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Effect of Retinol on the Mutagenicity of Benzo[a]pyrene and Benzo[a]pyrene 4,5-oxide

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ABSTRACT

The mutagenicity of benzo[a]pyrene (BP) towards *Salmonella typhimurium* strain TA98 was inhibited by non-toxic levels of retinol. The mutagen was activated using liver homogenates (9,000 × g supernatant), (S9), from β-naphthoflavone-induced rats. Mutagenicity of benzo[a]pyrene 4,5-oxide (BP 4,5-oxide), a direct acting mutagen produced upon metabolic activation of BP, was also inhibited by non-toxic levels of retinol. The presence and absence of an S9 activating system has a significant influence on the cytotoxic effect of retinol as assayed on nutrient agar plates.

Key words: Benzo[a]pyrene mutagenicity; Benzo[a]pyrene 4,5-oxide mutagenicity; Retinol; Vitamin A; Retinoids.

INTRODUCTION

"Vitamin A" is a generic term that designates any compounds possessing the biological activity of retinol, while the term "retinoids" includes both naturally occurring forms of vitamin A and the many synthetic analogs of retinol (IUPAC-IUB, 1981). All-trans retinol, the parent retinoid compound, is a primary alcohol with a molecular weight of 286. Considerable evidence demonstrates that vitamin A and its derivatives (the retinoids) may be effective in the treatment of a variety of human diseases, including cancer (Sporn et al., 1994; Bertram et al., 1991). Retinoid deficiency has been associated with increased susceptibility to chemical carcinogenesis in certain tissues (Slaga and DiGiovanni, 1984) and enhanced mutagenicity of several compounds (Narbonne et al., 1985; Qin and Huang, 1986; Alzieu et al., 1987). Studies using the Ames test have shown that retinol completely inhibits the mutagenicity of the carcinogen 2-fluorenamine (Baird and Birnbaum, 1979) and that vitamin A (retinol and some retinyl esters) inhibits the mutagenicities of a number of precarcinogens, e.g., aflatoxin B_1 (Busk and Ahlborg, 1980) and pyrolysate products (Busk et al., 1982). A strong inhibition by vitamin A palmitate of mutagenicity induced by polycyclic aromatic hydrocarbons in a human epithelial-like cell line (Rocchi et al., 1983) and by retinol and retinoic acid of 7,12-dimethylbenz[a]anthracene-induced cytotoxicity and mutagenicity in mammalian cells *in vitro* (Budroe et al., 1988) has also been demonstrated. The mechanism by which retinoids are able to elicit these diverse and complex effects ultimately resides in their ability to regulate gene expression at specific target sites within the body. Over the past ten years a number of developments in research

have helped in elucidating the link between vitamin A action and gene control. By the mid 1980's, an abundance of evidence had led researchers to believe that almost all of these effects were due to the metabolite retinoic acid and, more specifically all-trans retinoic acid. Vitamin A (retinol) is acquired from the diet and may be interconverted between its storage form in the liver or retinaldehyde. Retinaldehyde is not only required for the visual process but also serves as the precursor to all-trans retinoic acid through an irreversible reaction. Until recently, all-trans retinoic acid was thought to be responsible for all the effects of vitamin A seen in morphogenesis, growth, and development. This view has changed dramatically with the discovery of at least two classes of retinoid receptors (Mangelsdorf, 1994). As part of our efforts to investigate the mechanism by which some compounds inhibit the mutagenicity of benzo[a]pyrene (BP) [50-32-8] (Calle et al., 1978; Sullivan et al., 1980; Calle and Sullivan, 1982), the *Salmonella typhimurium* reversion test was used in this study to investigate the effectiveness of retinol (vitamin A alcohol) [68-26-8] as an inhibitor of the mutagenicities of BP, a carcinogenic polycyclic hydrocarbon which must be activated to exert its effect (Grover and Sims, 1968; Gelboin, 1969), and of BP 4,5-oxide (4,5-epoxide) [37574-47-3] a direct acting mutagen produced upon metabolic activation of BP.

MATERIALS AND METHODS

Retinol (synthetic all-trans, type X) was obtained from Sigma Chemical Co., St. Louis, Missouri. BP (99 + % purity), and β-naphthoflavone were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. BP 4,5-oxide was provided by IIT Research Institute, Chicago, Illinois. All samples were used without further purification.

