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Influence of Microsomal and Cytosolic Fractions from the Liver of 4 Animal Species and Man on the Mutagenicity of Carcinogenic Aminoazo Dyes and Nature of the Mutagenicity-Enhancing Factor in the Cytosol from Rat Liver[1)]

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The mutagenicity of 3′-methyl-*N,N*-dimethyl-4-aminoazobenzene (3′-Me-DAB) and 3′-CH_2OH-DAB, potent hepatocarcinogens, was examined. Microsomal and cytosolic fractions from rat, mouse, hamster, rabbit, and human livers were used for metabolic activation, with *Salmonella typhimurium* TA100 or TA98 as a tester strain. The mutagenicity of both aminoazo dyes mediated by liver microsomes from polychlorinated biphenyls (PCB)-induced rats was markedly enhanced by addition of the hepatic cytosol; it was also enhanced by addition of the hepatic cytosol from uninduced rats and phenobarbital- or 3-methylcholanthrene (3-MC)-induced rats. Similar mutagenesis enhancement was produced by cytosols from PCB-induced mice, hamsters, or female rats. The cytosols from rabbit and human liver enhanced the mutagenicity of 3′-CH_2OH-DAB but not that of 3′-Me-DAB. Heating of the cytosol from uninduced rat liver to 55 °C for 15 min caused a loss of most of the enhancement activity; at 40 °C, it was stable. About 30% of the enhancement activity for both aminoazo dyes was lost upon dialysis for 24 h at 4 °C. Protein fractions with the aminoazo dye mutagenicity-enhancing activity were obtained from the dialyzed cytosol by gel filtration through Sephadex G-100; the protein fraction with the greatest activity, representing an approximately 3-fold increase in specific activity, was assigned a molecular weight of 43000—47500. This protein preparation enhanced microsome-mediated mutagenesis by 3′-hydroxymethyl-*N*-methyl-4-aminoazobenzene and *o*-aminoazotoluene but not that by 3-MC, benzo[*a*]pyrene, or dimethylnitrosamine.

Keywords——mutagenicity; carcinogenic aminoazo dye; metabolic activation; liver microsomes; liver cytosol; cytosolic mutagenicity-enhancing effect

The *Salmonella* mutagenicity assay is the most widely used method for screening chemicals for potential carcinogenicity.[2–5)] This assay routinely employs a post-mitochondrial fraction (S9) which is required to activate many promutagens to their ultimate mutagenic forms. Some mutagens are known to be activated by the hepatic microsomes alone,[6,7)] and the hepatic microsome-mediated mutagenesis from some carcinogens is enhanced by addition of the cytosol.[8–10)] However, comparatively few studies have been undertaken on the enhancement of mutagenicity by the cytosol and further information is required concerning the precise mechanism(s) of mutagenic activation of carcinogens.

We have recently reported that the expression of mutagenicity of 3′-methyl-*N,N*-dimethyl-4-aminoazobenzene (3′-Me-DAB) and 3′-CH_2OH-DAB requires the presence of both microsomes and cytosol of rat liver as sources of enzymes as well as reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor[11)] and that microsomal cytochrome P-450 is involved in the mutagenic activation.[1)] In this paper, we report experiments designed to further elucidate the enhancement by cytosol of the mutagenicity of 3′-Me-DAB and 3′-CH_2OH-DAB. Another purpose of our studies was to determine whether the varying

abilities of liver S9 fractions from different species to activate both hepatocarcinogens to mutagens in the *Salmonella* assay[12] could be explained by differences in the microsomal enzyme activities of these S9 preparations. Some results with human liver S9 are included.

Materials and Methods

Chemicals——Sephadex G-100 and blue dextran 2000 were obtained from Pharmacia Fine Chemicals Inc., Uppsala, Sweden and Visking tubing from Sanko Chemicals Inc., Tokyo, Japan. Superoxide dismutase (Type I, SOD) from bovine blood, *Helix pomatia* sulfatase H-1, ovalbumin, cytochrome c, and *o*-iodosobenzoate were purchased from Sigma Chemical Co., St. Louis, Mo. and membrane filters (YM-10) from Amicon Ltd., Lexington, Mass. Bovine serum albumin (BSA) and 2-mercaptoethanol were from Tokyo Kasei Co., Tokyo, and *o*-aminoazotoluene (OAT), dimethylnitrosamine (DMN), *p*-nitrophenol, 3-methylcholanthrene (3-MC), and benzo[*a*]pyrene (BP) from Wako Pure Chemicals, Ltd., Osaka, Japan. Cofactors for the Ames test were from Oriental Yeast Co., Tokyo, and 3′-Me-DAB, 3′-CH_2OH-DAB, and 3′-hydroxymethyl-*N*-methyl-4-aminoazobenzene (3′-CH_2OH-MAB) were synthesized in our laboratory.[13] Prior to assays for mutagenicity, the purity of the aminoazo dyes was determined to be more than 99% by high-pressure liquid chromatography. All other chemicals and solvents were of the purest grade available.

