MECHANISM OF THE TOXIC ACTION OF BENZ(a)PYRENE AND ITS DERIVATIVES IN TISSUE CULTURE

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Polycyclic aromatic hydrocarbons (PAH) are oxidized by the cell enzyme systems with the formation of active compounds, whose interaction with components of the cell gives rise to effects such as mutagenesis and carcinogenesis [3]; the toxic action is also due to metabolic activation [2].

The biological activity of metabolites depends on the position of the functional group in the molecule. One approach to the study of the role of a particular position in the PAH molecule in the manifestation of its biological action is by blocking that position by various substituents.

This paper describes a study of the role of position 6 in the benz(a)pyrene (BP) molecule in the manifestation of its toxic action in cell culture.

EXPERIMENTAL METHOD

Indices of toxicity and metabolism were compared for a number of BP derivatives; 6-methyl-, 6-chloro-, and 6-methoxy-BP, and also BP derivatives with the same constituents, but in other positions: 4(5)-methoxy-BP and 12-methyl-BP.

Commercial preparations of BP (from Fluka, Switzerland) and of trichloropropene oxide (TCPO) (from Aldrich) were used in the experiments. The 6-methoxy-BP, 6-chloro-BP, and 4(5)-methoxy-BP were synthesized in the Oncologic Scientific Center, Academy of Medical Sciences of the USSR, by O. A. Pan'shin; the 12-methyl-BP was generously provided by M. Ya. Gubergrits (Institute of Chemistry, Academy of Sciences of the Estonian SSR).

Experiments to study the toxicity of BP derivatives were carried out on primary monolayer cultures of embryonic fibroblasts of C3HA mice. For this purpose, normal embryonic fibroblasts at the "zero" subculture were seeded in Carrel flasks at the rate of $750 \cdot 10^3$ cells per flask. The test hydrocarbon was added 24 h later in a concentration of 1-10 μg/ml. The number of cells was counted after 72 h on the TUP ZG2 Celloscope.

To determine the metabolic rate of the test PAH in cell culture, each compound was incubated for 72 h with a monolayer of cells, after which the medium was removed, extracted 3 times with benzene, and the quantity of residual PAH was determined by a spectral-fluorescence method [6].

In the experiments to determine metabolites of BP and its derivatives, liver microsomes from rats receiving a single intraperitoneal injection of 3-methylcholanthrene in a dose of 30 mg/kg body weight 24 h before sacrifice were used as the oxidizing system. Microsomes were isolated by differential centrifugation in 1.15% KCl [1]. The oxidation reaction was carried out in 50 mM phosphate buffer with 1 mM NADPH and 8 mM $MgCl_2$. The PAH was added in a dose of 10 μg/ml. After incubation for 20 min the medium was extracted 3 times with ethyl acetate, the solvent was evaporated, and the metabolites were fractionated by thin-layer chromatography in a system of benzene and ethanol (19:3). To determine dihydrodiols (DDO), in parallel tests the PAH was incubated in the presence of the epoxidase inhibitor TCPO, and depending on which spots were absent on the chromatogram with TCPO, the DDO were identified.

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TABLE 1. Toxic Action and Rate of Metabolism of BP and Its Derivatives in Cultures of Embryonic Fibroblasts (M ± m)

Index, % of control	Substance tested (1 μg/ml)					
	BP	6-methyl-BP	6-chloro-BP	6-methoxy-BP	12-methyl-BP	4(5)-methoxy-BP
No. of living cells after incubation for 72 h	56±1,89	63±2,06	78±0,5	88±6,0	65±4,8	55±3,5
Quantity of substance extracted from culture medium after incubation for 72 h	10,3±4,2	36,8±5,2	28±5,0	31,5±1,3	39,4±9,4	61,6±1,47

TABLE 2. Analytical Lines of Excitation and Fluorescence Spectra of BP Derivatives at 77°K

