MUTAGENIC ACTION OF BENZ(a)PYRENE AND ITS METABOLITES IN A Salmonella typhimurium – MICROSOME TEST SYSTEM in vitro

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Polycyclic aromatic hydrocarbons (PAH) are activated by nonspecific oxygenases to oxidation products: phenols, dihydrodiols (DDO), epoxides, and quinones. Primary oxidation products of PAH may be oxidized by the same enzymes as PAH themselves, with the formation of compounds such as epoxy-DDO or epoxyphenols [1, 8].

The action of a given metabolite depends on the position of the functional group in the PAH molecule. The properties of DDO-epoxides with functional groups arranged side by side have been studied the most extensively. It is considered that metabolites of this class are responsible for the transforming action of carcinogenic PAH [5].

However, the question of metabolism of DDO metabolites, the structure of whose PAH is such that the carbon atoms of the main chain, located alongside the DDO-group, cannot be oxidized to epoxy groups, such as 4,5-DDO-benz(a)pyrene (4,5-DDO-BP), has not been studied.

In the investigation described below the process of formation of active compounds during secondary oxidation of certain BP metabolites, namely 4,5-DDO-BP, 6-hydroxy-BP (6-OH-BP), and 3-hydroxy-BP) (3-OH-BP), was studied. Strains of Salmonella typhimurium were used as acceptor of the active metabolites [2, 6]. The index of activity of the metabolites was the number of colonies of mutant bacteria. It was also decided to study the contribution of the various classes of BP metabolites in the over-all mutagenic effect of BP itself. For this reason the possible modifying effect of glutathione and α-tocopherol on mutagenesis induced by BP and its metabolites was investigated.

EXPERIMENTAL METHOD

The BP used in the experiments was from Fluka, the 3-OH-BP from Midwest Research Institute, the 6-OH-BP and 4,5-DDO-BP were synthesized by the method described in [3, 4] by O. A. Pan'shin, NADPH and glutathione were from Reanal, and other agents were of Soviet manufacture and of the chemically pure grade.

Strains of Salmonella typhimurium TA 98, with counting frame-shift type of mutation in the histidine operon, and TA 100 with a mutation of base substitution type in the same operon [6], defective for histidine and biotin synthesis were used. The strains were obtained from the International Agency for the Study of Cancer (Lyon, France).

Experiments to determine mutagenic activity were carried out by the technique described previously [2]. The number of colonies of revertants counted 48 h after seeding was used as the index of mutagenicity. The rat liver homogenate was prepared as described previously [2]. To obtain 10 ml of activating mixture, 10 mg NADPH, 0.3 ml 0.5 M $MgCl_2$ solution, and 7 ml phosphate buffer, pH 7.0, were added to 3 ml of the liver homogenate.

To obtain the microsomal fraction the liver homogenate was centrifuged at 80,000g for 60 min and the residue thus obtained was suspended in isolation medium. 4,5-DDO-BP was oxidized in 50 mM phosphate

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TABLE 1. Effect of Glutathione and α-Tocopherol on Mutagenic Effect of BP and Its Metabolites

Compound	Dose, μg per dish	Activation	Number of his^+ revertants per dish	
			strain TA 98	strain TA 100
—	0	—	18	75
—	0	+	24	86
3-OH-BP	5	—	29	78
3-OH-BP	5	+	75	113
3-OH-BP	25	—	42	70
3-OH-BP	25	+	198	97
3-OH-BP + glutathione	25+1000	+	49	—
6-OH-BP	5	—	640	—
6-OH-BP	5	+	1120	—
6-OH-BP + α-tocopherol	5+500	—	61	—
6-OH-BP + α-tocopherol	5+500	+	67	—
BP		+	174	1161
BP + α-tocopherol	5+500	+	179	1200
BP + glutathione	5+1000	+	114	471
Glutathione	1000	+	20	91
α-tocopherol	500	+	26	85

buffer, pH 7.4, with the addition of NADPH (1 mM), $MgCl_2$ (8 mM), and oxidation substrate at the rate of 10 μg/ml incubation mixture. The mixture was incubated at 37°C for 15 min. Metabolites were extracted with ethyl acetate and fractionated by thin-layer chromatography on silica-gel. The position on the chromatogram was identified from fluorescence in UV light.

Depending on the experiment, homogenate from intact rats or from rats receiving an intraperitoneal injection of 3-methylcholanthrene (3-MCh) in oil in a dose of 30 mg/kg body weight, 48 h before sacrifice, to induce enzymes activating the test substance, was used.

EXPERIMENTAL RESULTS

In the presence of activating mixture BP effectively induced back mutations of the base-pair substitution type in strain TA 100 and mutations of counting frame-shift type in strain TA 98. The largest number of revertants appeared in response to BP in a dose of 5-25 μg per dish. With an increase in the dose to 100 μg per dish the number of revertants fell. The mutagenic activity of BP when liver homogenate from animals receiving 3-MCh was used was significantly higher than when homogenate from intact rats was used, in agreement with the results of earlier investigations [2].