The mutagenicities of BP and BP 4,5-oxide were determined in *Salmonella typhimurium* TA98 using the method described by Ames et al. (1975) with some slight modifications previously reported (Kittle et al., 1981). To 4.5 mL of molten agar maintained at 45°C, the following ingredients were added: 15 μL of a BP solution (4.0×10^{-4}M) or BP 4,5-oxide solution (1.7×10^{-4}M) in dimethyl sulfoxide, (DMSO), (the concentrations used were optimized previously by separate mutagenesis studies); 50 μL of a freshly prepared solution of retinol in DMSO; the amounts of retinol per plate ranged from equimolar (6.0 nmoles/plate for the BP containing plates and 2.6 nmoles/plate for the BP 4,5 oxide containing plates) to fifty times the amounts of hydrocarbon used per plate; 0.1 mL of the overnight culture of strain TA98 of *Salmonella typhimurium* (kindly supplied by Dr. B. Ames), and 0.5 mL of potassium phosphate buffer (0.2M, pH 7.4). The system used for the activation of BP consisted of 30 μL of the S9 fraction of a liver homogenate (protein *ca.* 38 mg/mL) prepared from β-naphthoflavone-induced male Sprague-Dawley albino rats weighing 260-300 g (Nebert et al., 1973), and 2.6 (moles NADPH. Toxicity tests were performed under the same conditions as the mutagenicity assay by plating 50 μL of an overnight culture, geometrically diluted by a factor of 10^{-4} with sterile 0.9% NaCl, on nutrient agar plates. All experiments were performed at least three times, and all determinations represent the average number of colonies found on five plates. The figures and table show data from a representative experiment.

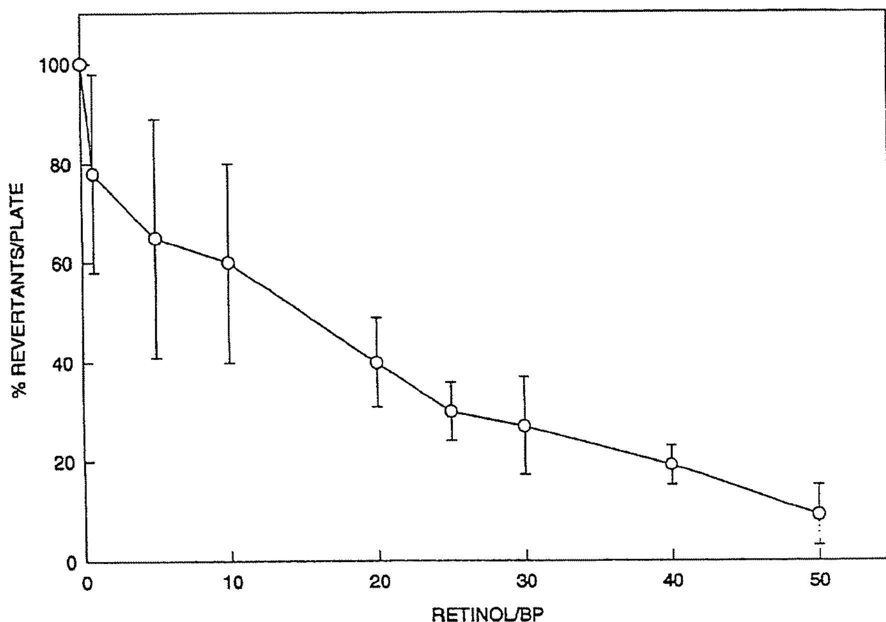

FIGURE 1. Inhibition of BP induced mutagenesis in *Salmonella typhimurium* strain TA98 by retinol. The amount of BP was kept constant at 6.0 nmoles/plate. Retinol was added in the range of 6.0 to 300 nmoles/plate. % Revertants/plate was calculated as the percentage change in the number of revertants as compared with the control value of BP revertants without retinol. A typical number of revertants in the control was 552 ± 63 colony forming units. Retinol/BP is the ratio of the concentrations of retinol and BP per plate. Error bars represent standard deviations from five replicate plates.

RESULTS AND DISCUSSION

The mutagenicity of BP towards *Salmonella typhimurium* strain TA98 was significantly decreased by the incorporation of retinol into the plates (Fig. 1). An increase in the amount of retinol, from equimolar up to fifty times the amount of the carcinogen per plate, resulted in the gradual increase of the inhibition of mutagenicity. At an amount of 86 μg/plate, the maximum amount of retinol added per plate, the mutagenicity of BP was completely inhibited.

