cornish-Bowden and Eisenthal [32] as modified by Porter and Trager [33].

† Determinations were performed at eight substrate concentrations in the range of 0.2 to 2 μM, and are expressed as μM for K_m and nmoles·mg^{-1}·min^{-1} for V_{max}.

3-MC-pretreated animals indicated that 7-MBAC behaved similarly to BP (Fig. 3). Both gave type I difference spectra, but that obtained with 7-MBAC was weaker than that found with BP. A similar difference spectrum was found for BP with microsomes from 7-MBAC-pretreated rats. When 7-MBAC was added to oxidized microsomes from 7-MBAC-pretreated animals, however, a weak atypical reverse type I spectrum was seen. In all cases 7-MBAC afforded weak difference spectra. The reduced cytochrome P-450 carbon monoxide complex [34] displayed a maximum in the difference spectrum against reduced microsomes at 448.3 nm for preparations from 7-MBAC-induced animals.

DISCUSSION

The radiochemical assay of 7-MBAC metabolism by liver microsomes offers a method to measure the total metabolism of the carcinogenic polycyclic aza-aromatic compound 7-MBAC. The assay method used is based on that developed by DePierre *et al.* [16] and Van Cantfort *et al.* [17], and omits an acid treatment step designed to convert arene oxides to phenols [35]. For BP, dihydrodiols, quinones and phenols are formed by liver microsomal preparations [26] and are measured by this assay method [17]. With 7-MBAC, metabolites identified or partially identified by synthetic and spectral techniques after

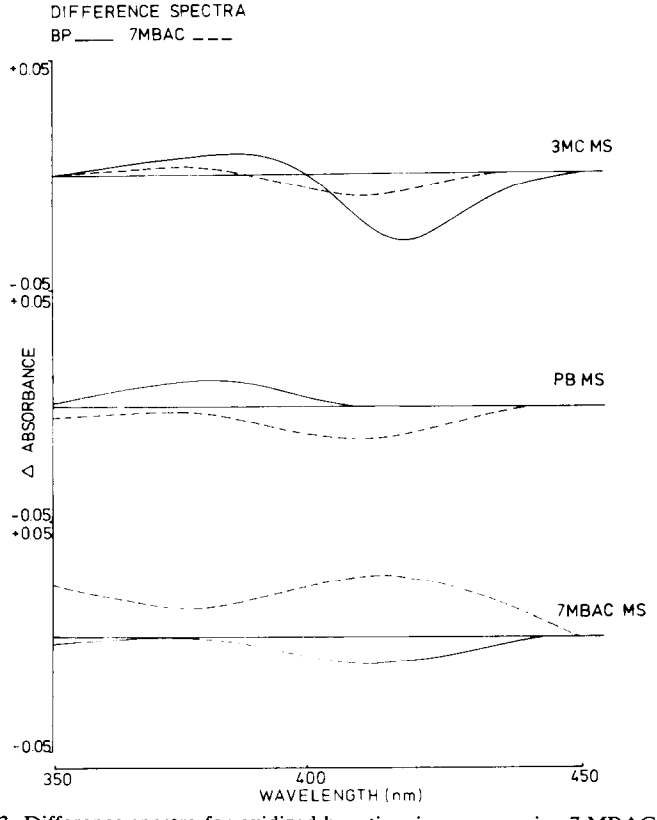

Fig. 3. Difference spectra for oxidized hepatic microsomes using 7-MBAC and BP.

high performance liquid chromatography (h.p.l.c.) are dihydrodiols, phenols, 7-hydroxymethylbenz[*c*]acridine, and further oxidation products of the latter. No evidence for the formation of arene oxide or quinone metabolite fractions has, as yet, been obtained.* Crossover of metabolites into the hexane phase was not detected, but the method has the disadvantage of relatively high blank values equivalent to 0.2 to 0.3 per cent of incubated substrate. This limited the sensitivity of the method to about 0.15 nmole per incubation when 40 μM 7-MBAC was used.

When BP metabolism was measured by the radiochemical method, several similarities to 7-MBAC metabolism were seen (Table 1). Induced levels of both activities showed the same-fold increases for microsomes from 3-MC- and PB-treated animals. Apparent Michaelis constants (Table 2) were similar when measured at low microsomal protein concentrations in the presence of extraneous protein [31]. Similar type I difference spectra were also observed with 3-MC-induced microsomes. These data suggest that 7-MBAC and BP are metabolized by the same or similar sets of liver microsomal enzymes. 7-MBAC, however, appears to be more readily oxidized than BP (see data of Table 1 and V_{max} values in Table 2). The apparent K_m and V_{max} values determined for BP agree with those determined previously under similar conditions [31], and are less than values determined by others [16, 36, 37]. The liver microsomal metabolites of 7-MBAC identified thus far include methyl modified compounds, phenols and dihydrodiols,* and are analogous to some of those formed from BP [26] and the methylbenzanthracenes [38]. As an inducer of MFO, 7-MBAC was analogous to other homocyclic PAH. A cytochrome P-448 was produced, and the metabolisms of BP and 7-MBAC were greatly increased.

We have shown that the carcinogenic nitrogen heterocycle 7-MBAC is comparable in its metabolism to the structurally similar carcinogenic PAH, BP. The method described may be expected to be of value in studies directed toward understanding the mutagenicity [39] and carcinogenicity of this and other polynuclear aza heteroaromatic compounds.

