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EFFECTS OF DIFFERENT CELL CONSTITUENTS ON METABOLIC ACTIVATION AND BINDING OF BENZO(a)PYRENE TO PURIFIED AND NUCLEAR DNA

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SUMMARY: The binding of metabolites of 9-hydroxybenzo(a)pyrene and 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene to DNA was compared in various incubation systems. The total binding was greatest when a system of purified DNA and liver microsomes was used. In this system the major binding species was derived from 9-hydroxybenzo(a)pyrene. When systems employing liver nuclei supplemented with microsomes, albumin or cytosol were used, the total binding to DNA was much lower and species derived from 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene predominated. Thus, binding in the purified system correlated poorly with binding in the nuclear system, and it appears that the majority of microsomally produced benzo(a)pyrene electrophiles may never reach the nucleus due to trapping by cytoplasmic nucleophiles. Nuclear metabolism may therefore play an important role in the binding of benzo(a)pyrene to DNA *in vivo*.

Expression of the carcinogenic properties of benzo(a)pyrene (BP) requires metabolic activation by the cytochrome P-450-dependent monooxygenase system. A large amount of evidence now suggests that certain electrophilic intermediates formed by one or several cytochrome P-450-dependent oxidations bind covalently to nuclear DNA and by so doing disrupt normal cell replication leading to cell transformation and tumor formation. Studies using such various systems as purified DNA and microsomes, isolated nuclei, isolated whole cells and isolated perfused organs have shown that the main DNA-binding species of BP are derived from the potent carcinogen 7,8-dihydro-7,8-dihydroxy-BP (BP-7,8-diol) and from the less carcinogenic 9-hydroxy-BP (9-OH-BP) (1-7). The specific DNA-binding species are BP-7,8-diol-9,10-oxides and, most likely, 9-OH-BP-4,5-oxide(s) (1,2,4,8).

It is obvious that binding of BP metabolites to DNA is the result of a multistep process where different activating steps may take place in different

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parts of the cell. The possible participation of nuclear monooxygenases in the formation of ultimate binding species, either from intermediate BP metabolites formed in the endoplasmic reticulum or from BP itself, has been previously discussed (9-14). In this study, we further investigate the significance of nuclear enzymes in the formation of active BP electrophiles, and compare the binding of the two major products to DNA, both in absolute terms and relative to each other, when various sources of DNA and activating enzymes are employed. We also show the effect of albumin and the cytosolic fraction on the binding pattern. We provide evidence that DNA may be modified very differently in different systems, and that nuclear metabolism may be highly significant for the final activation of BP to DNA-binding products.

MATERIALS AND METHODS

^{3}H-BP (~27 Ci/mmole) was purchased from The Radiochemical Center, Amersham, Bucks, England. Bovine serum albumin (fraction V), NADPH, 3-methylcholanthrene, unlabeled benzo(a)pyrene, calf-thymus DNA, protease type V, RNase type 1-A, DNase I, bacterial alkaline phosphatase and snake venom phosphodiesterase were purchased from Sigma Chemical Co, St. Louis, Mo. Sephadex LH-20 was obtained from Pharmacia, Uppsala, Sweden. Lumagel was obtained from Lumac Systems AG, Basel, Switzerland. Other chemicals were of analytical grade.

All liver cell fractions were isolated from rats pretreated once with 40 mg/kg 3-methylcholanthrene i.p. 40 hours before sacrifice. Microsomes were prepared as previously described in 50 mM Tris buffer, pH 7.5, containing 25 mM KCl and 5 mM $MgCl_2$ (11), and could be stored frozen at -70° for up to 3 months without apparent loss of activity. Nuclei were prepared fresh daily by the method of Blobel and Potter (15). Cytosol was collected as the supernatant from the 100,000xg sedimentation of the microsomal fraction. The cytosolic fraction was dialyzed against Tris-KCl-$MgCl_2$ buffer (see above) for one hour and the protein concentration was determined and adjusted before use to 50 mg/ml.

Purified DNA and/or cell fractions were incubated for 30 minutes with 20 µM ^{3}H-BP (2.7 Ci/mmole) and 1 mM NADPH. Incubations contained 12 mg of calf thymus DNA or $400x10^{6}$ nuclei in 10 ml of Tris-KCl-$MgCl_2$ buffer. In some experiments, 20 mg of microsomal protein was added, as was 100 mg of bovine serum albumin or 2 ml of cytosol (100 mg protein). DNA was isolated and purified as previously described (4). DNA recovery was calculated from the UV spectrum, and the DNA was hydrolyzed to deoxyribonucleosides (essentially as described (16)) by treatment with DNase (24 hours) followed by addition of phosphodiesterase and alkaline phosphatase (24 hours further).

