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USE OF A MIXED METABOLIC ACTIVATION SYSTEM IN THE *SALMONELLA* REVERSE MUTATION TEST OF CHEMICAL CARCINOGENS

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ABSTRACT: *The post-mitochondrial liver fractions (S-9) were prepared from rats and hamsters which have been treated with Aroclor 1254 (PCB) and the capacities of these S-9 fractions to generate mutagenic metabolites from several well known procarcinogens have been compared. Benzo(a)pyrene (B(a)P), 3-methylcholanthrene (3-MC), Aflatoxin B_1(AFB_1), 2-acetylaminofluorene(AAF), and 2-aminofluorene (AF) were employed as promutagens in the Salmonella reverse mutation tests. Results showed that the rat and hamster S-9 fractions had differential abilities to produce mutagenic metabolites from a given promutagen. With given doses of polyaromatic hydrocarbons (PAH), the rat S-9 fraction produced more of the mutagenic metabolites than the hamster S-9 fraction. Upon adding increased amounts of S-9 fraction to the test system, more of the mutagenic PAHs metabolites were produced, but without linear relationship. With other promutagens, this potentiating effects were not observed. Upon adding the mixture of the rat and hamster S-9 fractions, synergistic effects were observed with 3-MC and B(a)P, but antagonistic effects were observed with AFB_1, AAF, and AF. Although the addition of mixed S-9 fractions did not produce the strongest effects, the species differences in the obtained S-9 fractions for metabolic activation of promutagens. These results indicate that the S-9 fractions obtained from rat and hamster should be mixed and used as a general metabolic activation system in the bacterial mutagenesis tests for the screening of potential chemical carcinogens.*

Keywords: *Salmonella reverse mutation test, Mixed S-9 preparation, Promutagens.*

INTRODUCTION

The *Salmonella* reverse mutation test originally developed by Ames in 1971 has

been used to screen a wide range of chemicals for mutagenic activity. The value of the original assay method was considerably improved by the incorporation of liver S-9 preparation capable of metabolically activating those chemicals which are not direct-acting mutagens (Ames *et al.*, 1973; Ames *et al.*, 1975). For this purpose, liver S-9 preparations from rats which have been pretreated Aroclor 1254 (McCann *et al.*, 1975: McMahon *et al.*, 1979; Simmon, 1979). Muller *et al.* (1980) compared liver S-9 fractions obtained from seven different animal species and showed that there were species specific differences. They found that Aroclor 1254 induced fractions from all species produced positive effect with 5 mutagenic substances. However, Raineri *et al.* (1981a) reported that male hamster liver S-9 preparations were more active than those from male rats for the metabolic activation of nitrosamines in *Salmonella* reverse mutation test. In addition, they have also examined several polyaromatic hydrocarbons and aromatic amines and found that the species differences continued to exist between rats and hamsters (Raineri *et al.* 1981b). Even in the presence of these studies, Maron and Ames (1983) still recommended the use of Aroclor 1254 induced male rat liver S-9 fraction as a general metabolic activation system. As the result, general toxicity screening methods performed by many countries have followed the recommeded method of Maron and Ames (1983).

Our study was designed to examine the species differences between S-9 preparations of rats and hamsters and determine the effect of mixing the two S-9 preparations for metabolic activation of several promutagens by comparison with the individual unmixed S-9 preparations at two different concentrations. We have compared the mutagenicity of the known carcinogens such as 2-acetylaminofluorene(2-AAF), 2-aminofluorene(AF), 3-methylcholanthrene(3-MC), benzo(a)pyrene(B(a)P), aflatoxin B_1(AFB_1) using the Aroclor 1254 induced liver S-9 preparations obtained from Syrian golden hamsters and Sprague-Dawley rats.