Induction of different types of mutations is evidence that the mutagenic effect of BP is determined evidently by the action, not of one, but of several metabolites. The transforming action of BP is known to be due to the formation of epoxides [8]. It therefore seemed important to study the contribution of epoxides to the mutagenic action of BP. For this purpose the action of glutathione on the mutagenic effect of BP was studied. Glutathione interacts with epoxides and reduces their concentration, which could lead to a decrease in the number of revertants in the presence of glutathione, if the mutagenic effect of BP were due to epoxides. The results of the corresponding experiments are given in Table 1 and they show that addition of glutathione to the incubation medium reduced the number of his^+ revertants almost by half, in the case both of strain TA 98 and of strain TA 100. This means that the mutagenic effect of BP is due in a considerable measure to the epoxides formed during its metabolism, which can induce mutations of the two types described above.

It has been shown that the phenols 3-OH-BP and 6-OH-BP are among the metabolites of BP [1]. The study of the mutagenic action of these phenols showed that they can induce only frame-shift mutations. The mutagenic activity of 3-OH-BP was much less than that of 6-OH-BP and it was manifested only in the presence of an activating mixture. Glutathione almost completely abolished the mutagenic action of 3-OH-BP (Table 1). An epoxide is probably formed during further metabolism of 3-OH-BP.

Unlike 3-OH-BP, 6-OH-BP was found to be a mutagen with "direct" action and its effect was manifested in the absence of an activating mixture. In the presence of an activating mixture the number of his^+ revertants was increased, but this effect also was observed if the system consisted only of liver homogenate without NADPH. It was concluded that potentiation of the mutagenic action of 6-OH-BP in this case was not connected with its enzymic activation. The presence of glutathione in the incubation mixture did not change the number

of his$^+$ revertants. We know [7] that 6-OH-BP is broken down spontaneously with the formation of a highly reactive oxo radical, and that the presence of protein in the solution potentiates this process. It was logical to suppose that the observed mutagenic effect of 6-OH-BP was due to the formation of free radicals. To test this hypothesis we studied the mutagenic action of 6-OH-BP in the presence of α-tocopherol, an extinguisher of free-radical processes. The results of experiments on strain TA 98 are given in Table 1. α-Tocopherol almost completely abolished the mutagenic effect of 6-OH-BP both in the presence and in the absence of liver homogenate and of NADPH. The results of these experiments are evidence of the free-radical mechanism of action of 6-OH-BP.

To determine the contribution of 6-OH-BP to the total mutagenic effect of BP, the action of BP was studied in the presence of α-tocopherol. It was found that the antioxidant in a dose of 500 μg per dish had no effect on the mutagenic action of BP. This state of affairs can be explained on the grounds that the contribution of 6-OH-BP to the mutagenic action of BP is very small in the presence of other BP metabolites, and for that reason no difference could be found between the tests with and without α-tocopherol under these conditions.

Epoxides thus make the basic contribution to the over-all mutagenic effect of BP.

Oxidation products of PAH may act as substrate for enzymes oxidizing the original hydrocarbon. It has been shown [5] that DDO of PAH are oxidized with the formation of highly active DDO-epoxides. As has already been mentioned, the properties of diolepoxides with functional groups located side by side have been studied most extensively, but the question of the pathways of breakdown of DDO in which the formation of an epoxy group side by side with the dihydrodiol group is impossible has not been studied. One such DDO, formed by oxidation of BP, is 4,5-DDO-BP. The possible mutagenic effect of this compound was investigated and it was found that neither in the presence nor in the absence of an activating mixture did this substance lead to an increase in the number of his$^+$ revertants. To determine the reason for this fact, oxidation of 4,5-DDO-BP was studied in rat liver microsomes in vitro. The 4,5-DDO-BP was oxidized to form five products which were fluorescent in UV light. This process is connected with the function of a monooxygenase enzyme system, for in the absence of NADPH, the formation of these substances was not observed. To determine whether 4,5-DDO-BP is oxidized with the formation of epoxides, epoxycyclohexane (1 mM), an inhibitor of the enzyme epoxidase, was added to the reaction mixture. In the presence of epoxycyclohexane four substances were found to be formed, and none of the compound with low R_f value was formed. This means that this compound is oxidized through a stage of epoxidation and that it is diolepoxide. The results of these experiments are evidence that: 1) the formation of diolepoxides is not necessarily connected with the introduction of an epoxy group into the position alongside the DDO group in the PAH molecule; 2) the presence of a diolepoxide group does not necessarily endow the compound with mutagenic activity.

The investigation thus yielded indirect evidence that the main contribution to the combined mutagenic effects of BP metabolites is made by epoxides, which can induce mutations of both base pair substitution and frame shift types. No data on the contribution of phenols to the combined mutagenic effect of BP metabolites were obtained, although phenols which are metabolites of BP such as 6-OH-BP and 3-OH-BP are either mutagens themselves or become so during further metabolism. The mutagenic action of the metabolites studied was due to the position of the functional group in the molecule.

It can also be postulated on the basis of the study of 4,5-DDO-BP metabolism that a compound in which the epoxy and dihydrodioxy groups are spatially separated is not mutagenic.

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