Differences between Two Groups of Observations in Medical and Biological Research [in Russian], Moscow (1969), p.8.
3. L. M. Shabad, G. A. Belitskii, and T. A. Bogush, Z. Krebsforsch., 82, 13 (1974).
4. L. M. Shabad, G. A. Belitskii, T. A. Bogush, et al., Arzneimittelforsch., 24, 1177 (1974).
5. L. M. Shabad, T. A. Bogush, and G. A. Belitskii, Neoplasma, 11, 347 (1974).
6. L. M. Shabad, T. A. Bogush, I. A. Konopleva, et al., Neoplasma, 24, 147 (1977).
7. A. Alonso and G. Herranz, Naturwissenschaften, 57, 249 (1970).
8. J. M. Blunck, Life Sci., 9, 51 (1970).
9. J. M. Blunck, Chem. Biol. Interact., 2, 217 (1970).
10. J. M. Blunck, B. J. Lees, and N. P. Madsen, Chem. Biol. Interact., 4, 219 (1971/1972).
11. C. E. Crowther and H. M. Blunck, Res. Commun. Chem. Path. Pharmacol., 9, 97 (1974).
12. A. Lacassagne and L. Harst, Bull. Cancer, 54, 405 (1967).
13. T. Matsushima and J. H. Weisburger, Chem. Biol. Interact., 1, 211 (1969/1970).
14. J. H. Weisburger, Y. Shirasu, P. H. Grantham, et al., J. Biol. Chem., 242, 372 (1967).
15. W. A. Phillips and J. M. Blunck, Eur. J. Cancer, 13, 729 (1977).

MUTAGENIC AND CARCINOGENIC ACTION OF SOME POLYCYCLIC AROMATIC HYDROCARBONS

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The carcinogenic (on mice) and mutagenic (on bacteria *Salmonella typhimurium* TA-98 and TA-100) action of benz(a)pyrene (BP) and its derivatives: 6-methyl-, 6-formyl-, 6-chloro-, 6-hydroxy-, 6-acetoxy-, 6-methoxy-, and 4(5)-methoxy-BP was investigated. Powerful carcinogens (BP, 6-methyl- and 6-formyl-BP) were shown to cause mutations in both strains of bacteria. Weakly carcinogenic compounds [6-chloro-, 6-methoxy-, and 4(5)methoxy-BP] and noncarcinogenic (6-acetoxy- and 6-hydroxy-BP) compounds either were nonmutagenic or induced mutations in bacteria of only one of the two strains. Differences between the carcinogenic and mutagenic actions of the various compounds were not related to the velocity of their oxidation in an enzymic system.

KEY WORDS: Carcinogenesis; mutagenesis; polycyclic hydrocarbons

Compounds foreign to the body, including polycyclic aromatic hydrocarbons (PAH), are oxidized by enzymes of the endoplasmic reticulum of the cell with the formation of highly active compounds, which are responsible for the biological effect of the PAH. As a result of the high chemical activity of these metabolites and, consequently, of their instability *in vivo*, it is virtually impossible to isolate them. However, by using an appropriate acceptor system for active metabolites, in the presence of enzymes oxidizing PAH, it is possible to determine whether a particular compound, as a result of enzymic conversion, can form highly active agents. Various mutants of bacteria can be used as acceptors of active metabolites of PAH.

In this investigation the carcinogenic and mutagenic actions of benz(a)pyrene (BP) derivatives similar in structure but with different carcinogenic activity were compared. The following compounds were tested: BP, 6-methyl-BP, 6-formyl-BP, 6-chloro-BP, 6-hydroxy-BP, 6-acetoxy-BP, 6-methoxy-BP, and 4(5)-methoxy-BP. Bacteria of strains *Salmonella typhimurium* TA-98 and TA-100 were used as acceptors of active metabolites of PAH [8].

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TABLE 1. Results of Tests of Carcinogenic Action of Benz(a)-pyrene Derivatives

Compound	Number of animals					Latent period until appearance of tumor, days
	beginning of experiment	at time of appearance of first tumor (3 months)	at sacrifice (1-1.5 year)	with tumors at site of injection		
				absolute	%	
Benz(a)pyrene	30	30	2†	25	83,3	156±6,2
6-formyl-BP	20	19	2	16	84,2	153±16,8
6-methyl-BP	20	20	1	12	75,0	126±4,3
6-chloro-BP	20	20	10	6	30,0	193±17,1
6-methoxy-BP	20	19	16	1	5,3	312
6-acetoxy-BP	20	20	12	0		—
6-hydroxy-BP	20	20	13	0		—
Control	20	20	20	0		—

*On histological examination of the tumors a sarcoma was diagnosed, mainly of polymorphocellular type.
†The mice were sacrificed after 1 year.