MATERIALS AND METHODS

Preparation of Liver Post-Mitochondrial Fractions(S-9 Fractions)

Ten weeks old male Syrian golden hamsters and eight weeks old male Sprague-Dawley rats were used for the preparation of liver S-9 fractions. The induction procedure was similar to the methods of Maron and Ames (1983). Aroclor 1254 was diluted in corn oil to a concentration of 200 mg/m*l* and a single i.p. injection of 500 mg/kg was administered to each rat and hamster 5 days before sacrifice. The method employed for preparation of the liver S-9 fraction was based on the procedure of Garner *et al.*, (1972). All steps of the procedure were carried out at 0-4°C using cold, sterile solutions and glass wares. The freshly excised livers were placed in preweighed beakers containing approximately 1 m*l* of chilled 0.15M KCl per gram of wet liver. After weighing, the livers were washed several times in the freshly prepared chilled KCl solution. The washed livers were than transfered to a beaker contining 3 vol. of 0.15M KCl, minced with sterile scissors, and hamogenized using a Polytron homogenizer. The homogenate was centrifuged for 10 min. at 9,000g (12,000 rpm in Eppendorf microcentrifuge) and the supernatant was collected and used as the S-9 fraction. This fraction was frozen immediately in liquid N_2 and stored at -80°C untill use.

Salmonella Reverse Mutation Test(Ames' test)

The *Salmonella* mutagenicity assay was performed using the preincubation procedure essentially as described by Maron and Ames (1983). The bacterial strain used in this experiment was *Salmonella typhimurium* TA98, a frameshift mutant strain. Their genotypes were confirmed by histidine requirement, *rfa* mutation, *uvrB* mutation and R-factor plasmid. Reaction mixture contained 20 or 40 μl S-9 fraction and 16.5 μmole KCl, 4 μmole $MgCl_2$, 2.5 μmole glucose-6-phosphate, 2 μmole NADP, 25 μl 0.2 M phosphate buffer (pH 7.4) and 0.1 m*l* of the overnight bacterial culture. In the case where mixed S-9 preparations was used combination of 20 μl of rat S-9 preparation and of 20 μl of hamster S-9 preparation were added to the reaction mixture. Reaction volume was then adjusted to 0.6 m*l* with distilled water. Mutagenic chemicals were dissolved in 6 μl(AFB_1) or 60 μl(other chemicals) of dimethylsulfoxide(DMSO) and then added to the reaction mixture. In the negative control group, same volume DMSO containing no chemicals was added. This mixture was incubated in shaking water bath at 37 °C for 20 min., added to the 2 m*l* of molten top agar and then poured evenly onto the hardened minimal glucose agar plate. After 48 hours of incubation, the colonies which have reverted to histidine prototrophy were counted. Results were expressed as the average of two or three determinations.

RESULTS

Mutagenicity Studies

Liver S-9 preparations from male hamsters and rats treated with Aroclor 1254 were used in the Ames' test to determine their effectiveness in metabolically activating the selected chemical carcinogens such as two polycyclic aromatic hydrocarons (i.e., B(a)P and 3-MC), two aromatic amines (*i.e.*, 2-AAF and AF) and one mycotoxin(AFB_1).

Polycyclic Aromatic Hydrocarbons

With B(a)P, the numbers of revertants produced by rat S-9 preparations were greater than that of the revertants obtained with the hamster preparation (Fig. 1). With increased amounts of S-9 preparation added in reaction mixture, the greater mutagenicity was expressed but the species difference continued to exist (Fig. 2). Mixed S-9 preparation has produced more revertants than with the individual components. When the mutagenicity produced with mixed S-9 preparation was compared with same amount of either the rat or the hamster S-9 preparations, median numbers of revertants were produced. Similar results of cancelling out of species differences of S-9 fractions could be seen with 3-MC (Fig. 3). Rat S-9 preparation was more effective than that of the hamster in metabolic activation of 3-MC. Upon adding increased amounts of S-9 fraction, mutagenicity was augmented, but the species difference still remained (Fig. 4). Synergistic effect of producing more revertants was also seen upon adding the equal volume of S-9 fractions obtained from two species. When the produced mutagenicity of this condition was compared using equal volume of individual S-9 fractions, the mixed S-9 preparation showed similar mutagenic activity as with rat

