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REDUCTION OF *N*-HYDROXY-2-ACETYLAMINOFLUORENE BY LIVER MICROSOMES

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Abstract—The reduction of *N*-hydroxy-2-acetylaminofluorene(*N*-hydroxy-AAF) to 2-acetylaminofluorene and 2-aminofluorene by liver microsomes was studied. The reductase preferred NADPH rather than NADH as a cofactor and was strongly inhibited by carbon monoxide and oxygen. Further, the inhibitors of hepatic mixed function oxidase effectively inhibited the reductase activity. Pretreatments of rats with 3-methylcholanthrene and PCB markedly induced the reductase activity. From these results it was suggested that the reduction of *N*-hydroxy-AAF was catalysed by cytochrome P-450, especially by cytochrome P-448. The reductase activity was also detected in microsomes from lung, kidney and small intestinal mucosa, and the rates of the reduction were about 6, 7 and 27 per cent, respectively, of that in liver microsomes. Species difference in the reductase activity of liver microsomes was also examined. The highest activity of the reductase was found in hamsters, followed by guinea pigs and rabbits.

A wide variety of carcinogenic and mutagenic compounds have been well recognized as exerting their toxic effects after undergoing metabolic activation. The metabolites thus formed are not believed to remain in the body for an appreciable period since there are some other mechanisms involved in the detoxication of the active metabolites. Among the numerous metabolic reactions, the *N*-hydroxylation reaction is a common reaction which activates carcinogenic aromatic amine compounds. Regarding the detoxication of the active metabolite of 2-acetylaminofluorene (AAF), *N*-hydroxy-2-acetylaminofluorene (*N*-hydroxy-AAF), reduction of this metabolite to form the parent compound by an enzyme(s) present in the liver of experimental animals has been known.

Lotlikar *et al.* [1] and Grantham *et al.* [2] reported that *N*-hydroxy-AAF was reduced to AAF and 2-aminofluorene (AF) by liver homogenates in an atmosphere of nitrogen. However, no reports are available describing the enzyme system mediating the reduction of *N*-hydroxy-AAF. We have reported that the reduction of tertiary amine *N*-oxides [3, 4] and benzo[*a*]pyrene 4,5-oxide [5, 6] were catalysed by microsomal cytochrome P-450. In this report, we characterized the enzymatic mechanism which catalysed the reduction of *N*-hydroxy-AAF.

MATERIALS AND METHODS

Animals and treatments. Unless otherwise stated, male rats of the Sprague–Dawley strain (6–8 weeks old) were used. When necessary, the animals were treated intraperitoneally (i.p.) with phenobarbital (PB), in saline, or with 3-methylcholanthrene (MC), dissolved in corn oil, for three successive days at a daily dose of 80 and 40 mg/kg, respectively. A commercial polychlorinated biphenyl (PCB) mixture, KC-500, dissolved in corn oil, was given to rats at a dose of 500 mg/kg i.p., seven days before the rats were killed. Adult male New Zealand white rabbits, Hartley guinea pigs, Syrian golden hamsters and ICR mice were also used. All animals were fasted for approximately 18 hr before being killed.

Preparation of microsomes. The animals were stunned by a blow on the head and decapitated. The livers were immediately perfused with 1.15% potassium chloride solution from inferior vena cava to portal vein according to the method previously described [7]. Liver microsomes obtained after sequential centrifugation at 9000 *g* for 20 min and 105,000 *g* for 1 hr were washed once by resuspension with 1.15% potassium chloride solution and recentrifugation. Pulmonary microsomes were prepared according to the method described by Capdevila *et al.* [8]. Intestinal mucosa microsomes were prepared by the method of Hoensch *et al.* [9].

Assay methods. The incubation mixture for determination of *N*-hydroxy-AAF-induced NADPH oxidation rate contained 3 mg protein of liver microsomes and 150 μmoles of Na,K-phosphate (pH 7.4) in a final volume of 3.0 ml. The mixture was bubbled with oxygen-free nitrogen for 5 min, followed by addition of NADPH (0.3 μmole) and *N*-hydroxy-AAF (3 μmoles). The NADPH oxidation rate was measured by recording the decrease of absorption at 340 nm using a glass cuvette sealed with a rubber cup. When necessary, carbon monoxide was bubbled through the mixture for 30 sec after introducing the nitrogen gas. NADPH oxidation rate was calculated using a molar extinction coefficient of 6.2 mM^{-1} cm^{-1}. The incubation mixture for the assay of the reductive reaction of *N*-hydroxy-AAF to AAF and aminofluorene (AF) contained 1 mg protein of microsomes, 0.8 μmole of NADP, 8 μmoles of glucose 6-phosphate, 1 I.U. of glucose 6-phosphate dehydrogenase, 6 μmoles of $MgCl_2$ and 50 μmoles of Na,K-phosphate (pH 7.4) in a final volume of 1.0 ml.