BP-modified nucleosides were analyzed (essentially as described (17)) by Sephadex LH-20 chromatography. Samples were applied to 20x1.6 cm columns and eluted with a 45%-100% linear gradient of methanol in water, at a flow rate of 1 ml/min. One hundred 2.5 ml fractions were collected, and 1 ml aliquots of each fraction were counted in 10 ml Lumagel. Between runs, the column was washed with 180 ml methanol, then reequilibrated with 60 ml of 45% methanol.

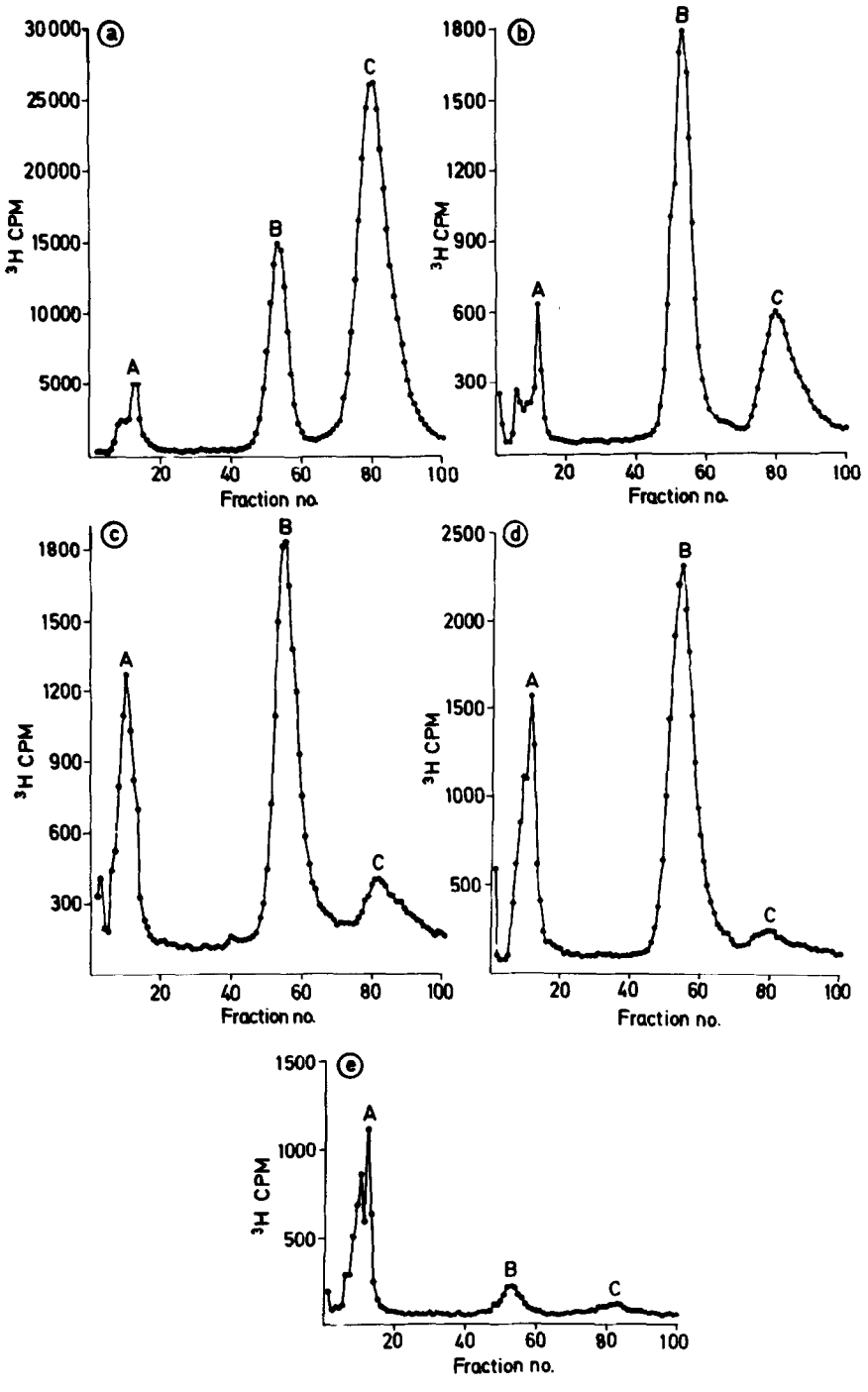

Fig. 1 a-e. Sephadex LH-20 chromatograms of hydrolysates of BP-modified DNA. The incubates contained ^{3}H-BP, NADPH and (a) microsomes and calf thymus DNA, (b) nuclei, (c) nuclei and microsomes, (d) nuclei, microsomes and bovine serum albumin, and (e) nuclei, microsomes and cytosolic fraction.