Substance	Fluorescence (maxima, in nm)	Excitation (maxima, in nm)
BP	403, 408, 427, 431, 454	298, 333, 350, 368, 385, 390
4,5-DDO of BP	367, 387, 411	
7,8-DDO of BP	393, 405, 415, 436	295, 337, 353, 367, 373
9, 10-DDO of BP	401, 405, 424, 455	300, 317, 330, 347
6-Methyl-BP	408, 411, 431, 458	302, 338, 357, 377, 398, 407
DDO No. 1	367, 388, 403, 411	
DDO No. 2	399, 422, 449	284, 297, 338, 355, 372, 380
6-Chloro-BP	411, 417, 423, 436, 440, 463	304, 324, 341, 359, 380, 387, 401, 410
DDO	401, 5, 424, 449	295, 338, 355, 372, 380
6-Methoxy-BP	412, 417, 436, 440, 463	301, 342, 357, 376, 386, 396, 409,
DDO	406, 416, 430, 445	302, 320, 336, 351

Spectra of DDO were recorded on the MPF-44a spectrofluorometer (Hitachi—Perkin-Elmer).

EXPERIMENTAL RESULTS

It will be clear from Table 1 that the toxic action of the BP derivatives depends on the nature of the substituents and the position which it occupies in the molecule. Among the BP derivatives studied with a substituent in position 6, the one with toxicity closest to that of BP was the methyl derivative. The chlorine and methoxy derivatives were less toxic. Increasing the dose of the PAH from 1 to 10 μg/ml did not change the toxic action of the substances. 12-Methyl-BP was indistinguishable in toxicity from 6-methyl-BP, but 4(5)-methoxy-BP, the isomer of 6-methoxy-BP, was equal in toxicity to unsubstituted BP.

The methyl group thus did not change the toxic properties of the substance, in whatever position it was introduced into the BP molecule. The effect of the methoxy group was determined by which carbon atom it blocked in the BP molecule.

The toxic effect of a PAH is determined by the compounds formed from it as a result of enzymic oxidation in the cell. Introduction of a substituent may change the rate of enzymic breakdown of the substance, as a result of which the steady-state concentration of active metabolites responsible for toxicity of the compound is altered. The substituent may perhaps change the character of metabolism, and the metabolites thus formed may differ in their biological activity from the compound without a substituent. In order to test these hypotheses, attempts were made to discover the cause of the difference in toxicity of BPs substituted in position 6.

The results of comparison of the data for the rates of oxidation and toxicity of the test substances in cell culture (Table 1) enable the following conclusions to be drawn. First, there was no correlation between the rate of oxidation of the BP derivatives and their toxic action. Second, introduction of a substituent into the region of the BP mole-

cule with highest electron density (position 4, 5) caused slowing of oxidation of the substance. The difference in the toxic action of the BP derivatives was thus not due to differences in their rate of oxidation in the cell culture. The next step was to investigate products formed by oxidation of the test substances in enzyme preparations.

In the first stage of enzymic destruction of PAH, phenols, quinones, and epoxides are formed in the cell [3]. It has been suggested that the carcinogenic [3] and mutagenic [5] effects of PAH are due to the formation of this class of compounds. Accordingly, in the present investigation the toxic action of the test compounds was compared with their ability to form epoxy derivatives in an enzyme system. Epoxy derivatives of BP are unstable compounds and in biological systems they are converted by the action of the enzyme epoxidase into DDO, which can easily be identified by their position on the chromatogram. For that reason, epoxide formation was judged from the formation of the corresponding DDO. As Table 2 shows, during oxidation of toxic BP and 6-methyl-BP, 3 and 2 DDO (and, correspondingly, the same number of epoxides) were formed: 6-chloro- and 6-methoxy-BP each formed 1 DDO. It follows from these data that in the case of 6-methyl-BP one of the positions where oxygen is incorporated during oxidation of BP becomes "prohibited." During oxidation of the nontoxic 6-chloro- and 6-methoxy-BP, there are two such "prohibited" positions. In the case of 6-methyl-BP, among the two epoxides formed there was evidently a toxic metabolite, whereas during oxidation of 6-chloro-BP and 6-methoxy-BP no toxic metabolite was formed.

All 6-substituted BPs have excitation and fluorescence spectra shifted a little into the region of longer wavelengths than unsubstituted BPs. The maxima of the spectra of the corresponding DDO of the 6-substituted BPs must also be shifted into the region of longer wavelengths than the corresponding DDO of BP.

It will be clear from Table 2 that DDO No. 1, formed from 6-methyl-BP, has the fluorescence spectrum of the 4,5-DDO of BP. Hence it follows that DDO No. 1 for 6-methyl-BP is the 6-methyl-4,5-DDO of BP.