Similar results were obtained when retinol was incorporated into the BP 4,5-oxide containing plates (Fig. 2). BP 4,5-oxide does not require activation for mutagenicity and therefore phosphate buffer was added to the plates instead of the S9 mix. Addition of amounts of retinol equimolar to the BP 4,5-oxide in this case produced a larger decrease in the mutagenicity than that observed under similar conditions for BP. After the initial drop in the number of his$^+$ revertants in the presence of equimolar amounts of retinol, the addition of retinol up to fifty times the amount of the mutagen per plate did not result in further significant decreases in the number of his$^+$ revertants per plate.

The incorporation of retinol into the plates in the absence of either mutagen had no appreciable effect on the spontaneous reversion frequency. In order to further confirm that the inhibitory effect of retinol on the mutagenic activities of

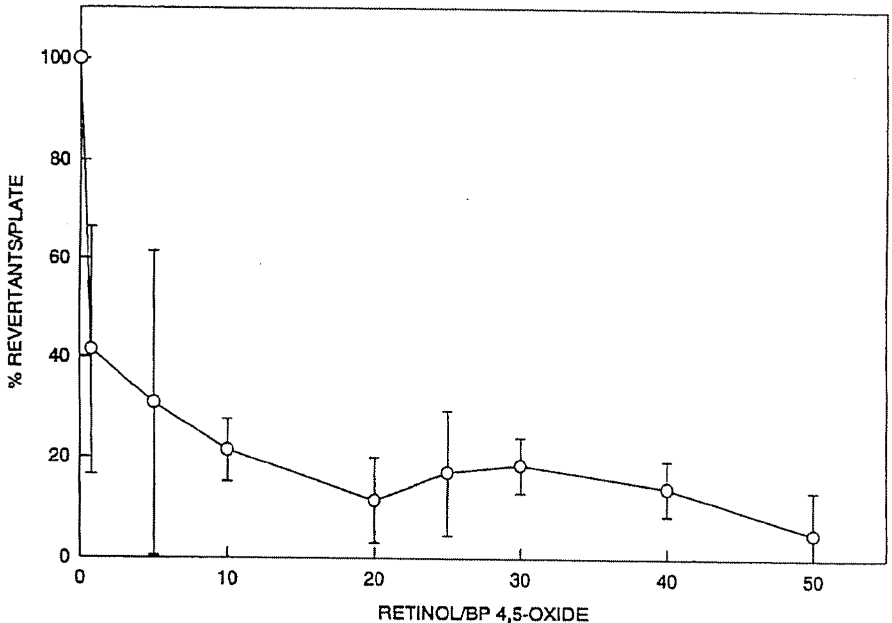

FIGURE 2. Inhibition of BP 4,5-oxide induced mutagenesis in strain TA98 by retinol. The amount of BP 4,5-oxide used was 2.6 nmoles/plate. Retinol was added in the range of 2.6 to 130 nmoles/plate. % Revertants/plate was calculated as the percentage change in the number of revertants as compared with the control value of BP 4,5-oxide revertants without retinol. Retinol/BP 4,5-oxide is the ratio of the concentrations of retinol and BP 4,5-oxide per plate. A typical number of revertants in the control is 399 ± 46 colony forming units. Error bars represent standard deviations from five replicate plates.

BP and BP 4,5-oxide was not an artifact, cytotoxic effects of retinol alone were investigated using nutrient agar plates. The toxicity tests for BP and BP 4,5-oxide differed in that buffer instead of S9 mix was used with the latter. When bacteria were exposed to the same amounts of retinol used in the BP experiment, an increasingly toxic effect was observed in the absence of S9. However, when S9 was incorporated into the system (Table 1), the toxicity was almost completely reversed although a slight but consistent decline in the bacterial count was observed with amounts of up to 105 μg/plate of retinol. This reversal of toxicity by the S9 fraction could be due to a transformation of the retinol to a non-toxic derivative or to an S9 binding effect. Nevertheless, the toxic effect of retinol, as assayed on nutrient agar plates, is clearly distinguishable from the observed inhibitory effect on the mutagenicity of BP.

BP 4,5-oxide does not require activation by the S9 fraction and therefore the inhibition of mutagenicity in this system at concentrations of retinol larger than approximately 3μg/plate may be an artifact caused by killing of the bacteria by retinol (Table 1). However, the inhibitory effect of retinol on the mutagenicity of BP 4,5-oxide caused by lower concentrations of retinol ($<3\mu$g/plate) is approximately four times larger than the inhibitory effect observed for the same amount of retinol on the mutagenicity of BP.

