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Use of Metabolic Activation Systems of Tulip Bulbs in the Ames Test for Environmental Mutagens

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It has been reported that polycyclic aromatic hydrocarbons in the environmental pollutants adsorb on the surface of plant or absorb into plant tissues(BAUM 1978). It is, therefore, important to examine the effects of these trace amounts of polycyclic aromatic hydrocarbons on the carcinogenesis of animals and human beings and also on the ecology of plants. To examine the metabolic fate of environmental mutagens in the plants, the development of reliable method would be of considerable importance. We have searched such a *in vitro* system originated in plant and so far succeeded in confirming the presence of metabolic activation systems only for potent carcinogens.

MATERIALS AND METHODS

Tulip bulbs (*Tulipa gesnerana* L., Frasquita) were purchased directly from a local cultivator and stored in the dark at room temperature (18-25°C). Bulbs were sliced and homogenized in a blender with 1.5 vol of 0.1 M sodium phosphate buffer, pH 7.4, in the presence of 10 mM mercaptoethanol, 1 mM EDTA, 0.4 % bovine serum albumin and 0.6 M mannitol. The crude extract was filtered through cheesecloth and centrifuged at 10,000 x g for 10 min. The supernatant obtained was filtered through a membrane filter (pore size 1μm) twice under aseptic conditions and used as S9. To prepare microsomes, the unfiltered supernatant was centrifuged at 105,000 x g for 1 h in a 50-Ti rotor(Beckman Instr.Inc).

The content of cytochrome P-450 (cyt.P-450) was determined as described by OMURA & SATO(1964) using a CARY 17D spectrophotometer. Microsomes were washed once with 0.05 M sodium pyrophosphate buffer, pH 7.4, containing 0.1 mM EDTA before SDS-polyacrylamide gel electrophoresis (LAEMMLI 1970). Rat liver-microsomes as a marker were prepared from the animals after treatment with phenobarbital for three days(80 mg/kg of body weight, each day). After staining the gel with coomassie brilliant blue,the gel was scanned by a Joyce-Loebl microddensitometer.

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Either benzo(a)pyrene or aflatoxin B1 was preincubated with Salmonella TA98 and reaction mixtures containing S9 at 37°C for 20 min and poured onto a soft agar (YAHAGI et al. 1975). The plates were incubated at 37°C for two days,after which the number of colonies appearing was counted (AMES et al. 1975). Benzo(a)-pyrene was purchased from Sigma Chem. Co. and aflatoxin B1 was from Aldlich Chem. Co.

RESULTS AND DISCUSSIONS

Microsomes were prepared from several plants and the contents of cyt.P-450 were determined (Table 1). The microsomes of both tulip bulbs and avocado had significantly higher levels of cyt.P-450 than those of other plants tested. Induction of microsomal cyt.P-450 in both cauliflower buds and Jerusalem artichoke tubers were observed after slicing these tissues and aeration of the disks in water at 25°C (BENVENISTE et al. 1977, HIGASHI et al. 1981). Attempt to induce microsomal cyt.P-450 in the disks of tulip bulbs was unsuccessful after the same treatment employed for artichoke tuber (data not shown), although the content of microsomal cyt.P-450 in the uninduced tulip bulbs was originally higher than those of the induced microsomes in either cauliflower buds or artichoke tubers. We decided to use uninduced tulip bulbs as the source of S9 from plants due to the high content of cyt. P-450 and easy availability of fresh materials.

Environmental contamination elevates the polycyclic hydrocarbon content of vegetation (BAUM 1978). Leafy

Table 1. Contents of cyt.P-450 in the microsomes of various tissues

Sources	Cyt.P-450(nmol/mg protein)
Cauliflower(bud)	
Uninduced	0.068
Induced*	0.101
Jerusalem artichoke(tuber)	
Uninduced	0.060
Induced*	0.154
Maize(seed)	0.186
Avocado (mesocarp)	0.837
Tulip(bulb)	0.838

* Disks(0.5-1 mm thick) were vigorously shaken in the water at 25°C for 16 h.

Table 2. Mutagenicity of metabolites of benzo(a)pyrene or aflatoxin B1 by S9 of tulip bulbs

A) Benzo(a)pyrene

S9	B(a)P*	Number of revertants/plate
mL	µg/plate	
0.3	10	73** (35,43,53,70,166)
0.3	20	74 (51,55,76,92,100)
0.5	10	61 (52,55,59,69,71)
0.5	20	163 (57,144,181,201,234)
0.6	10	134 (83,106,121,157,204)
0.6	20	79 (59,68,70,82,114)
0.5	-	60 (57,62)
-	20	24 (8,39)
-	-	19 (17,21)

* B(a)P: benzo(a)pyrene, ** Average values

B) Aflatoxin B1

S9	Afl-B1*	Number of revertants/plate
mL	µg/plate	
0.5	5	59** (38,48,68,82)
0.5	10	118 (109,110,115,117,141)
0.5	20	160 (117,123,167,195,196)
0.5	50	364 (253,320,362,404,479)
0.5	-	36 (33,38)
-	20	54 (47,61)
-	-	19 (17,21)

* Afl-B1: aflatoxin B1, ** Average values

plants, such as kale and spinach are found to contain high levels of benzo(a)pyrene (D'ARRIGO & LAGHI 1972). In the beginning, we examined the metabolic activation of benzo(a)pyrene by S9 from tulip bulbs by the Ames test. In this case, there are optimal levels of S9 and benzo(a)pyrene to obtain the maximum number of colonies. The combination of either S9(0.5 mL) and benzo-(a)pyrene 20 µg/plate or S9(0.6 mL) and benzo(a)pyrene 10 µg/plate was optimal (Table 2-A). The plate with other combinations did not show constantly the positive results in the case of benzo(a)pyrene.