The incubation tube was sealed with a rubber serum cup and the mixture was bubbled through a needle with oxygen-free nitrogen gas for 5 min. NADPH generating system was added after bubbling with the nitrogen gas. The reaction was started by addition of *N*-hydroxy-AAF (1 μmole in 20 μl of acetone) after preincubation at 37° for 2 min. Incubations were carried out at 37° for 10 min with appropriate shaking and terminated by addition of 1.5 N potassium hydroxide (0.5 ml). Diethylether (5.0 ml) and internal standard solution (0.1 ml) containing 100 nmoles of phenacetin were added to the mixture and shaken for 5 min. A portion (4.0 ml) of the organic layer was transferred to another tube and then evaporated to dryness under a current of nitrogen gas. The residue was resolved in 100 μl of acetonitrile and an aliquot (15 μl) was subjected to high performance liquid chromatography (h.p.l.c.). For the h.p.l.c. analysis reported here, a Waters GPC/ALC model 204 liquid chromatograph equipped with μBondapak C_{18} column (30 cm × 4 mm) and u.v. detector model 440 was used. A precolumn (2.5 cm × 4 mm) containing μBondapak C_{18} was also used to prevent deterioration of the separation column. A mobile phase of acetonitrile–water (50:50) was utilized at a flow rate of 1.2 ml/min. AAF, AF and phenacetin were eluted from the column with retention times of 5.2, 6.5 and 3.0 min, respectively. The samples were injected to the column using WISP model 710 automatic sampler and the chromatogram was recorded with a dual pen recorder (Nippon Denshi Kagaku, model V-225M).

Mass spectrometry. The mass spectrum of *N*-hydroxy-AAF was obtained by direct probe insertion of the sample into JEOL JMS-D 300 instrument (electron energy 30 eV, ionizing current 250 μA, ion source pressure 2×10^{-7} Torr., ion source temperature 250°). Mass spectra of AAF, AF and *N*-chloro-*N*-trifluoroacetylaminofluorene were obtained by gas chromatography–mass spectrometry using the same instrument equipped with JEOL 20K gas chromatograph fitted with a glass column (1 m × 2 mm i.d.) of 3% OV-1 on chromosorb W HP(80/100 mesh) at 205° with helium as the carrier gas (30 ml/min). The instrument was operated in the e.i. mode (electron energy 25 eV).

Chemicals. NADH, NADPH, NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast, Tokyo, Japan. Glucose oxidase was obtained from Sigma Chemical Co., St. Louis, MO. AAF, 2-nitrofluorene, nitrosobenzene and *n*-octylamine were from Tokyo Kasei Kogyo, Tokyo, Japan and α-naphthoflavone (ANF) and phenacetin were from Wako Pure Chemicals, Osaka, Japan. AF and methylviologen (*NN'*-dimethyl-r,r'-dipyridium dichloride) were purchased from Nakarai Chemicals, Kyoto, Japan. DPEA (2,4-dichloro-6-phenyl-phenoxyethylamine), tiaramide *N*-oxide (4-{[5-chloro-2-oxo-3(2H)-benzothiazolyl]-acetyl}-1-piperazine ethanol-l-oxide) and SKF 525-A were generous gifts from Eli Lilly Research Laboratories (Indianapolis, IN), Fujisawa Research Laboratories (Osaka, Japan) and Smith Kline & French Laboratories (Philadelphia, PA), respectively. *N*-Hydroxy-AAF was synthesized from nitrofluorene by the modification of the method described by Hinson *et al.* [10] for the synthesis of *N*-hydroxyphenacetin. The mass spectrum of *N*-chloro-*N*-trifluoroacetyl-derivative of *N*-hydroxy-AAF was identical with the spectrum reported previously [11]. *N*-Hydroxy-AAF was recrystallized sequentially from acetone and benzene before use.