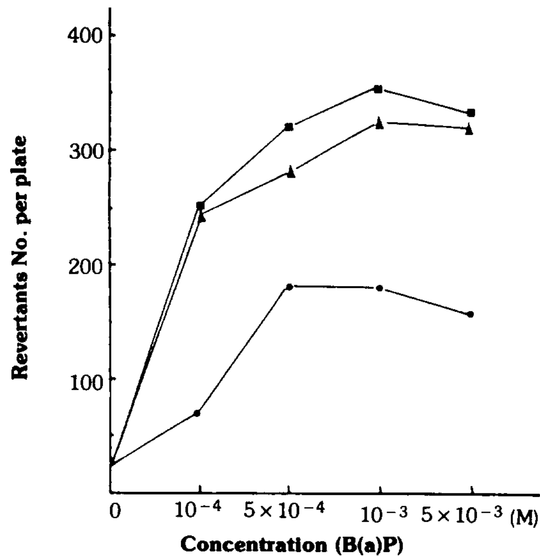

Fig. 1. Effects of using the mixed S-9 fraction for the metabolic activation of Benzo(a)pyrene. *Salmonella typhimurium* TA98 and various dose of Benzo(a)pyrene were incubated in the presence of liver S-9 fractions which were made from Aroclor 1254 induced animals. The S-9 fractions used in this test were as follow: rat, 20 μl (▲-▲); hamster, 20 μl (● - ●); equal mixture of rat and hamster S-9 fractions, 40 u*l* (■ - ■).

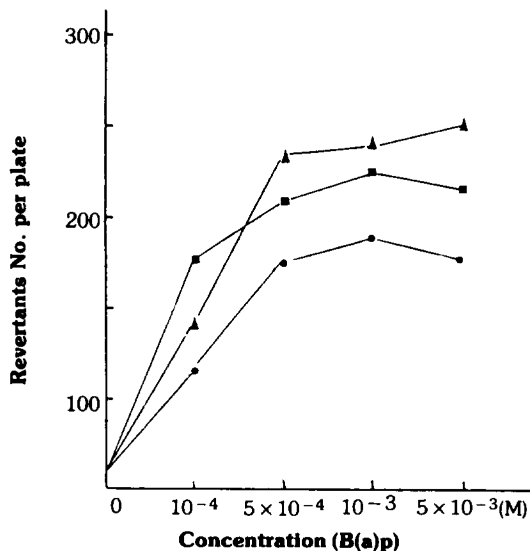

Fig. 2. Effects of using the mixed S-9 fraction for the metabolic activation of Benzo(a)pyrene. *Salmonella typhimurium* TA98 and various dose of Benzo(a)pyrene were incubated in the presence of liver S-9 fractions which were made from Aroclor 1254 induced animals. The S-9 fractions used in this test were as follow: rat, 40 μl(▲-▲); hamster, 40 μl(● - ●); equal mixture of rat and hamster S-9 fractions, 40 μl(■ - ■).

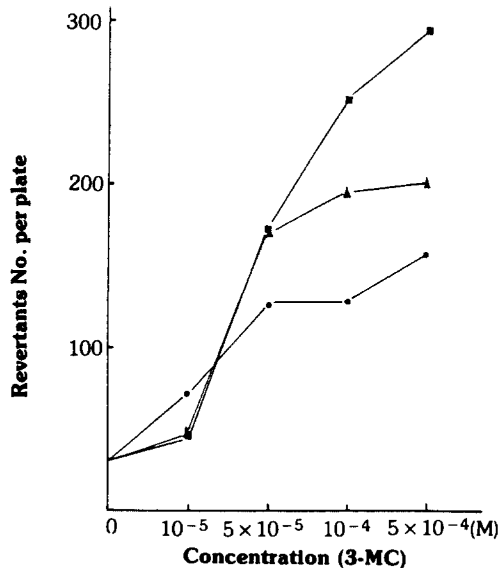

Fig. 3. Effects of using the mixed S-9 fraction for the metabolic activation of 3-Methylcholanthrene. *Salmonella typhimurium* TA98 and various dose of 3-Methylcholanthrene were incubated in the presence of liver S-9 fractions which were made from Aroclor 1254 induced animals. The S-9 fractions used in this test were as follow: rat, 20 μl(▲-▲); hamster, 20 μl(● - ●); equal mixture of rat and hamster S-9 fractions, 40 μ l(■-■).