TABLE. ABSOLUTE AND RELATIVE BINDING OF BENZO(a)PYRENE METABOLITES TO DNA IN SEVERAL INCUBATION SYSTEMS

System	BP-7,8-diol metabolites bound (pmole/mg DNA)	9-OH-BP metabolites bound (pmole/mg DNA)	Ratio of binding BP-7,8-diol/9-OH-BP
Calf Thymus DNA + Microsomes	23	67	0.34
Nuclei Alone	4.6	2.1	2.2
Nuclei + Microsomes	7.2	1.1	6.5
Nuclei + Microsomes + Albumin	8.2	0.39	21.0
Nuclei + Microsomes + Cytosol	0.53	0.13	4.2

The total amount of each metabolite bound to DNA was calculated by integrating peaks "B" and "C" on the graphs shown in Figs 1a-e. The final results were corrected for the total volume of elution, the amount of DNA added to the column, and the specific activity of the ^{3}H-BP substrate. However, no correction was made for ^{3}H loss during metabolism which may be estimated to affect the measure of bound products from 9-OH-BP (20-30%) but not that of products from BP-7,8-diol (23).

RESULTS AND DISCUSSION

When purified DNA and microsomes were incubated with ^{3}H-BP and the modified DNA analyzed by LH-20 chromatography, the results shown in Fig. 1a and the Table were obtained. The two peaks of interest, shown here as "B" and "C", reflect the binding of products of BP-7,8-diol and 9-OH-BP, respectively. In the case of peak "B", the binding species have been identified as BP-7,8-diol-9,10-oxides; the chromatographic system used in this study does not resolve the isomers of BP-7,8-diol-9,10-oxides (18). In the case of peak "C", the binding species has not been as rigorously identified, but good evidence has shown it to be 9-OH-BP-4,5-oxide(s) (2). The identity of peak "A" has not been satisfactorily determined but it may contain nuclease-resistent phosphotriesters, unhydrolyzed nucleotides and/or exchange-tritiated unmodified nucleosides (17,19,20). In general, the chromatographic pattern corresponds well to the pattern seen in other laboratories (3,5,21).

When isolated nuclei alone were used as the source of DNA and activating enzymes, the results shown in Fig. 1b and the Table were obtained. The same

binding species are seen here, but both the absolute amounts bound and their ratio to each other differ from the purified system. As the table shows, the binding of both species is decreased, and the amount of 9-OH-BP product bound is decreased the most. These results are in contrast to a previous study, where little qualitative or quantitative differences were noted between purified and nuclear systems (3). In the previous study, however, a much higher substrate concentration was used (80 μM BP). The difference observed here in DNA modification by BP between the nuclear system and the purified DNA - microsomal system may occur for one of the following reasons: (1) A qualitative difference in the metabolic capacities of nuclei and microsomes which results in different relative rates of formation of various metabolites, (2) a greater susceptibility of the 9-OH-BP product towards trapping or conjugation by non-DNA molecules in the nucleus, or (3) the presence of active DNA-repair systems in the nuclei which preferentially remove adducts derived from 9-OH-BP. The first possibility seems unlikely, since previous studies have shown that the patterns of BP metabolites produced by nuclei and microsomes are quite similar. In fact, nuclei appear to produce a greater proportion of phenolic products, including 9-OH-BP, to total metabolites than do microsomes (3,11,22). The other two hypotheses seem more credible.

When nuclei were incubated with BP in the presence of microsomes, the DNA-binding pattern seen in Fig. 1c resulted. Although a greater amount of 9-OH-BP is produced in this system than by nuclei alone, the amount of product eventually bound to DNA is actually lower. The DNA-binding pattern seen in Fig. 1c is more similar to that of Fig. 1b (nuclei alone) than that of Fig. 1a (purified DNA and microsomes). It is conceivable that although more 9-OH-BP and the corresponding binding intermediate is formed, it is rapidly bound to microsomal nucleophilic sites and never reaches the nucleus. The binding product of BP-7,8-diol, however, may be less susceptible to immediate nucleophilic attack and may therefore be more likely to reach the nucleus. When the system was supplemented with albumin, the binding of 9-OH-BP product to nuclear DNA was

further reduced, but the binding of BP-7,8-diol product was more or less unaffected (Fig. 1d and the Table). Replacing albumin with a corresponding amount of cytosolic protein resulted in an almost total reduction of bound 9-OH-BP product and a marked reduction in the amount of BP-7,8-diol product bound (Fig. 1e and the Table). It appears, then, that under our experimental conditions most of the 9-OH-BP formed by the microsomes never reaches the nucleus in the form of an electrophilic intermediate, while the electrophile formed from BP-7,8-diol seems to be more resistent to attack by microsomal or cytosolic nucleophiles and may reach the nucleus in an active form.