RESULTS

Effects of N-*hydroxy-AAF on the rate of NADPH oxidation.* To know if NADPH can be an electron donor required for the reduction of *N*-hydroxy-AAF, the effect of *N*-hydroxy-AAF on the rate of NADPH-oxidation was measured under an anaerobic atmosphere. NADPH was oxidized at a slow rate in the absence of the substrate while the addition of the substrate brought about an increased oxidation rate of NADPH. It has been reported that reductive reactions such as those from *p*-nitrobenzoate to *p*-aminobenzoate [12], from benzo[*a*]pyrene 4,5-oxide to benzo[*a*]pyrene [5, 6] and from tiaramide *N*-oxide to tiaramide [3, 4] are catalysed by cytochrome P-450. To look into the possibility of whether *N*-hydroxy-AAF is also reduced by cytochrome P-450, the effect of carbon monoxide on the rate of NADPH oxidation induced by *N*-hydroxy-AAF was measured. As shown in Fig. 1, *N*-hydroxy-AAF-induced NADPH oxidation was effectively inhibited by carbon monoxide. The inhibition was about 67 per cent.

Isolation and identification of reductive metabolites of N-*hydroxy-AAF.* The reductive metabolites of *N*-hydroxy-AAF were separated by h.p.l.c. as described in Materials and Methods. The elution profile of the metabolites is shown in Fig. 2. Two metabolite peaks formed during anaerobic incubation (p-1, p-2) were identified as AAF and AF, respectively, using gas chromatography–mass spectrometry (data not shown). The formation of AAF and AF have also been observed previously, utilizing liver homogenates as the enzyme source [1, 2]. AF is assumed to be formed mainly by deacetylation of AAF. Thus, in some cases the activity of microsomal *N*-hydroxy-AAF reductase was represented by the sum of these metabolite formation reactions. The properties of microsomal *N*-hydroxy-AAF reductase were examined (Table 1). In the presence of

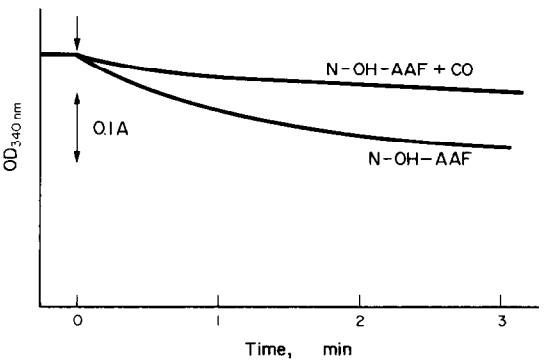

Fig. 1. Effect of carbon monoxide on the rate of *N*-hydroxy-AAF-induced NADPH oxidation. The reaction was started by addition of *N*-hydroxy-AAF at the time indicated. Other experimental conditions were as described in Materials and Methods.

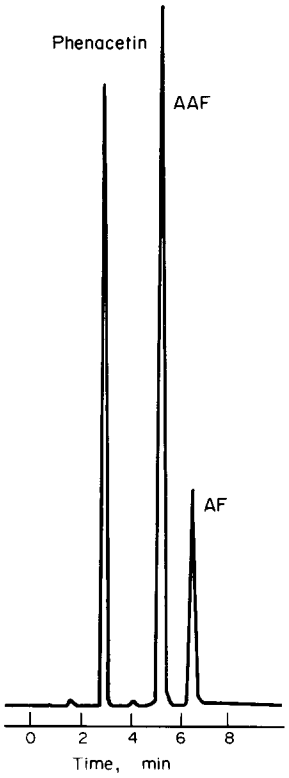

Fig. 2. Separation by high performance liquid chromatography of AAF, AF and phenacetin (internal standard). The peaks were detected by their absorbance at 280 nm.

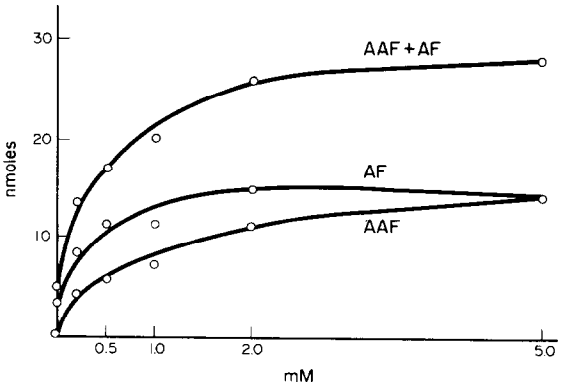

Fig. 3. Effect of methylviologen on the rate of AAF and AF formations as a function of methylviologen concentrations. The experimental conditions were as described in Materials and Methods, except that various amounts of methylviologen were added to the incubation mixture. Each value is the mean of duplicate determinations.