TABLE 1. Viability of *Salmonella typhimurium* strain TA98 on nutrient agar plates in the presence of retinol.

Retinol[a], $\frac{\mu g}{plate}$	Bacterial[b] viability, %	Bacterial[c] viability, %
None	100	100
2	92 ± 8	93 ± 10
11	62 ± 3	99 ± 11
21	44 ± 5	98 ± 13
42	39 ± 3	82 ± 8
53	27 ± 1	84 ± 8
63	18 ± 6	80 ± 13
84	23 ± 2	79 ± 14
105	21 ± 7	64 ± 6

a Retinol was added to the surface of the agar in 50 μL DMSO.
b Buffer instead of S9 mix was added to the surface of the agar.
c S9 was added to the surface of the agar.

The fact that the presence or absence of S9 affected the toxicity of retinol towards the bacteria (Table 1) points out the importance of examining bacterial viability in mutagenicity assays using the Ames test. Routine examination of the background lawn of bacterial growth, resulting from the trace of histidine added to the agar surface (Ames et al., 1975), as the only aid in determining the toxic effects, may not reveal the toxic effects that are observed by culturing the bacteria on nutrient agar plates in the presence of the suspect cytotoxic substance. During the course of this investigation, it was also noticed that the toxic effects observed on nutrient agar plates were more pronounced as the master plates, from which the overnight cultures were grown, aged.

The facts that non-toxic concentrations of retinol inhibit the mutagenicities of BP and BP 4,5-oxide, and that retinol toxicity is affected by the metabolizing system, suggest that retinol can alter carcinogen/mutagen metabolism. Retinol might directly interfere with carcinogen/mutagen metabolism either by inhibition of a common rate-limiting step or by altering specific pathways. Support for this hypothesis comes from the observation that retinoids can inhibit the in vitro microsomal oxidation of a number of compounds including carcinogens (Hill and Shih, 1974; Colby et al., 1975; Genta et al., 1974). Indirect evidence for the possible modulation of carcinogen/mutagen activating enzymes by retinyl acetate is also presented by the observations of Genta et al. (1974). Another possible explanation that can be based on the observed inhibition of mutagenicity of BP 4,5-oxide by non-toxic concentrations of retinol, is that retinol inhibits the binding to target macromolecules. If binding is predominantly a function of metabolic activation of the hydrocarbon, it is possible that this mechanism may also account, in part, for the inhibitory effect of retinol on the mutagenicity of BP. Supporting evidence for the above suggestion comes from studies with β-retinyl acetate (Yuspa et al., 1977) which showed that this retinoid can markedly alter carcinogen metabolism and binding of activated products to macromolecules. Baird and Birnbaum (1979) reported that retinol inhibits the mutagenicity of the carcinogen 2-fluorenamine,

which requires activation, thus supporting our results with BP. However, they observed no inhibition by retinol on the mutagenicity of the direct acting mutagen adriamycin in contrast to our observations with BP 4,5-oxide. This suggests that the mechanism by which retinol inhibits mutagenicity may differ for different compounds.

The results of our studies to date suggest the usefulness of the Ames test in investigating the mechanism by which retinoids exhibit anticancer and/or antimutagenic activity.

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LITERATURE CITED

Alzieu, P., P. Cassand, C. Colin, P. Grolier, and J.F. Narbonne. 1987. Effect of vitamin A, C and glutathione on the mutagenicity of benzo[a]pyrene mediated by S9 from vitamin A-deficient rats. Mutation Res. 192: 227-231.

Ames, B.N., J. McCann, and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Res. 31: 347-364.

Baird, M.B., and L.S. Birnbaum. 1979. Inhibition of 2-fluorenamide-induced mutagenesis in *Salmonella typhimurium* by vitamin A. J. Natl. Cancer Inst. 63: 1093-1096.

Bertram, J.S., A. Pung, M. Churley, T.J. Kappock, L.R. Wilkins, and R.V. Cooney. 1991. Diverse carotenoids protect against chemically induced neoplastic transformation. Carcinogenesis 12: 671-703.

Burdroe, J.D., H.M. Schol, J.G. Shaddock, and D.A. Casciano. 1988. Inhibition of 7,12-dimethylbenz[a]anthracene-induced genotoxicity in Chinese hamster ovary cells by retinol and retinoic acid. Carcinogenesis 9: 1307-1311.

Busk, L., and U.G. Ahlborg. 1980. Retinol (vitamin A) as an inhibitor of the mutagenicity of aflatoxin B_1. Toxicol. Lett. 6: 243-249.