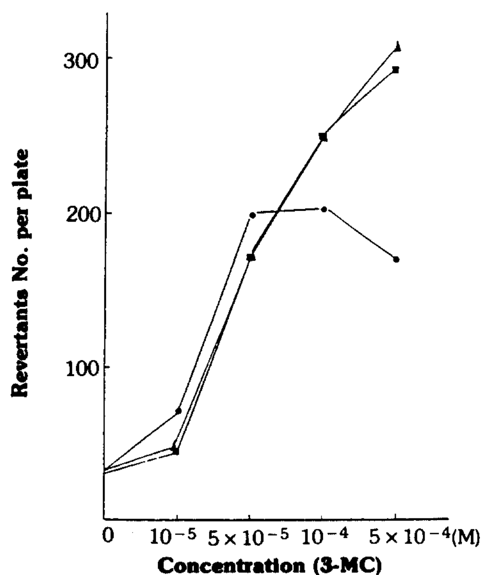

Fig. 4. Effects of using the mixed S-9 fraction for the metabolic activation of 3-Methylcholanthrene. *Salmonella typhimurium* TA98 and various dose of 3-Methylcholanthrene were incubated in the presence of liver S-9 fractions which were made from Aroclor 1254 induced animals. The S-9 fractions used in this test were as follow: rat, 40 μl(▲-▲); hamster, 40 μl(● - ●); equal mixture of rat and hamster S-9 fractions, 40 μ l(■-■).

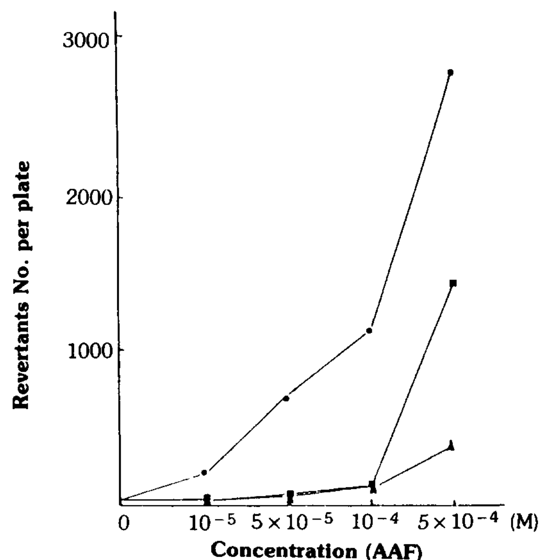

Fig. 5. Effects of using the mixed S-9 fraction for the metabolic activation of 2-Acetylaminofluorene. *Salmonella typimurium* TA98 and various dose of 2-Acetylaminofluorene were incubated in the presence of liver S-9 fractions which were made from Aroclor 1254 induced animals. The S-9 fractions used in this test were as follow: rat, 20 μl(▲-▲); hamster, 20 μl(● - ●); equal mixture of rat and hamster S-9 fractions, 40 μl(■ - ■).