Animals and Human——Male and female Sprague-Dawley rats (body weight, 150—200 g), male ddY mice (20—30 g), male Syrian golden hamsters (50—80 g), and male Japanese white rabbits (2 kg) were obtained from Shizuoka Laboratory Animal Center, Hamamatsu, Japan, and used as the source of livers to prepare S9 fractions. Induction with polychlorinated biphenyls (PCB), 3-MC, or phenobarbital (PB) was performed as described previously.[11a] The animals were starved for 18 h prior to sacrifice. Samples of human liver with no pathological lesions were obtained from females and males either for diagnostic purposes or an surgical specimens.

Preparation of S9, Microsomes, and Cytosol——S9, microsomes, and cytosol of various organs from each of the 4 animal species or of liver from surgery patients were prepared as described previously.[12] Pools of tissues from 3—10 animals or 4 individual biopsy samples were used; the liver S9's had protein concentrations of 27—35 mg/ml.[11a,12] Microsomal fractions were washed twice with 1.15% KCl and resuspended again in a volume of cold sterile 1.15% KCl equal to that of the original S9. All hepatic preparations were stored at −80 °C. No difference in mutagenic activity as determined with fresh liver preparations and with those stored for 6 months at −80 °C could be detected. Protein contents in S9, microsomes, and cytosol were determined by the method of Lowry *et al.*[14] using BSA as the standard.

Mutation Assay——Each test was carried out according to the standard method of Ames *et al.*[15] as modified by Nagao *et al.*[16] and Mori *et al.*[17] Tester strains employed were *S. typhimurium* TA100 and TA98. The S9 mixture contained 0.05—0.45 ml of liver preparations, 4 μmol of $MgCl_2$, 16.5 μmol of KCl, 2.5 μmol of glucose 6-phosphate, 0.25 unit of glucose-6-phosphate dehydrogenase, 2 μmol of NADPH, 2 μmol of reduced nicotinamide adenine dinucleotide, 2.5 μmol of adenosine-5′-triphosphate, and 50 μmol of sodium phosphate buffer (pH 7.4).[16] 3′-Me-DAB, 3′-CH_2OH-DAB, and 3′-CH_2OH-MAB (1 μmol in 0.1 ml of dimethyl sulfoxide) and 0.1 ml of bacterial culture were added, and the mixture was preincubated at 37 °C for 1 min with or without 0.2 mg of norharman (0.05 ml in dimethyl sulfoxide).[15] Other test compounds were preincubated for 20 min without norharman.

Chromatography on Sephadex G-100——All procedures were conducted at 4 °C. To purify a soluble protein fraction with enhancement activity, frozen cytosol from uninduced male rats was thawed and dialyzed against 300 volumes of 0.1 M sodium phosphate buffer (pH 7.4) for 24 h. Nine ml of the dialyzed cytosol was loaded onto a 3 × 73-cm Sephadex G-100 column equilibrated with 0.1 M sodium phosphate buffer (pH 7.4) and eluted with the same buffer. Fractions of 8.3 ml were collected at a flow rate of 12.5—20 ml/h, and the elution profile of protein was monitored by measurement of the absorbance at 280 nm; it required about 48 h to obtain the final protein fraction. BSA (MW = 68000), ovalbumin (43000), and cytochrome c (13000) were used as molecular weight standards. Two fractions (16.6 ml) of the eluates were combined and concentrated to 3 ml with membrane filter. The protein fraction (0.3 ml/assay) thus obtained was used for the mutation assay.

Results

A previous study[17] demonstrated a linear dose-dependence of mutagenicity in TA100 for 3′-Me-DAB and 3′-CH_2OH-DAB over the concentration range of 0.05 to 1 μmol per plate. Figure 1 shows the effect of different amounts of S9, microsomes, and cytosol on the mutagenic activities of both aminoazo dyes in strain TA100. A clear dose-dependence on the liver S9 was observed for both aminoazo dyes over the range of 50 to 200 μl per plate. The aminoazo dyes showed no mutagenicity in the presence of either cytosol or microsomes alone,

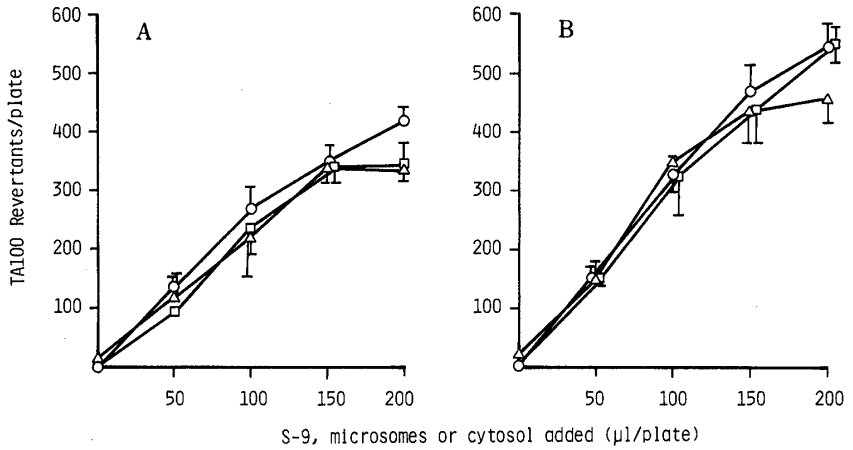

Fig. 1. Effect of Various Amounts of S9, Microsomes, or Cytosol on the Mutagenicity of 3′-Me-DAB (A) and 3′-CH_2OH-DAB (B) in Strain TA100.