NADPH-generating system, *N*-hydroxy-AAF was reduced to AAF and AF at a rate of 9.73 nmoles/mg/10 min. No detectable reduction was observed in the absence of microsomes, and the activity was decreased to only 2 per cent of control when microsomes were heated at 70° for 5 min prior to addition to the incubation mixture. NADH (1 mM) was about 35 per cent as effective as NADPH in supporting the reductase activity. Further, the addition of NADH to the incubation mixture containing NADPH-generating system did not show any synergistic effects on the reductase activity. To obtain higher anaerobicity in the incubation medium, glucose and glucose oxidase were added. The addition of these reagents, however, did not lead to further increase in the reductase activity. In accordance with the results shown in Fig. 1, the reductase activity as measured by the product formation was also inhibited markedly by carbon monoxide. The decrease in the activity was also seen when incubations were carried out aerobically, in accord with the results observed by Lotlikar *et al.* [1] with liver homogenates. The effects of inhibitors of the drug metabolizing enzymes on the *N*-hydroxy-AAF reductase activity were investigated (Table 2). DPEA and *n*-octylamine, which are known to exhibit type II difference spectra when bound to cytochrome P-450, inhibited the reductase activity while SKF 525-A, a well known type I compound, inhibited less effectively. α-Naphthoflavone, a widely used specific inhibitor of cytochrome P-448, slightly enhanced the activity. It is of interest that the reductase activity was almost completely inhibited by tiaramide *N*-oxide, which is reduced by cytochrome P-450 as

Table 1. Properties of microsomal *N*-hydroxy-AAF reductase*

Gas phase	Addition	Reductase activity: AAF plus AF formed (nmole/mg protein/10 min)	Reductase activity: %
N_2	Microsomes plus NADPH† (control)	9.73	100
	Heat-treated microsomes‡ plus NADPH	0.19	2.0
	Microsomes plus NADH (1 mM)	3.38	34.7
	Microsomes plus NADPH and NADH (1 mM)	8.18	84.1
	Microsomes plus NADPH and glucose–glucose oxidase system§	8.25	84.8
CO	Microsomes plus NADPH	0.90	9.3
Air	Microsomes plus NADPH	1.28	13.2

* The activities were calculated by subtracting the amounts of the products without incubation. Results are expressed as the mean of duplicate determinations.

† NADPH was added as an NADPH-generating system as described in Materials and Methods.

‡ Microsomes were heated at 70° for 5 min.

§ Glucose–glucose oxidase system contained 2.2 μmoles of glucose and 7 units of glucose oxidase.

Table 2. Effects of various reagents on *N*-hydroxy-AAF reductase activity*

Addition	Reductase activity: AAF plus AF formed nmole/mg protein/10 min	%
None (control)	8.78	100
n-Octylamine (1 mM)	3.47	39.6
DPEA (0.1 mM)	2.34	25.5
SKF-525A (0.5 mM)	7.90	90.0
α-Naphthoflavone (0.02 mM)	10.13	115.4
Tiaramide *N*-oxide (1 mM)	0.00	0.0
Nitrosobenzene (1 mM)	1.33	15.2

* The incubation conditions are the same as described in Materials and Methods except that a reagent noted above is contained. Results are expressed as the mean of duplicate determinations.

Table 3. Effects of pretreatments of rats with phenobarbital (PB), 3-methylcholanthrene(MC) and PCB on *N*-hydroxy-AAF reductase activity

Pretreatment	Reductase activity nmoles/mg protein/10 min formed: AAF	AF	Total	%
None (control)	0.21 ± 0.12	6.92 ± 2.71	7.13 ± 2.81	100
PB	0.98 ± 0.72	10.51 ± 1.30	11.48 ± 1.99	160.3
MC	9.22 ± 2.46	8.11 ± 4.30	17.33 ± 6.57	242.0
PCB	10.63 ± 4.18	6.06 ± 0.66	16.70 ± 3.52	233.0

* Results are expressed as the mean ± S.D. of triplicate determinations.