METHODS

The 6-hydroxy-, methoxy-, acetoxy-, formyl-, and methyl-BP were synthesized by known methods [6, 7]. The 6-chloro-BP was obtained by treatment of BP with anhydrous cupric chloride by known methods for anthracene and pyrene [3], and the product was identical with that described in [5]. The 4(5)-methoxy-BP was obtained by boiling 4,5-dihydro-4,5-dihydroxyl-BP [1] with dimethyl sulfate in methanol followed by treatment with aqueous alkali; the product was identical with that described in [9].

Experiments to detect carcinogenic activity was carried out on male (C57Bl × CBA)F_1 hybrid mice. The compound was injected into the animals in two doses, each of 1 mg in 0.5 ml olive oil, at an interval of one week (total dose 2 mg per mouse). All animals which died were autopsied. All surviving animals were killed after 1.5 years and examined.

The ability of the compounds to induce mutations in bacteria was determined by the method of Ames et al. [4]. Strains of *S. typhimurium* auxotrophic for histidine and biotin were used: TA-98 with a frame shift mutation in the histidine operon, and TA-100, with a base substitution type of mutation in the same operon. The bacteria were grown in nutrient broth for 18 h, reseeded in fresh nutrient broth, incubated for 5 h at 37°C, sedimented by centrifugation, and the residue was resuspended to a density of $(2-3) \cdot 10^9$ cells/ml in salt medium (sodium phosphate 0.1 M, NaCl, 0.15 M; pH 7.0). To 2 ml semiliquid agar (Difco agar 0.6% NaCl 0.6%) with histidine (10 μg/ml) and biotin (5 μg/ml), melted and cooled to 45°C, 0.1 ml of bacterial suspension, 0.1 ml of the test substance in acetone, and 0.5 ml of activating mixture (0.25 ml of submicrosomal fraction of rat liver, NADP 2 mM, glucose-6-phosphate 2.5 mM, $MgCl_2$ 4 mM, and sodium phosphate 0.05 mM; pH 7.2) were added. The mixture was spread over the surface of glucose—salt agar (1.3%), poured previously in a volume of 15 ml into Petri dishes. The dishes were incubated for 48 h at 37°C, after which the number of colonies of his^+ revertants was counted as an indicator of the mutagenic activity of the compound.

To obtain the submicrosomal fraction liver tissue from male rats was homogenized in cold KCl solution (0.15 M) in the proportion of 1 g tissue to 3 ml solution. The homogenate was centrifuged at 10,000g for 15 min. The supernatant was used in the experiment either immediately or it was stored at −10°C and thawed before the experiment at 20°C. Microsomes were obtained by centrifugation of the submicrosomal fraction at 80,000g for 1 h followed by resuspension of the residue in KCl solution (0.15 M).

In the experiments to study enzymic degradation of the PAH the following incubation medium was used: phosphate buffer pH 7.5, NADPH 1 mM, $MgCl_2$ 8 mM, microsomes 1.5-2.5 mg protein/ml. The test PAH was added in ethanol in a final concentration of 35 nmoles/ml. Incubation was carried out at 37°C for 15 min. Unoxidized PAH was extracted with benzene and determined quantitatively by a spectral fluorescence method [2].

To induce a monooxygenase enzyme system, 3-methylcholanthrene was injected intraperitoneally into the rats 48 h before sacrifice in a dose of 15 mg/kg.

TABLE 2. Carcinogenic and Mutagenic Action of BP Derivatives

Compound	Carcinogenicity of substance	Mutagenicity of substance			
		dose, μg/dish	activation	number of revertants	
				TA-98	TA-100
Control	—	—	+	15	80
BP	+++	5	—	17	78
		5	+	252	787
6-formyl-BP	+++	5	—	12	84
		5	+	545	748
6-methyl-BP	++	5	—	12	90
		5	+	1847	1267
6-chloro-BP	+	25	—	24	105
		25	+	102	136
6-methoxy-BP	±	25	—	18	80
		25		41	132
6-acetoxy-BP	—	50	—	29	75
		50	+	33	73
6-hydroxy-BP	—	5	—	540	130
		5	+	1120	194
4(5)-methoxy-BP	Not exhibited	25	—	24	75
		25	+	100	550

TABLE 3. Rate of Oxidation of BP and Its Derivatives in Rat Liver Microsomes (M ± m)

Compound	Oxidation of substance in 10 min, nmoles/mg protein
BP	10,41±1,81
6-methyl-BP	15,14±3,1
6-methoxy-BP	16,20±3,0
6-chloro-BP	16,40±2,85

RESULTS

Position 6 in the BP molecule is the most reactive in substitution reactions, and for that reason the introduction of a substituent in this position in the BP molecule changes its biological action in a manner depending on the nature of the substituent. As will be clear from Table 1, introduction of a methyl or formyl group preserves the carcinogenic properties of the compound, introduction of a chlorine atom reduces the carcinogenic effect, and introduction of a methoxy, acetoxy, or hydroxy group makes the substance noncarcinogenic.