Liver S9 and microsomes were obtained from PCB-induced rats and cytosol from uninduced rats. The aminoazo dye (1 μmol) was preincubated with 0.05—0.2 ml of S9 (○) or of microsomes (△) or cytosol (□) in the presence of 0.15 ml of cytosol or microsomes (equivalent to 37.5 mg wet weight of liver), respectively. The number of spontaneous revertants was subtracted. Each point represents the mean ± S.D. (n=2—4).

TABLE I. Effect of Mixing Liver Microsomes and Cytosol from Uninduced Rats and PCB-, 3-MC-, or PB-Induced Rats on the Mutagenicity of 3′-Me-DAB and 3′-CH_2OH-DAB

Liver fraction(s) added	Revertants[a]/plate			
	3′-Me-DAB		3′-CH_2OH-DAB	
	TA98	TA100	TA98	TA100
Uninduced cytosol (experiment 1)	0	0	6± 5	9± 3
+uninduced microsomes	15± 1	7±23	19± 1	13±31
+PB-microsomes	1± 1	0	11± 1	0
+3-MC-microsomes	96± 1	165± 1	144± 1	188±14
+PCB-microsomes	296± 7	324±34	329±27	428±70
PCB-microsomes (experiment 2)	77±36	30± 1	120±39	43±17
+PB-cytosol	303±15	313± 7	349±13	382±28
+3-MC-cytosol	318±10	341± 4	343±14	416± 3
+PCB-cytosol	325±75	330±42	331±54	391±18

a) Spontaneous revertant counts (TA98: 35, TA100: 130) subtracted. The aminoazo dye (1 μmol) was preincubated in the presence of 0.15 ml of liver microsomes and/or 0.15 ml of cytosol (equivalent to 37.5 mg wet weight of liver). Data represent the mean values ± S.D. (n=2—4).

and the activities were dependent on the additions of cytosol as well as microsomes; the addition of cytosol or microsomes (in the presence of microsomes or cytosol, respectively) was approximately equally as effective as that of S9 up to 150 μl in stimulating the mutagenicity of both aminoazo dyes.

To test the idea that a microsomal enzyme rather than an enzyme present in the cytosol is responsible for the mutagenic activation, the effects of induction of rats with PCB, 3-MC, and PB on the activation of 3′-Me-DAB and 3′-CH_2OH-DAB were then examined (Table I). When tested with uninduced cytosol (experiment 1), the number of revertant colonies in the presence of liver microsomes from uninduced or PB-induced rats was less than twice the spontaneous rate in all cases. The mutagenic activities were induced to the highest extent by

TABLE II. Effect of Mixing Liver Microsomes and Cytosol from 4 Animal Species and Man on the Mutagenicity of 3′-Me-DAB and 3′-CH_2OH-DAB in Strain TA100

Liver fraction(s)[a] added	TA100 revertants[b]/plate	
	3′-Me-DAB	3′-CH_2OH-DAB
Rat cytosol (experiment 1)	0	9± 3
+rat microsomes (control)	341± 8 (100)	434±56 (100)
+mouse microsomes	37± 5 (11)	58± 8 (13)
+hamster microsomes	47±32 (14)	61±16 (14)
+rabbit microsomes	13±15 (4)	1±14 (0)
+female rat microsomes	236± 2 (69)	383±13 (88)
Rat microsomes (experiment 2)	77±36	43±17
+mouse cytosol	342±27 (100)	481±70 (111)
+hamster cytosol	347±13 (102)	472±71 (109)
+rabbit cytosol	109±32 (32)	402±36 (93)
+female rat cytosol	338±15 (99)	489±34 (113)
+human cytosol (4y, male)	115± 4 (34)	193±27 (44)
+human cytosol (34y, male)	116± 2 (34)	166±16 (38)
+human cytosol (34y, female)	92±11 (27)	226±30 (52)
+human cytosol (37y, female)	102±11 (30)	214±15 (49)

a) Liver microsomes (0.15 ml) from rats, mice, and hamsters were obtained from PCB-induced animals and other liver preparations (0.15 ml) from uninduced animals.
b) Spontaneous revertant counts (130) subtracted. Data represent the mean values±S.D. ($n=2$—7) and values in parentheses are percentages of the control.

PCB followed by 3-MC in both strains. On the other hand, when tested with PCB-induced microsomes (experiment 2), cytosol derived from PB, 3-MC, or PCB-induced rats produced approximately as much mutagenic activities with both aminoazo dyes as did uninduced cytosol in both strains. 3′-Me-DAB and 3′-CH_2OH-DAB showed weak mutagenicity in strain TA98 in the presence of PCB-induced microsomes alone. The cytosol from kidney of uninduced rats was approximately equally as effective as that from the liver in stimulating the mutagenicity of 3′-Me-DAB and 3′-CH_2OH-DAB, whereas the cytosols from lung, brain, heart, and small intestine were not effective.