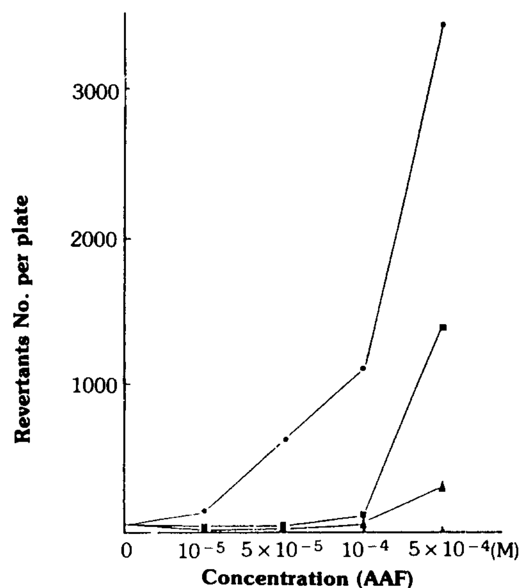

Fig. 6. Effects of using the mixed S-9 fraction for the metabolic activation of 2-Acetylaminofluorene. *Salmonella typhimurium* TA98 and various dose of 2-Acetylaminofluorene were incubated in the presence of liver S-9 fractions which were made from Aroclor 1254 induced animals. The S-9 fractions used in this test were as follow: rat, 40 μl(▲-▲); hamster, 40 μl(● - ●); equal mixture of rat and hamster S-9 fractions, 40 μl(■ - ■).

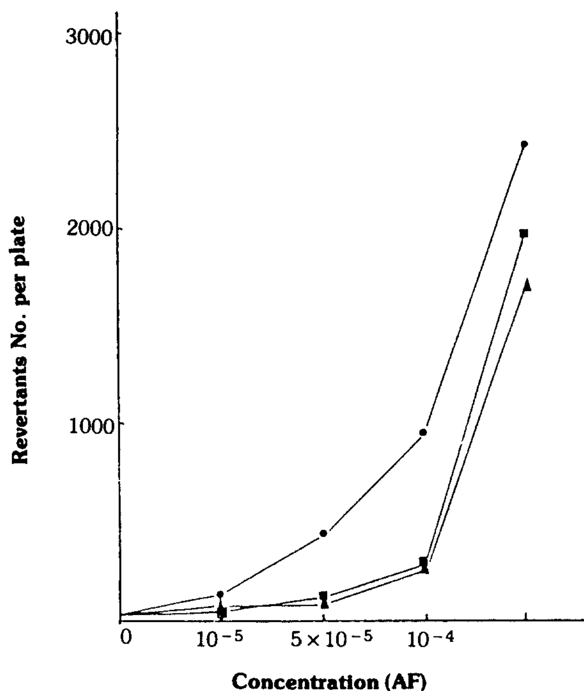

Fig. 7. Effects of using the mixed S-9 fraction for the metabolic activation of 2-Aminofluorene. *Salmonella typhimurium* TA98 and various dose of 2-Aminofluorene were incubated in the presence of liver S-9 fractions which were made from Aroclor 1254 induced animals. The S-9 fractions used in this test were as follow: rat, 20 μl(▲–▲); hamster, 20 μl(●–●); equal mixture of rat and hamster S-9 fractions, 40 μl(■–■).

S-9 but higher activity than the hamster S-9 preparation.

Aromatic Amines

With AAF, hamster S-9 preparation exerted strong ability for metabolic activation, but the rat S-9 had only a weak metabolic activity (Fig. 5). Even when the volume of S-9 fraction was doubled up, the numbers of obtained revertants were not increased (Fig. 6). When two fractions were mixed, strong antagonistic effect was observed with all doses tested. At 10^{-5}-10^{-4} M concentration ranges, the mixed S-9 preparation having 2x the S-9 volume produced only similar numbers of revertants as the rat S-9 preparation. However, at 5×10^{-4} M AAF, median numeric value of revertants was produced. In the case of AF, the pattern of all data was similar to those obtained with AAF (Fig. 7 and 8). But the degree of species difference was somewhat less than that observed with AAF. With the use of mixed S-9 preparation the antagonistic cancelling out effect of species difference can still be seen with all doses of AF tested. Thus the results obtained with aromatic amines were quite different from the pattern observed with polyaromatic hydrocarbons.