The study of the mutagenic action of the compounds showed (Table 2) that the powerful carcinogens (BP, 6-methyl-BP, 6-formyl-BP), in the presence of activation enzymes, induced a strong mutagenic effect on both strains of bacteria used, i.e., they were able to produce mutations both of the base substitution type and of the frame shift type. Such activity on strains with different types of mutations can be regarded as evidence that during the oxidation of these compounds at least two different mutagenic metabolites are formed. Of these compounds, 6-methyl-BP had the greatest activity.

Of the other 6-substituted BP studied, 6-chloro-BP had slight mutagenic activity on strain TA-98, whereas 6-hydroxy-BP had strong mutagenic activity on the same strain. The latter compound was the only one of those tested which had mutagenic activity in the absence of an activation system. Neither 6-acetoxy-BP nor 6-methoxy-BP was able to induce mutations in the strains of bacteria used.

One explanation of the considerable differences found between the carcinogenic and mutagenic action of the 6-substituted BP with indirect action could be differences in the rate of their activation in the enzyme system and corresponding differences in the quantity of active metabolites formed. To test this possibility, the rate of decomposition of the various BP derivatives in the microsomal fraction of rat liver was studied. As Table 3 shows, introduction of a substituent in position 6 accelerates oxidation of the BP derivatives compared with BP cells; the rate of oxidation of the powerful carcinogen and mutagen 6-methyl-BP was

comparable with the rate of oxidation of the weaker carcinogen and mutagen 6-chloro-BP and of the biologically inactive compound 6-methoxy-BP. These observations suggest that differences in the biological activity of the compounds studied were due, not to differences in the rate of their oxidation in the enzyme system, but to differences in the reactivity of the metabolites formed.

Among the compounds tested which oxidize cytochrome P-450 (Table 3), only 6-methoxy-BP is not converted into a active compound. This effect of the methoxy group may be connected with its introduction in position 6 only, or this group in any position in the BP molecule may perhaps reduce the ability to form active metabolites. Investigation of the mutagenic activity of an isomer of 6-methoxy-BP, namely 4(5)-methoxy-BP, showed (Table 2) that the latter compound is a powerful mutagen when tested on strain TA-100 but has little activity when tested on strain TA-98. This shows that ability to form active metabolites in the process of oxidation is due to the position occupied by the methoxy group in the BP molecule. As regards the carcinogenic properties of 4(5)-methoxy-BP, according to data in the literature [9] this compound is a weak carcinogen, approximately on the same level as 6-methoxy-BP.

A certain parallel can thus be traced between the carcinogenic and mutagenic actions of the various BP derivatives tested. Strongly carcinogenic compounds induced mutations in both strains of bacteria, whereas noncarcinogenic and weakly carcinogenic derivatives of BP are nonmutagenic or induced mutations in only one strain of bacteria. In order to exhibit their carcinogenic action, BP derivatives must perhaps form several chemically active metabolites, which induce mutations in different strains of bacteria, or several different chemically active groups must be formed in one molecule of a BP derivative.

LITERATURE CITED

1. T. P. Ryabykh, V. A. Koblyakov and O. A. Pan'shin, Tsitologiya, No. 8, 1003 (1976).
2. I. A. Khitrovo, A. Ya. Khesina, V. A. Koblyakov, et al., Kazan. Med. Zh., No. 5, 39 (1974).
3. L. Fieser and M. Fieser, Reagents for Organic Synthesis [Russian translation], Vol. 2, Moscow (1970), p. 246.
4. B. N. Ames, W. E. Durston, E. Yamasaki, et al., Proc. Natl. Acad. Sci. USA, 70, 2281 (1973).
5. F. Dewhurst and D. A. Kitchen, J. Chem. Soc. Perkin 1, 710 (1972).
6. L. F. Fieser and E. B. Hershberg, J. Am. Chem. Soc., 60, 2542 (1938).
7. L. F. Fieser and E. B. Hershberg, J. Am. Chem. Soc., 61, 1565 (1939).
8. J. McCann, N. E. Springarn, J. Kobory, et al., Proc. Natl. Acad. Sci. USA, 72, 979 (1975).
9. R. Schoental and M. A. Head, Brit. J. Cancer, 9, 457 (1955).