Table II summarizes the number of revertants obtained in strain TA100 after activation of 3′-Me-DAB and 3′-CH_2OH-DAB by liver preparations from various animal species and man. When tested with rat liver cytosol (experiment 1), the aminoazo dyes showed negative mutagenicity in the presence of microsomes from mice, hamsters, or rabbits; human microsomes were also ineffective for the activation of aminoazo dyes (data not shown). Only liver microsomes from male and female rats markedly activated the aminoazo dyes to mutagens. The mutagenic activity of 3′-Me-DAB in the presence of liver microsomes from female rats was significantly ($p<0.01$) lower than that obtained with liver microsomes from the male; the activity of 3′-CH_2OH-DAB was at the same level in both cases. The cytosol derived from female rat, mouse, hamster, or rabbit liver was approximately equally as effective as that derived from male rat liver in stimulating the mutagenicity of 3′-Me-DAB and 3′-CH_2OH-DAB in the presence of rat liver microsomes (experiment 2), except for the enhancement of 3′-Me-DAB mutagenicity by rabbit cytosol. The number of revertant colonies in the presence of rabbit cytosol was not greater than twice the spontaneous rate with 3′-Me-DAB. As in the case of rabbit cytosol, human cytosol had an effect on the activation of 3′-CH_2OH-DAB, although the activity was lower than that found with the animal cytosols.

Some of the properties of the cytosolic component(s) are shown in Table III.

TABLE III. Effect of Temperature on the Stability of Mutagenesis-Enhancing Activity in the Cytosol

Liver fraction(s)[a] added (conditions)	TA100 revertants[b]/plate	
	3′-Me-DAB	3′-CH_2OH-DAB
Microsomes	45±4	60±40
+cytosol (control)	330±15 (100)	421±51 (100)
+cytosol (37 °C, 1 h)	308±35 (93)	419±51 (96)
+cytosol (40 °C, 15 min)	295±35 (89)	398±68 (95)
+cytosol (50 °C, 15 min)	191±4 (58)	332±53 (79)
+cytosol (55 °C, 15 min)[c]	17±22 (5)	36±18 (9)
+cytosol (4 °C, 24 h)	283±28 (86)	414±35 (98)
+cytosol (4 °C, 72 h)	281±6 (85)	390±57 (93)
+dialyzed cytosol (4 °C, 72 h)	218±34 (66)	286±26 (68)
+0.3 ml dialyzate (4 °C, 24 h)	40±32 (12)	32±20 (8)
+4.1 mg BSA	49±31 (15)	54±39 (13)

a) Liver microsomes were obtained from PCB-treated rats and cytosol from uninduced rats; 150 μl of each fraction, corresponding to 4.1 mg of cytosolic protein, was added unless otherwise indicated.
b) Spontaneous revertant counts (130) subtracted. Data represent the mean values±S.D. (n=2—8) and values in parentheses are percentages of the control.
c) When the cytosol was heated to 55 °C for 15 min, the resulting precipitates were removed by centrifugation prior to the Ames test.

Enhancement factor(s) for microsome-mediated mutagenesis from 3′-Me-DAB and 3′-CH_2OH-DAB were essentially unaffected by maintaining the cytosol either at 37 °C for 1 h or at 40 °C for 15 min. However, it should be noted that the cytosolic enhancement activity was labile; heating the cytosol at 50 °C for 15 min caused loss of 42% of the enhancement activity for 3′-Me-DAB, and the enhancement activity for both 3′-Me-DAB and 3′-CH_2OH-DAB was completely lost after heating to 55 °C for 15 min. About 24% of the activity for both aminoazo dyes was lost during storage at −80 °C for 12 months (data not shown). Dialysis of the cytosol against phosphate buffer for 24 h at 4 °C caused loss of about 30% of the enhancement activity for both aminoazo dyes, whereas essentially all of the enhancement activity was retained with the cytosol upon maintaining the cytosol at 4 °C for up to 72 h. The decrease of activity during dialysis was not restored by addition of 1 mM 2-mercaptoethanol to phosphate buffer. On the other hand, when 0.3 ml (all of the fraction) of the dialyzate or 4.1 mg of BSA instead of the cytosol was added to the preincubation mixture, no mutagenic activity was observed with either 3′-Me-DAB or 3′-CH_2OH-DAB.

The mutagenic activities of both 3′-Me-DAB and 3′-CH_2OH-DAB (Tables I—III) are rather weak for testing the activation of both aminoazo dyes to mutagens by cytosolic fractions after Sephadex G-100 chromatography. Thus, the use of a comutagen, norharman, was then examined; when 0.15 ml of liver S9 from PCB-induced rats was used for metabolic activation, the activities of 3′-Me-DAB and 3′-CH_2OH-DAB in strain TA98 were increased 5.5- and 12.5-fold, respectively. Figure 2 shows the effect of different amounts of the cytosol and dialyzed cytosol on the mutagenic activities of both aminoazo dyes in the presence of norharman and liver microsomes from PCB-induced rats. Norharman had a comutagenic effect on both aminoazo dyes in the presence of microsomes alone; the activities of 3′-Me-DAB and 3′-CH_2OH-DAB were enhanced 6.9- and 4.1-fold, respectively, by norharman. However, the mutagenic activities were greatly enhanced by addition of the cytosol, like those observed without norharman (Fig. 1). A clear dose-dependence on the cytosol was observed for both aminoazo dyes over the range of 0.05 to 0.15 ml per plate. When the cytosol was used after dialysis for 24 h followed by holding at 4 °C for 48 h, approximately half of the