Table 4. Microsomal reductase activity of *N*-hydroxy-AAF in various rat tissues*

Tissue	Reductase activity nmoles/mg protein/10 min formed: AAF	AF	Total	%
Liver	0.18	6.81	6.99	100
Kidney	0.16	0.35	0.51	7.3
Lung	0.07	0.37	0.44	6.3
Small intestinal mucosa	0.59	1.31	1.90	27.2

* Each value is the mean of two separate determinations.

reported previously [3, 4]. Thus, it seems reasonable to assume that tiaramide *N*-oxide competes with *N*-hydroxy-AAF for cytochrome P-450 at the active site located very close to the heme iron. In support of this assumption, nitrosobenzene, which is known to interact with cytochrome P-450 in the heme region, was found to inhibit the reductase activity strongly.

Previous reports from this laboratory have shown that cytochrome P-450-mediated reduction of tertiary amine *N*-oxide [13], benzo[*a*]pyrene 4,5-oxide [14] and carbon tetrachloride [15] is markedly enhanced by addition of methylviologen to the incubation mixture. The enhancement by methylviologen is probably due to the fact that methylviologen increases the electron flow to cytochrome P-450 [13]. The effects of methylviologen on the *N*-hydroxy-AAF reductase activity were also examined (Fig. 3). As was expected, the reductase activity increased markedly with increasing the amount of methylviologen added. The maximal rate of the reduction was obtained at the concentration of methylviologen of 5 mM. The maximal rate calculated as

Table 5. Microsomal reductase activity of *N*-hydroxy-AAF in various species

Species	Reductase activity nmoles/mg protein/10 min formed: AAF	AF	Total	%†
Rat (6)	0.15 ± 0.02	5.08 ± 1.37	5.23 ± 1.22	100
Rabbit (6)	1.06 ± 0.19	12.01 ± 2.62	13.06 ± 2.44	249.6
Hamster (5)	15.32 ± 3.00	79.80 ± 25.69	95.12 ± 28.59	1817.3
Guinea pig (5)	1.06 ± 0.92	55.61 ± 29.45	56.67 ± 30.35	1082.8
Mouse (4)	0.19 ± 0.06	1.83 ± 0.30	2.02 ± 0.34	38.7

* Results are expressed as the mean ± S.D. Number of experiments given in parentheses.
† Per cent of total activity in rat.

sum of AAF and AF formations was 27.6 nmoles/mg protein/10 min. The enhancement was more pronounced in AAF formation than in AF formation. Thus, AAF formation in the presence of 5 mM methylviologen was 139 times higher than that in the absence of methylviologen.

Effects of inducers of the drug metabolizing enzymes on N-hydroxy-AFF reductase activity. Effects of pretreatment with PB, MC and PCB on *N*-hydroxy-AAF reductase activity were examined. As can be seen in Table 3, pretreatment of rats with these inducers resulted in an increase of the reductase activity. MC was the most effective inducer, followed by PCB and PB. It should be noted that MC and PCB increased AAF formation more than AF formation. The rate of AAF formation was enhanced approximately 44- and 51-fold by MC and PCB, respectively.

N-Hydroxy-AAF reductase activity of rat hepatic and extra-hepatic organs. The highest reductase activity with respect to formation rate of AAF was observed in microsomes of small intestinal mucosa (Table 4). The highest activity of formation of AF was seen in the liver. The ratios of formation of AAF to AF thus varied considerably from one organ to another. The variation may be caused by the difference in the *N*-deacetylase activity among these organs. The reductase activity in various tissues were not in proportion to the content of cytochrome P-450. The content in kidney, lung and small intestinal mucosa was 0.04, 0.02 and 0.01 nmole/mg protein, respectively.

Species difference in the N-hydroxy-AAF reductase activity. Species difference in the reductase activity was investigated using rats, rabbits, hamsters, guinea pigs and mice. As shown in Table 5, microsomes from hamster livers had the highest reductase activity with respect to either or both AAF and AF formations. The ratio of formation of AAF to AF varied among the animal species, though AF formation was faster than AAF formation in all animal species employed.