In conclusion, the present study has shown that the purified DNA-microsomal system, while it probably reflects the rate of microsomal formation of DNA-binding BP metabolites, only poorly reflects the amount of metabolites eventually bound in the nucleus. Microsomally formed reactive intermediates may not all reach the nucleus due to non-selective or selective trapping by microsomal and cytosolic nucleophiles. Attempts to identify these components are presently in progress in our laboratory.

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REFERENCES

1. Sims, P., Grover, P.L., Swaisland, A., Pal, K. and Hewer, A. (1974) Nature 252, 326-328
2. King, H.W.S., Thompson, M.H. and Brookes, P. (1976) Int. J. Cancer 18, 339-344
3. Alexandrov, K., Brookes, P., King, H.W.S., Osborne, M.R. and Thompson, M.H. (1976) Chem. Biol. Interactions 12, 269-277
4. Jernström, B., Orrenius, S., Undeman, O., Gräslund, A. and Ehrenberg, A. (1978) Cancer Res. 38, 2600-2607
5. Fahl, W.E., Shen, A.L. and Jefcoate, C.R. (1978) Biochem. Biophys. Res. Commun. 85, 891-899
6. Deckers-Schmelzle, B., Klaus, E., Kahl, R. and Kahl, G.F. (1978) Naunyn-Schmiedeberg's Arch. Pharmacol. 303, 303-307
7. Kahl, G.F., Klaus, E., Legraverend, C., Nebert, D.W. and Pelkonen, O. (1979) Biochem. Pharmacol. 28, 1051-1056
8. Ivanovic, V., Geacintov, N.E. and Weinstein, I.B. (1976) Biochem. Biophys. Res. Commun. 70, 1172-1179

9. Rogan, E.G. and Cavalieri, E. (1974) Biochem. Biophys. Res. Commun. 58, 1119-1125
10. Pezzuto, J.M., Lea, M.A. and Yang, C.S. (1976) Cancer Res. 36, 3647-3653
11. Jernström, B., Vadi, H. and Orrenius, S. (1976) Cancer Res. 36, 4107-4113
12. Bresnick, E., Vaught, J.B., Chuang, A.H.L., Stoming, T.A., Bockman, D. and Mukhtar, H. (1977) Arch. Biochem. Biophys. 181, 257-269
13. Pezzuto, J.M., Lea, M.A. and Yang, C.S. (1977) Cancer Res. 37, 3427-3433
14. Jernström. B., Vadi, H. and Orrenius, S. (1978) Chem. Biol. Interactions 20, 311-321
15. Blobel, G. and Potter, V.R. (1966) Science 154, 1662-1665
16. Jeanette, K.W., Jeffrey, A.M., Blobstein, S.H., Beland, F.A., Harvey, R. G. and Weinstein, I.B . (1977) Biochemistry 16, 932-938
17. Baird, W.H., Harvey, R.G. and Brookes, P. (1975) Cancer Res. 35, 54-57
18. King, H.W.S., Osborne, M.R., Beland, F.A., Harvey, R.G. and Brookes, P. (1976) Proc. Natl. Acad. Sci. USA 73, 2679-2681
19. Bastman, A., Sweetenham, J. and Bresnick, E. (1978) Chem. Biol. Interactions 23, 345-353
20. Philips, D.H., Grover, P.L. and Sims, P. (1979) Int. J. Cancer 23, 201-208
21. Pelkonen, O., Boobis, A.R., Yagi, H., Jerina, D.M. and Nebert, D.W. (1978) Molecul. Pharmacol. 14, 306-322
22. Pezzuto, J.M., Yang, C.S., Yang, S.K., McCourt, D.W. and Gelboin, H.V. (1978) Cancer Res. 38, 1241-1245
23. Osborne, M.R., Thompson, M.H., King, H.W.S. and Brookes, P. (1975) Int. J. Cancer 16, 659-664