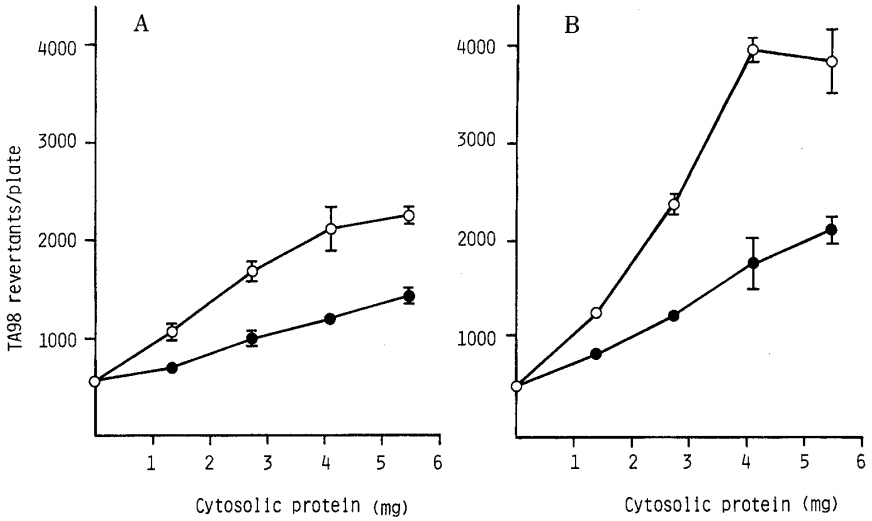

Fig. 2. Enhancement of Microsome-Mediated 3′-Me-DAB (A) and 3′-CH_2OH-DAB (B) Mutagenesis in the Presence of Norharman by Cytosol (○) or Dialyzed Cytosol (●)

Liver microsomes from PCB-induced rats and cytosol from uninduced rats were used. The cytosol was dialyzed at 4 °C for 24 h and used as a dialyzed cytosol after storage at 4 °C for 48 h. TA98 His^+ revertants per plate were determined with 0.05, 0.1, 0.15, and 0.2 ml of cytosol or dialyzed cytosol (expressed as mg cytosolic protein/plate) in the presence of 0.15 ml of microsomes. Each point represents the mean ± S.D. ($n=2$—4).

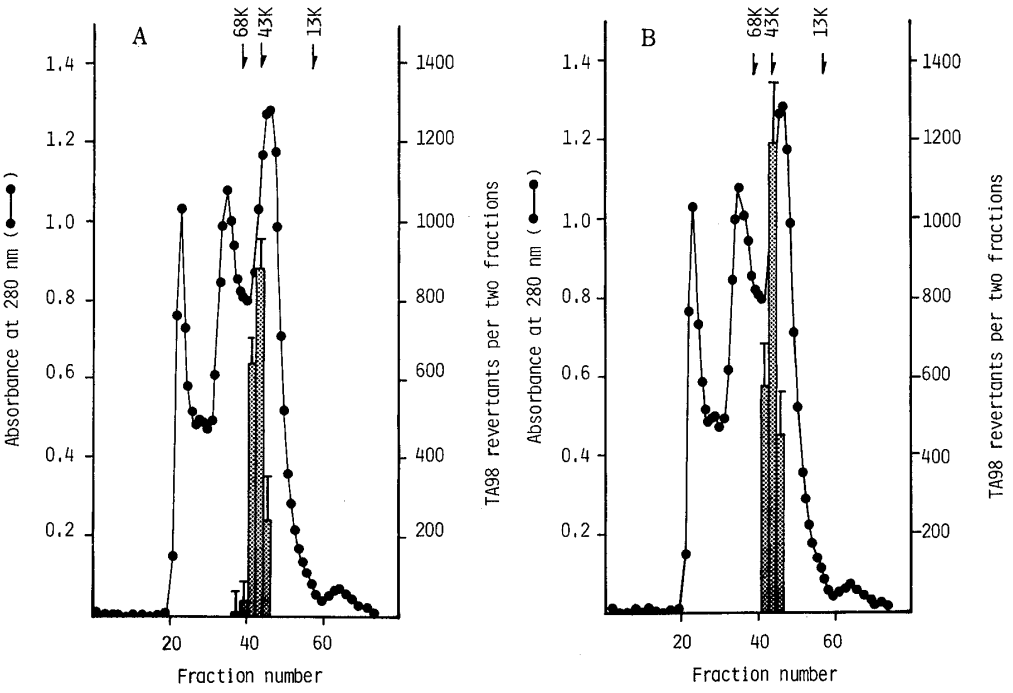

Fig. 3. Purification of Enhancing Activity in Rat Liver Cytosol by Sephadex G-100 Column Chromatography

The procedure for mutation assay was the same as in Fig. 2, except for the use of eluted fraction (1.37 mg protein/plate) instead of cytosol. The numbers of TA98 revertants with 3′-Me-DAB (A) and 3′-CH_2OH-DAB (B) in the presence of microsomes and norharman (Fig. 2) were subtracted. Mutation data (▨) represent the mean values ± S.D. ($n=2$—3). BSA (68000), ovalbumin (43000), and cytochrome c (13000) were used as molecular weight standards.