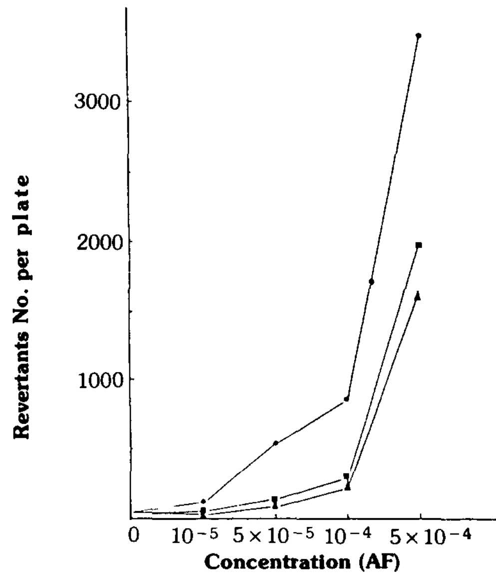

Fig. 8. Effects of using the mixed S-9 fraction for the metabolic activation of 2-Aminofluorene. *Salmonella typhimurium* TA98 and various dose of 2-Aminofluorene were incubated in the presence of liver S-9 fractions which were made from Aroclor 1254 induced animals. The S-9 fractions used in this test were as follow: rat, 40 μl(▲ - ▲); hamster, 40 μl(● - ●); equal mixture of rat and hamster S-9 fractions, 40 μl(■ - ■).

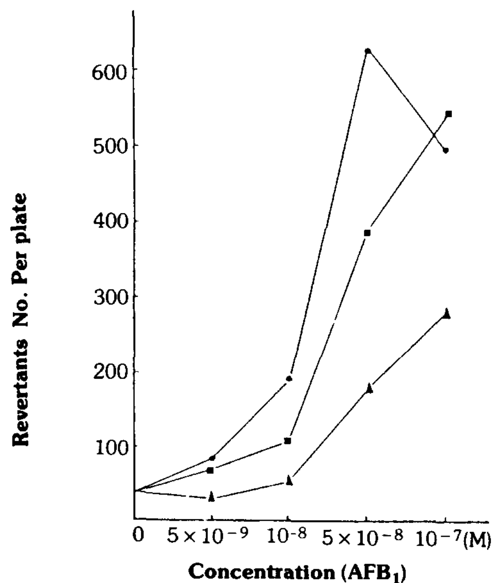

Fig. 9. Effects of using the mixed S-9 fraction for the metabolic activation of Aflatoxin B_1. *Salmonella typhimurium* TA98 and various dose of Aflatoxin B_1 were incubated in the presence of liver S-9 fractions which were made from Aroclor 1254 induced animals. The S-9 fractions used in this test were as follow: rat, 20 μl(▲ - ▲); hamster, 20 μl(● - ●); equal mixture of rat and hamster S-9 fractions, 40 μl(■ - ■).

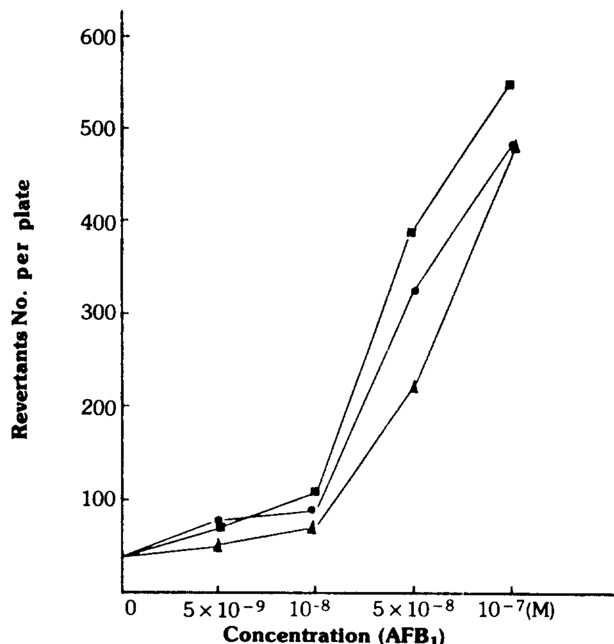

Fig. 10. Effects of using the mixed S-9 fraction for the metabolic activation of Aflatoxin B_1. *Salmonella typhimurium* TA98 and various dose of Aflatoxin B_1 were incubated in the presence of liver S-9 fractions which were made from Aroclor 1254 induced animals. The S-9 fractions used in this test were as follow: rat, 40 μl(▲-▲); hamster, 40 μl(● - ●); equal mixture of rat and hamster S-9 fractions, 40 μl(■ - ■).