Busk, L.,. U.G. Ahlborg, and L. Albanus. 1982. Inhibition of protein pyrolysate mutagenicity by retinol (vitamin A). Food Chem. Toxicol. 20: 535-539.

Calle, L.M. and P.D. Sullivan. 1982 Screening of antioxidants and other compounds for antimutagenic properties towards benzo[a]pyrene-induced mutagenicity in strain TA98 of *Salmonella typhimurium*. Mutation Res. 101: 99-114.

Calle, L.M., P.D. Sullivan, M.D. Nettleman, I.J. Ocasio, J. Blazyk, and J. Jollick. 1978. Antioxidants and the mutagenicity of benzo[a]pyrene and some derivatives. Biochem. Biophys. Res. Commun. 85: 351-356.

Colby, H.D., R.E. Kramer, J.W. Greiner, D.A. Robinson, R.F. Krause, and W.J. Canady. 1975. Hepatic drug metabolism in retinod-deficient rats. Biochem. Pharmacol. 24: 1644-1646.

Gelboin, H.V. 1969. A microsomal-dependent binding of benzo[a]pyrene to DNA, Cancer Res. 29: 1972-1976.

Genta, V.M., D.G. Kaufman, C.C. Harris, J.M. Smith, M.B. Sporn, and U. Saffiotti. 1974. Vitamin A deficiency enhances binding of benzo[a]pyrene to tracheal epithelial DNA. Nature 247: 48-49.

Grover, P.L. and P. Sims. 1968. Enzyme catalyzed reactions of polycyclic hydrocarbons with deoxyribonucleic acid and protein *in vitro*. Biochem. J. 110: 159-160.

Hill, D.L. and T. Shi. 1974. Vitamin A compounds and analogs as inhibitors of mixed-function oxidases that metabolize carcinogenic polycyclic hydrocarbons and other compounds. Cancer Res. 34: 564-570.

IUPAC-IUB Joint Commission on Biochemical Nomenclature. Nomenclature of retinoids. Recommendations 1981. Eur. J. Biochem. 1982, 129: 1-5.

Kittle, Jr. J.D., L.M. Calle, and P.D. Sullivan. 1981. The effect of substituted phenothiazines on the mutagenicity of benzo[a]pyrene. Mutat. Res. 80: 259-264.

Mangelsdorf, D.J. 1994. Vitamin A receptors. Nutr. Rev. 52: S32-S44.

Narbonne, J.F., P. Cassand, M. Daubeze, and P. Alzieu. 1985. Carence en vitamine A et activation du benzo[a]pyrene. Sci. Aliments, 5, no. hors serie V, 41-46.

Nebert, D.W., J.K. Heidema, H.W. Strobel,. and M.J. Coon. 1973. Genetic expression of aryl hydrocarbon hydroxylase induction. J. Biol. Chem. 248: 7631-7636.

Qin, S., and C.C. Huang. 1986. Influence of mouse liver stored vitamin A on the induction of mutations (Ames tests) and SCE of bone marrow cells by aflatoxin B_1, benzo[a]pyrene, or cyclophosphamide. Environ. Mutagen. 7: 137-146.

Rocchi, P., G. Arfellini, A. Capucci, M.P. Grilli, and G. Prodi. 1983. Effect of vitamin A palmitate on mutagenesis induced by polycyclic aromatic hydrocarbons in human cells. Carcinogenesis 4: 245-247.

Slaga, T.J. and J. DiGiovanni. Inhibition of chemical carcinogenesis, in: C.E. Searle (Ed.), 1984. Chemical Carcinogens, volume 2, American Chemical Society, Washington, D.C., pp. 1300-1302.

Sporn, M.B., Roberts, A.B., and Goodman, D.S. (Eds.), 1994. The retinoids. Second Edition. Raven Press, New York. 679 p.

Sullivan, P.D., L.M. Calle, I.J. Ocasio, J.D Kittle Jr., and L.E. Ellis. 1980. The effect of antioxidants on the mutagenicity of benzo[a]pyrene and derivatives *in*: A. Bjorseth and A.J. Dennis (Eds.), Polynuclear Aromatic Hydrocarbons: Chemistry and Biological Effects, Batelle, Columbus, pp. 163-175.

Yuspa, S.H., K. Elgjo, M.A. Morse, and F.J. Wiebel. 1977. Retinyl acetate modulation of cell growth kinetics and carcinogen-cellular interaction in mouse epidermal cell cultures. Chem-Biol. Interactions 16: 251-264.