TABLE IV. Effect of the Fractionated Cytosol on Hepatic Microsome-Mediated Mutagenesis by Several Mutagens or/and Carcinogens

Liver fraction(s)[a] added	Revertants[b]/plate				
	3′-CH_2OH-MAB on TA98	OAT on TA100	3-MC on TA100	BP on TA98	DMN on TA100
S9	8763±644	1672± 85	2302±429	867±130	926± 38
Cytosol	0	32± 5	11± 13	7± 11	—
Microsomes	2926±322	431±183	900±137	192± 42	259± 68
+cytosol	8619±961	1717± 35	2118±299	791±162	910±133
+dialyzed cytosol (4 °C, 72 h)	6630±822	1416±150	1814±441	454± 55	677± 70
+1.37 mg fractionated cytosol	7495±721	964± 23	1099±163	239± 29	259± 43
+4.1 mg BSA	2500±335	308± 43	—	—	—

a) Liver S9 and microsomes were obtained from PCB-induced rats, except for the case of DMN (from PCB-induced hamsters), and cytosol from untreated rats.
b) Spontaneous revertant counts (TA98: 35, TA100: 130) subtracted. OAT (0.25 μmol), 3-MC (0.1 μmol), BP (0.02 μmol) or DMN (135 μmol) was preincubated with liver preparations for 20 min, and 3′-CH_2OH-DAB (1 μmol) was preincubated for 1 min in the presence of norharman. Data represent the mean values±S.D. (n=2—7).

enhancement activity at 4.1 mg protein per plate was lost, as in the case of the results obtained without norharman (Table III). A clear cytosolic protein-dependence was also observed for both aminoazo dyes over the concentration range of 1.4 to 5.5 mg per plate. A typical elution profile of protein in the dialyzed cytosol from a Sephadex G-100 column and the mutagenic activities of both aminoazo dyes in the presence of the fractionated cytosol are shown in Fig. 3. The mutagenic activities were detected only in the presence of protein fractions between 41 to 46, and the eluate fraction of 357 to 365 ml (fraction No. 43—44) contained the greatest activity with both aminoazo dyes; this purification was approximately 2.7- and 3.5-fold for 3′-Me-DAB and 3′-CH_2OH-DAB, respectively, based on the data of Fig. 2. Consequently, the molecular weight of the protein fraction with the greatest mutagenicity enhancement activity was determined to be 43000—47500 by chromatography with Sephadex G-100 using three standard proteins, BSA, ovalbumin, and cytochrome c.

To further explore the basis for the mutagenesis enhancement shown in Fig. 3, the experiment summarized in Table IV was conducted; mutagenesis by these mutagens or/and carcinogens is known to be enhanced by cytosolic components in the *Salmonella* assay system.[8b-10] As shown in Table IV, all the mutagens showed little or no mutagenicity in the presence of either cytosol or microsomes alone when compared with the activities observed with S9, whereas in the presence of the combined fractions of microsomes and cytosol the activities were restored. When the dialyzed cytosol was used for the assay, the enhancement activity with all the mutagens was slightly lower compared to that of the cytosol, as in the case of the results obtained with 3′-Me-DAB and 3′-CH_2OH-DAB (Table III and Fig. 2). The protein preparation from Sephadex G-100 chromatography (Fig. 3) enhanced microsome-mediated mutagenesis from 3′-CH_2OH-MAB and OAT, but did not enhance that from 3-MC, BP, and DMN.

Sulfotransferase and SOD in rat liver cytosol are known to enhance the microsome-mediated mutagenesis from 7,12-dimethylbenz[*a*]anthracene[18] and several amines.[19] The effects of sulfotransferase inhibitors,[20] sulfatase, and SOD on the mutagenicity of 3′-Me-DAB and 3′-CH_2OH-DAB were then examined. The aminoazo dye was preincubated with liver S9 from PCB-induced rats in the presence of 100 units of *Helix pomatia* sulfatase,[18] 0.25 mM *o*-iodosobenzoate, or 1.0 mM *p*-nitrophenol; these showed no inhibitory effect on the mutagenic activities of the aminoazo dyes. The rat liver cytosol contains 4480 units of SOD

per g liver,[21] therefore, 168 units of SOD will be present in 0.15 ml of liver S9. The aminoazo dye was preincubated with liver microsomes from PCB-induced rats in the presence of 56, 113, 168, and 281 units of bovine blood SOD or human liver SOD.[22] However, no enhancement activity was seen for the microsome-mediated mutagenesis by 3′-Me-DAB or 3′-CH_2OH-DAB, even in the presence of norharman.[19]

Discussion

We have reported previously that rat liver cytosol enhances microsome-mediated bacterial mutagenesis by 4 aminoazo dyes,[11] and specificity in regard to species, sex, and organ has also been observed in the mutagenic effects of 3′-Me-DAB and 3′-CH_2OH-DAB in the presence of S9 fraction.[12] The data shown in this communication confirm and expand upon these findings. Only liver microsomes from PCB- or 3-MC-induced rat could activate the aminoazo dyes to mutagens in the presence of liver cytosol (Tables I and II), whereas no species difference was observed in the mutagenicity-enhancing effects of cytosol, except for the absence of 3′-Me-DAB mutagenesis enhancement by rabbit liver cytosol (Table II). Human liver cytosol also had enhancement activity for mutagenesis by 3′-CH_2OH-DAB, although 3′-CH_2OH-DAB mutagenicity was not activated by human liver S9.[12] The cytosolic enhancement activity was not induced by PCB, PB, or 3-MC (Table I), and was present in a protein fraction obtained by Sephadex G-100 chromatography from the dialyzed cytosol (Fig. 3).