As regards DDO No. 2 for 6-methyl-BP, this compound is evidently the 6-methyl-7,8-DDO of BP, for the fluorescence and excitation spectra are shifted a little toward the region of longer wavelengths than the corresponding spectra of the 7,8-DDO of BP, whereas the fluorescence spectrum of this compound lies in the region of shorter wavelengths than the fluorescence spectrum of the 9,10-DDO of BP, and for that reason this compound is not the 9,10-DDO of 6-methyl-BP.

In the case of the two other 6-substituted BPs the only DDO formed from each compound is evidently not the 4,5-DDO, for their fluorescence spectra were shifted into the region of longer wavelengths than that of the 4,5-DDO of BP. The writers showed previously that the DDO of 5-methoxy-BP is 9,10-DDO-6-methoxy-BP [4]. To determine in what position of the molecule (7-8 or 9-10) oxygen is incorporated during the formation of a DDO from 6-chloro-BP, the same arguments are valid as in the case of DDO No. 2 for 6-methyl-BP. On this basis it can be concluded that 7,8-DDO-6-chloro-BP is formed from 6-chloro-BP.

A common metabolite for the substances studied which have a toxic action was thus 4,5-DDO, which is not formed from the nontoxic 6-methoxy- or 6-chloro-BP. Within the limits of the present investigation, it can evidently be considered that the 4,5-epoxy-derivative is responsible for the toxic action of BP derivatives.

The following conclusion can be drawn from these results. The toxic effect of BP derivatives with a substituent in position 6 depends on the nature of the substituent and not on the fact that this position in the BP molecule is blocked. The toxic action of BP and its derivatives may be connected with the formation of the 4,5-epoxy-derivative. The methoxy group and the chlorine atom in the BP molecule make position 4-5 (the K region) inaccessible for the oxygen addition reaction, and this is responsible for their weak toxicity in cell culture. The effect of the methyl group is independent of the position into which it is introduced in the BP molecule.

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ACTION OF EPIDERMAL CHALONES ON PROLIFERATION AND DIFFERENTIATION OF MOUSE LOWER LIP TUMOR CELLS

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The discovery of tissue-specific regulators of cell proliferation, or chalones [9], has raised the urgent question of their use as tumor inhibitors. The first research in this direction has already been undertaken and some hopeful results obtained [3, 6, 10].

The object of the present investigation was to study the effect of epidermal chalones (EC) on the onset and development of lower lip tumors induced in mice by methylcholanthrene (MCh).

EXPERIMENTAL METHOD

Altogether 110 male CC57BR mice, divided into five groups, were used. A 0.5% solution of MCh in acetone was applied 3 times a week for 2 months to the mucosa of the lower lip of all the animals. The mice of group 1 received no other treatment. The mice of groups 2 and 4 received a preparation of EC consisting of an alcoholic extract of the epidermis of rat skin. The method of isolation of the extract and its activity, reflecting the presence of epidermal G_1- and G_2-chalones, was described previously [4]. The choice of method of administration of the chalone was based on data showing that systemic administration of chalone-containing extracts can cause death of animals [8], whereas local application is safe [6]. The EC extract was infiltrated into the tissues of the lower lip in a dose of 100 μg in 0.1 ml of 0.9% NaCl. This dose proved effective previously in inhibiting carcinogenesis in mice [6].

To study the effect of EC on the onset of tumors, the extract was injected into the mice of group 2 1 h after each application of MCh, and thereafter 3 times a week for 1 month after the end of MCh application. To investigate the effect of EC on neoplasms already formed, EC began to be given to the mice of group 4 1 week after the end of MCh application, when 50% of the mice had developed a neoplasm of the lower lip. In that case the EC preparation was injected 3 times a week for 3 months. As controls, the mice of groups 3 and 5 received a similar ethanol extract of liver containing G_1- and G_2-hepatic chalones (HC), in the same way and in the same dose as the animals of groups 2 and 4 respectively [5].

EXPERIMENTAL RESULTS

Application of MCh induced the appearance of multiple papillomas and of carcinoma of the mucosa and skin of the lower lip in all mice. The degree of keratinization was estimated in accordance with the known classifications [1, 2].

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