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REFERENCES

1. E. Sawicki, S. P. McPherson, T. W. Stanley, J. Meeker and W. C. Elbert, *J. Air Water Pollut.* **9**, 515 (1965).
2. A Lacassagne, N. P. Buu Hoi, R. Daudel and F. Zajdela, *Adv. Cancer Res.* **4**, 315 (1956).
3. B. L. VanDurren, J. A. Bilbao and C. A. Joseph, *J. natn. Cancer Inst.* **25**, 53 (1960).
4. *I. A. R. C. Monograph on the Evaluation of Carcinogenic Risk of Chemicals to Man* **3**, 241 (1972).
5. R. J. Lewis and R. L. Tatken, *Registry of Toxic Effects of Chemical Substances* , U.S. Department of Health and Human Services, National Institute of Occupational Safety and Health, Cincinnati, Ohio (1980).
6. J. W. DePierre and L. Ernster, *Biochim. biophys. Acta* **473**, 149 (1978).
7. D. R. Thakker, H. Yagi, H. Akagi, M. Koreeda, A. Y. H. Lu, W. Levin, A. W. Wood, A. H. Conney and D. M. Jerina, *Chem. Biol. Interact.* **16**, 281 (1977).
8. M. R. Osborne, *Trends biochem. Sci.* **4**, 213 (1979).
9. P. Brookes, *Cancer Lett.* **6**, 285 (1979).
10. H. Bartsch, C. Malaveille, B. Tierney, P. L. Grover and P. Sims, *Chem. Biol. Interact.* **26**, 185 (1979).
11. M. K. Buening, P. G. Wislocki, W. Levin, H. Yagi, D. R. Thakker, H. Akagi, M. Koreeda, D. M. Jerina and A. H. Conney, *Proc. natn. Acad. Sci. U.S.A.* **75**, 5358 (1978).
12. T. Okano, M. Haga, K. Vjiie and N. Motohashi, *Chem. pharm. Bull. Tokyo* **26**, 2855 (1978).
13. L. Bahna, V. Podany, M. Benesova, A. Godal and A. Vachalkova, *Neoplasma* **25**, 641 (1978).
14. D. M. Jerina and R. E. Lehr, in *Microsomes and Drug Oxidations* (Eds. V. Ullrich, A. Hildebrandt, I. Roots, R. W. Estabrook and A. H. Conney), pp. 709–20. Pergamon Press, Oxford (1977).
15. I. A. Smith and P. Seybold, *J. heterocyclic Chem.* **16**, 421 (1979).
16. J. W. DePierre, M. S. Moron, K. A. M. Johannesen and L. Ernster, *Analyt. Biochem.* **63**, 470 (1975).
17. J. Van Cantfort, J. DeGraeve and J. E. Gielen, *Biochem. biophys. Res. Commun.* **79**, 505 (1977).
18. O. Tsugu, N. Nishinshara and M. Tashiro, *Bull. chem. Soc. Japan* **36**, 1477 (1963).
19. G. M. Holder, P. J. Little and A. J. Ryan, *Biochem. Pharmac.* **25**, 2747 (1976).
20. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
21. L. W. Wattenberg, J. L. Leong and P. J. Strand, *Cancer Res.* **22**, 1120 (1962).
22. D. W. Nebert and H. V. Gelboin, *J. biol. Chem.* **243**, 6242 (1968).
23. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3**, 113 (1967).
24. K. Kumaki, M. Sato, H. Kon and D. W. Nebert, *J. biol. Chem.* **253**, 1048 (1978).
25. G. Holder, H. Yagi, W. Levin, A. Y. H. Lu and D. M. Jerina, *Biochem. biophys. Res. Commun.* **65**, 1363 (1975).
26. G. Holder, H. Yagi, P. Dansette, D. M. Jerina, W. Levin, A. Y. H. Lu and A. H. Conney, *Proc. natn. Acad. Sci. U.S.A.* **71**, 4356 (1974).
27. A. P. Alvares, G. Schilling and W. Levin, *J. Pharmac. exp. Ther.* **175**, 4 (1970).
28. H. Vainio and A. Aitio, *Acta pharmac. tox.* **34**, 130 (1974).
29. A. R. Hansen and J. R. Fouts, *Biochem. Pharmac.* **20**, 3125 (1971).
30. A. R. Hansen and J. R. Fouts, *Chem.–Biol. Interact.* **5**, 167 (1972).
31. K. M. Robie, Y-M. Cha, R. E. Talcott and J. B. Schenkman, *Chem.–Biol. Interact.* **12**, 285 (1976).
32. A. Cornish-Bowden and R. Eisenthal, *Biochem. J.* **139**, 721 (1974).
33. W. R. Porter and W. F. Trager, *Biochem. J.* **161**, 293 (1977).
34. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2379 (1964).
35. S. Nesnow, W. E. Fahl and C. R. Jefcoate, *Analyt. Biochem.* **80**, 258 (1977).
36. D. L. Hill and T-W. Shih, *Cancer Res.* **34**, 564 (1974).
37. M. Vore and E. Soliven, *Biochem. Pharmac.* **28**, 3659 (1979).
38. A. D. MacNicoll, P. M. Burden, O. Ribeiro, A. Hewer, P. L. Grover and P. Sims, *Chem.–Biol. Interact.* **26**, 121 (1979).
39. R. S. U. Baker, A. Bonin, I. Stupans and G. M. Holder, *Mutation Res.* **71**, 43 (1980).

* G. M. Holder, C. M. Ireland, D. J. Wright and A. J. Ryan, unpublished results.