Aflatoxin B_1(AFB_1)

As with the results of aromatic amines, when AFB_1 was employed as promutagen, hamster S-9 preparation produced greater number of revertants than the rat preparation (Fig. 9). When the concentration of S-9 preparation was increased by two fold, rat S-9 showed increased mutagenic activity but the mutagenicity induced by hamster S-9 was decreased to about half scale (Fig. 10). When compared with individual components, the use of mixed S-9 preparation produced the cancelling out species difference. But, the mixed preparation produced the highest number of revertants when compared with the same volume of individual S-9 preparation.

DISCUSSION

The *Salmonella* reverse mutation test has served important role in the screening of toxicity of wide variety of chemicals because the test method has shown good correlations with the conventional carcinogenicity testing (Ray *et al.*, 1987). The use of metabolic activation systems in conjunction with the original Ames' test and other *in vitro* mutagenesis assays has been proven necessary for the effective detection of chemical promutagens (Ames *et al.*, 1975; McCann *et al.*, 1975). However, as reported earlier, varying results have been obtained caused by the species difference of S-9 fractions. This was also confirmed in the present study. Generally, with polyaromatic hydrocar-

bons, the numbers of revertants were somewhat larger when the rat S-9 fraction was used. However, with aromatic amines and AFB_1, the use of hamster S-9 fraction has produced greater number of revertants. In contrast to our results, Raineri *et al.* (1981b) did not find any species differences of S-9 fractions with B(a)P and 3-MC. This may have been due to the use of Aroclor 1254 induced S-9 fractions as compared to the use of uninduced S-9 fractions in other studies published earlier (Polley *et al.*, 1980; Muller *et al.*, 1980; Bartsch *et al.*, 1980; Raineri *et al.*, 1981b) (confirmed by our unpublished data). Maron and Ames (1983) recommended 20 u*l* of S-9 preparation per plate for the general reaction mixture and 50 u*l* per plate for high concentration reaction mixture. But, as shown in our study, using the increased amount of S-9 fraction in reaction mixture did not produce a uniform potentiation in the number of revertants obtained. The degree of changes were different depending on the chemicals tested and also on the animal species from which the S-9 fractions were prepared (Maron and Ames, 1983; Muller *et al.*, 1980; Bartsch *et al.*, 1980).

Upon mixing the Aroclor 1254 induced liver S-9 fractions obtained from rat and hamster, the ability for metabolic activation was synergistic for 3-MC and B(a)P, but was antagonistic for 2-AAF, AF and AFB_1. The synergistic effect observed with PAH can be explained by the fact that enzyme concentrations which participate in the formation of ultimate mutagenic metabolites such as diol epoxide and dihydrodiol were increased (Gelboin, 1980; Glatt *et al.*, 1981; Pelkonen and Nebert, 1982; Conney, 1982). The antagonism observed with aromatic amines can be partially explained in two ways. Firstly, the increased amounts of detoxication enzymes present in one S-9 fraction could participate in the degradation of mutagenic entities produced by another S-9 fraction in which there were insufficient amount of detoxification enzymes. The detoxication enzymes have been well known to be greatly induced by the Aroclor 1254 treatments in earlier reports (Thorgeirsson *et al.*, 1984; Roebuk and Wogan, 1977). Secondly, the mixing of S-9 fractions causes an increase in the concentrations of molecules such as DNA and RNA which can bind easily with the highly reactive mutagenic metabolites produced by the mixed function oxidase (Croy *et al.*, 1979).

As shown in the presented results, the use of mixed S-9 preparation produced median numbers of revertants and the species differences could be cancelled out. So, even if the use of mixed S-9 fractions did not produce the strongest metabolic activation, a mixed S-9 preparation could be use as the metabolic activation system which is highly useful in the general mutagenicity screening tests

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