The soluble protein fraction is known to inhibit lipid peroxidation under some conditions, as is ethylenediaminetetraacetic acid.[23,24] When BSA was added instead of cytosol, no enhancing activity was observed (Table III), and moreover, the soluble protein fraction could not be replaced by addition of 1 mM ethylenediaminetetraacetic acid to the preincubation mixture (data not shown) as regards the mutagenicity enhancement. Accordingly, these results appear to exclude the possibility that the cytosolic enhancement results simply from nonspecific protection of microsomes from degradative processes.

Watabe *et al.*[18] reported that 7-hydroxymethyl-12-methylbenz[*a*]anthracene was transformed by liver cytosolic sulfotransferase to the 7-hydroxymethylsulfate ester, which is mutagenic toward strain TA98 without metabolic activation. The activation of carcinogenic aminoazo dyes such as DAB and 3′-Me-DAB to ultimate forms by rat liver is considered to require three metabolic reactions; N-demethylation and N-hydroxylation of the amino group, followed by sulfate conjugation.[20,25] Furthermore, Degawa *et al.*[26] reported that the acyl esters of N-hydroxylated aminoazo dyes (model compounds for reactive sulfates) are direct mutagens in *Salmonella*. However, sulfotransferase inhibitors and sulfatase showed no inhibitory effects on the mutagenic activation of 3′-Me-DAB or 3′-CH_2OH-DAB. The molecular weight of aryl sulfotransferase in rat liver cytosol is reported to be 61000 to 68000,[27] excluding the possibility that the enhancement in rat liver cytosol is due to sulfotransferase. Similarly, the cytosolic factor required for 3′-Me-DAB and 3′-CH_2OH-DAB mutagenesis could not be replaced by SOD.[19]

Earlier work has shown that ligandin,[28] h2-5S,[29] and protein A[28,30] bind 3′-Me-DAB and its metabolites with high affinity and/or covalently. It is also known that binding of 3′-Me-DAB or its metabolites in rat liver cytosol occurs mainly to 4 proteins in addition to ligandin, and the cytosol protein with a molecular weight of 47000 exhibits the highest binding of 3′-Me-DAB.[31] Tierney *et al.*[32] described a protein with a molecular weight of 46000 in rat liver cytosol which is involved in translocation of 3-MC from the cytoplasm to the nucleus. Since glutathione-S-transferases play a role in carcinogenic azo dye transport in the cytoplasm and even in the nucleus,[33] the extensive binding of 3′-Me-DAB, 3′-CH_2OH-DAB, and its metabolites to these enzymes may constitute a step in the process of tumor induction, and

possibly also in the process of mutagenesis by aminoazo dyes in *Salmonella*. In this context, further detailed studies are required. Nevertheless, the present results show that a soluble protein with a molecular weight of 43000—47500, which appears to be present in liver cytosols from 4 animal species and man, is involved in the mutagenic activation of 3′-Me-DAB and 3′-CH_2OH-DAB. It should be noted that the mutagenesis enhancement by this soluble protein fraction showed specificity for aminoazo dyes; this protein enhanced microsome-mediated mutagenesis by 3′-CH_2OH-MAB and OAT but not that from carcinogenic polycyclic hydrocarbons and *N*-nitrosamine (Table IV). Although the mechanism whereby the soluble protein fraction enhances the mutagenicity of aminoazo dyes is not yet known, the soluble protein may be similar to the cytosol factor detected in rat liver by Degawa *et al.*[10]; they showed that the factor could enhance bacterial mutagenesis by *N*-hydroxy-OAT. This may differ from the cytosolic protein fraction with *N*-hydroxy-2-aminofluorene[8b] and DMN[9] mutagenicity-enhancing activity, since this activity is unaffected by heating at 60 °C for 10 min and one fraction was assigned a molecular weight of 33500 on the basis of gel filtration through Sephadex G-100.[8a] It was also reported that this protein fraction does not enhance microsome-mediated mutagenesis by aflatoxin B_1.[8b] These results indicate that mutagenesis enhancement by the soluble protein fraction may proceed by a variety of mechanisms which could include effects on microsomal enzymes, effects on solubility and/or stability of metabolites, and enhanced migration of active compounds across bacterial membranes.