DISCUSSION

Kadlubar *et al.* [16] have demonstrated that there is an NADH-dependent flavin enzyme which reduces hydroxylamine compounds in porcine liver microsomes. The hydroxylamine reductase is not likely to be identical to *N*-hydroxy-AAF reductase since the hydroxylamine reductase has been reported to be insensitive to carbon monoxide and oxygen, and since the reductase prefers NADH rather than NADPH as an electron donor. In fact, Kodlubar *et al.* noted that several carcinogenic hydroxylamines were not reduced by the porcine hydroxylamine reductase [16]. From the evidence that carbon monoxide inhibited *N*-hydroxy-AAF reductase activity, it was postulated that the reduction was catalysed by cytochrome P-450. In support of this hypothesis, several inhibitors of hepatic mixed function oxidase effectively inhibited the reductase activity. It is noteworthy that tiaramide *N*-oxide inhibited the reductase strongly since tiaramide *N*-oxide has already been confirmed to be reduced by cytochrome P-450 by the authors [4, 17]. Among the inducers employed, PCB and MC induced *N*-hydroxy-AAF reductase activity more than PB. This result suggests that cytochrome P-448 induced by PCB and MC is more active than other cytochrome P-450 species in the reduction of *N*-hydroxy-AAF. In accordance with this idea, our preliminary studies have shown that cytochrome P-448 purified from MC-treated rats is more active than cytochrome P-450 purified from PB-treated rats (unpublished results). Nevertheless, we found that the specific inhibitor of cytochrome P-448, α-naphthoflavone, enhanced rather than inhibited the reductase activity. The reason why α-naphthoflavone does not inhibit cytochrome P-448-mediated reduction reaction is obscure at present.

In the present experiment, we found a marked species difference in the reduction of *N*-hydroxy-AAF by liver microsomes. It is of interest that guinea pig liver microsomes showed high activity in the reduction of *N*-hydroxy-AAF, since AAF is known to be a low or non-active carcinogen in guinea pigs [18]. Although the rates of *N*-hydroxylation and/or enzymatic sulfate conjugation must be considered, it is possible that the high activity in *N*-hydroxy-AAF reduction may be one of the factors responsible for the low carcinogenic activity in guinea pigs. Microsomes from hamster liver have the highest reductase activity among the types examined, while AAF has been shown to be carcinogenic in this species, probably due to its high *N*-hydroxylase activity [19].

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REFERENCES

1. P. D. Lotlikar, E. C. Miller, J. A. Miller and A. Margreth, *Cancer Res.* **25**, 1743 (1965).
2. P. H. Grantham, E. K. Weisburger and J. H. Weisburger, *Biochim. biophys. Acta* **107**, 414 (1965).
3. M. Sugiura, K. Iwasaki and R. Kato, *Molec. Pharmac.* **12**, 322 (1975).
4. M. Sugiura, K. Iwasaki and R. Kato, *Biochem. Pharmac.* **26**, 489 (1977).
5. R. Kato, K. Iwasaki, T. Shiraga and H. Noguchi, *Biochem. biophys. Res. Commun.* **70**, 681 (1976).
6. Y. Yamazoe, M. Sugiura, T. Kamataki and R. Kato, *Fedn. Eur. biochem. Soc. Lett.* **88**, 337 (1978).
7. T. Kamataki and H. Kitagawa, *Jap. J. Pharmac.* **24**, 195 (1974).
8. J. Capdevila, S. W. Jakobsson, B. Jernström, O. Helia and S. Orrenius, *Cancer Res.* **35**, 2820 (1975).
9. H. Hoensch, C. H. Woo and R. Schmid, *Biochem. biophys. Res. Commun.* **65**, 399 (1975).
10. J. A. Hinson and J. R. Mitchell, *Drug Metab. Disp.* **4**, 430 (1976).
11. C. Razzouk, G. Lhoest, M. Roberfroid and M. Mercier, *Analyt. Biochem.* **83**, 194 (1977).
12. J. R. Gillette, J. J. Kamm and H. A. Sasame, *Molec. Pharmac.* **4**, 541 (1968).
13. F. J. Wiebel and H. V. Gelboin, *Biochem. Pharmac.* **24**, 1511 (1975).
14. Y. Yamazoe, M. Sugiura, T. Kamataki and R. Kato, *Jap. J. Pharmac.* **29**, 715 (1979).
15. S. S. Thorgeirsson, S. A. Atlas, A. R. Boobis and J. S. Felton, *Biochem. Pharmac.* **28**, 217 (1979).
16. F. F. Kadlubar, E. M. Mckee and D. M. Ziegler, *Archs. Biochem. Biophys.* **156**, 46 (1973).

17. K. Iwasaki, H. Noguchi, R. Kato, Y. Imai and R. Sato, *Biochem. biophys. Res. Commun.* **77**, 1143 (1977).
18. J. H. Weisburger, P. H. Grantham, E. Vanhorn, N. H. Steigbigel, D. P. Rall and E. K. Weisburger, *Cancer Res.* **24**, 475 (1964).
19. P. D. Lotlikar, M. Enomoto, J. A. Miller and E. C. Miller, *Proc. Soc. exp. Biol. Med.* **125**, 341 (1967).