The metabolic pathway of benzo(a)pyrene to its ultimate form of mutagen,that is,7,8-diol,-9,10-epoxide (THAKKER et al. 1981) is known to be multi-step.

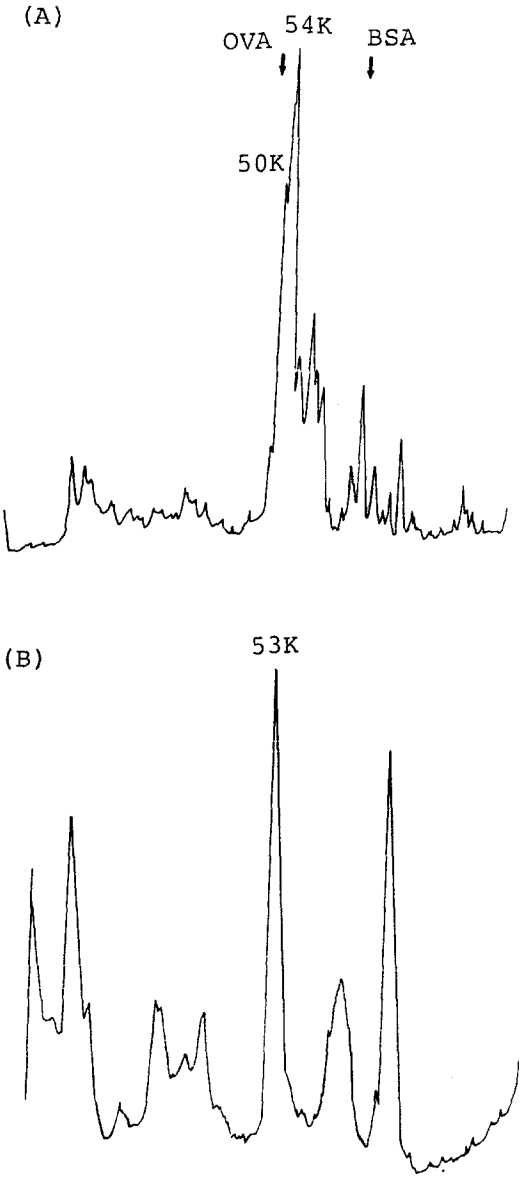

Figure 1. Densitometric scan(550 nm) of an SDS-polyacrylamide gel after electrophoresis of microsomal proteins of phenobarbital-treated rat liver(A) and tulip bulb(B). The gel was stained with coomassie blue. Arrows indicate electrophoretic mobility of ovalbumin (OVA) and bovine serum albumin(BSA).

Optimal levels of S9 and other factors to obtain a maximal number of revertants must varied with individual chemicals,which required multi-step activation. On the contrary, the activation of aflatoxin B1 is simpler than that of benzo(a)pyrene. The 2,3-oxide form of aflatoxin B1 was reported as a probable ultimate carcinogenic metabolites (SWENSON et al. 1974). As shown in Table 2-B, reproducible dose-response relationship was observed in the case of aflatoxin B1.

Microsomal cyt.P-450 in the higher plants have been generally considered as analogous to animal cyt.P-450 (RICH & BENDALL 1975). The existence of multiple forms of cyt.P-450 in mammalian tissues has been widely accepted (GUENGERICH 1978, GUENTHNER & NEBERT 1978). We wanted to see whether plant cells have merely low activity of cyt.P-450 or have a less number of isozymes of cyt.P-450. To resolve this problem, the microsomal proteins of tulip bulbs were analyzed on SDS-polyacrylamide gel electrophoresis in parallel with a sample of rat liver microsomes (Fig. 1).

According to TOFTGARD et al.(1980),induction of two forms with apparent molecular weight of 54,000 and 50,000 was obtained with phenobarbital. The major peak located in the middle of the gel corresponds to these induced cyt.P-450 (Fig. 1-A). Densitometric scan of gel for tulip bulbs showed a rather simple peak at the corresponding region (molecular weight 53,000). We have confirmed that there was a peroxidase-staining band in the presumptive cyt.P-450 locus of tulip bulbs,although there were several other proteins having peroxidase activities (data not shown). This preliminary result suggests that the species of cyt.P-450 in the microsomes of plants is rather less heterogenous as compared with those found in animal cells.

In coclusion, the use of S9 prepared from uninduced tulip bulbs for examining the general environmental mutagens in the Ames test has apparent limitations,as compared with that of rat liver after Aroclor-treatment (AMES et al. 1975). We have shown, however, at least the metabolic activation of chemical carcinogens by plant microsomes. Development of *in vitro* metabolic systems, which originated in plants, is quite important in connection with human exposure to environmental contamination through vegetations and also the control of the use of both herbicide and pesticide (PLEWA 1978). On the other hand, cyt.P-450 in plants must play important roles to detoxify the environmental mutagens.

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