References and Notes

1) This constitutes Part XXI of a series entitled "Carcinogenic Azo Dyes," Part XX: Y. Mori, T. Niwa, and K. Toyoshi, *Carcinogenesis*, **4**, 1487 (1983).
2) J. McCann, E. Choi, E. Yamasaki, and B. N. Ames, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 5135 (1975).
3) I. F. H. Purchase, E. Longstaff, J. Ashby, J. A. Styles, D. Anderson, P. A. Lefevre, and F. R. Westwood, *Nature* (London), **264**, 624 (1976).
4) T. Sugimura, S. Sato, M. Nagao, T. Yahagi, T. Matsushima, Y. Seino, M. Takeuchi, and T. Kawachi, "Fundamentals in Cancer Prevention," ed. by P. N. Magee, S. Takayama, T. Sugimura, and T. Matsushima, University of Tokyo Press, Tokyo, 1976, pp. 199—215.
5) S. J. Rinkus and M. S. Legator, *Cancer Res.*, **39**, 3289 (1979).
6) Y. Hashimoto, H. K. Watanabe, and M. Degawa, *Gann*, **72**, 921 (1981).
7) K. Ishii, Y. Yamazoe, T. Kamataki, and R. Kato, *Cancer Res.*, **40**, 2596 (1980).
8) *a*) G. T. P. Saccone, B. R. DasGupta, and M. W. Pariza, *Cancer Res.*, **41**, 4600 (1981); *b*) G. T. P. Saccone and M. W. Pariza, *Mutation Res.*, **88**, 135 (1981).
9) M. J. Prival and V. D. Michell, *Cancer Res.*, **41**, 4361 (1981).
10) M. Degawa, C. Kanazawa, and Y. Hashimoto, *Carcinogenesis*, **3**, 1113 (1982).
11) *a*) Y. Mori, T. Niwa, and K. Toyoshi, *Gann*, **74**, 483 (1983); *b*) *Idem, Mutat. Res.*, **122**, 13 (1983).
12) Y. Mori, T. Niwa, K. Toyoshi, K. Hirano, and M. Sugiura, *Mutat. Res.*, **121**, 95 (1983).
13) S. Baba, Y. Mori, and K. Toyoshi, *Yakugaku Zasshi*, **92**, 1364 (1972); Y. Mori, T. Hori, and K. Toyoshi, *Gann*, **69**, 757 (1978).
14) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
15) B. N. Ames, J. McCann, and E. Yamasaki, *Mutat. Res.*, **31**, 347 (1975).
16) M. Nagao, T. Yahagi, M. Honda, Y. Seino, T. Kawachi, and T. Sugimura, *Cancer Lett.*, **3**, 339 (1977).
17) Y. Mori, T. Niwa, T. Hori, and K. Toyoshi, *Carcinogenesis*, **1**, 121 (1980).
18) T. Watabe, T. Ishizuka, M. Isobe, and N. Ozawa, *Science*, **215**, 403 (1982).
19) M. Nagao, M. Ochiai, K. Wakabayashi, and T. Sugimura, "Proceedings of the Japanese Cancer Association," Japanese Cancer Association, Tokyo, 1982, p. 67.
20) F. F. Kadlubar, J. A. Miller, and E. C. Miller, *Cancer Res.*, **36**, 2350 (1976).
21) C. Peeters-Joris, A. M. Vandevoorde, and P. Baudhuin, *Biochem. J.*, **150**, 31 (1975).
22) B. J. Carrico and H. F. Deutsch, *J. Biol. Chem.*, **244**, 6087 (1969).
23) T. J. Player and A. A. Horton, *FEBS Lett.*, **89**, 103 (1978); T. Kamataki, N. Ozawa, M. Kitada, H. Kitagawa, and R. Sato, *Biochem. Pharmacol.*, **23**, 2485 (1974); A. N. Kotake, L. B. Deloria, V. S. Abbott, and G. J. Mannering, *Biochem. Biophys. Res. Commun.*, **63**, 209 (1975).
24) M. Sato, Y. Amikura, S. Suzuki, and T. Murata, *Yakugaku Zasshi*, **9**, 1152 (1978).

25) *a*) A. H. Conney, R. R. Brown, J. A. Miller, and E. C. Miller, *Cancer Res.*, **17**, 628 (1957); *b*) F. F. Kadlubar, J. A. Miller, and E. C. Miller, *ibid.*, **36**, 1196 (1976).
26) M. Degawa, S. Miyairi, and Y. Hashimoto, *Gann*, **69**, 367 (1978).
27) S. G. Wu and K. D. Straub, *J. Biol. Chem.*, **251**, 6529 (1976); R. D. Sekura and W. B. Jakoby, *Arch. Biochem. Biophys.*, **211**, 352 (1981).
28) B. Ketterer and E. Tipping, "Conjugation Reactions in Drug Biotransformation," ed. by A. Aitio, Amsterdam Elsevier/North Holland Biomedical Press, 1978, pp. 91—100.
29) S. Sorof, B. P. Sani, V. W. Kish, and H. P. Meloche, *Biochemistry*, **13**, 2612 (1977); S. Sorof and E. M. Young, *Cancer Res.*, **33**, 2010 (1974).
30) B. Ketterer, E. Tipping, D. Beale, and J. A. T. P. Meuwissen, "Metabolism and Function," Vol. 6, Raven Press, New York, 1976, pp. 317—338.
31) N. Ohmi, M. Bhargava, and I. M. Arias, *Cancer Res.*, **41**, 3461 (1981).
32) B. Tierney, D. Weaver, N. H. Heintz, W. I. Schaeffer, and E. Brensnick, *Arch. Biochem. Biophys.*, **200**, 513 (1980).
33) G. A. Bannikov, V. I. Guelstein, and T. A. Tckipysheva, *Int. J. Cancer*, **11**, 398 (1973).