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METABOLIC ACTIVATION OF THE HEPATOCARCINOGEN 3′-METHYL-4-DIMETHYLAMINOAZOBENZENE BY A RAT LIVER CELL-FREE SYSTEM

EVIDENCE SUGGESTING THE FORMATION OF AT LEAST TWO ELECTROPHILIC METABOLITES

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Abstract—A cell-free system obtained from rat liver is described, which catalyzes the covalent binding of [^{3}H]-3′-methyl-4-dimethylaminoazobenzene (3′MeDAB)* to both yeast RNA and endogenous tissue protein. The binding to yeast RNA requires the presence of microsomes and cytosol as source of enzymes as well as an NADPH/NADH generating system and 3′-phosphoadenosine-5′-phosphosulphate (PAPS) or a PAPS generating system as cofactors. In contrast, the binding to tissue protein requires the presence of microsomes and the NADPH/NADH generating system only. Gassing the incubation mixtures with N_2 or CO, or the addition of α-naphthylthiourea (α-NTU) or reduced glutathione (GSH) inhibited the covalent binding to both RNA and protein. The addition of cysteine, however, inhibited the binding to RNA only. The results suggest the formation of at least two electrophilic metabolites in this system, one of which binds preferentially to yeast RNA and the other to tissue protein.

Despite the great diversity in structure of chemical carcinogens, one common property is the requirement for the carcinogens to exist in electrophilic forms, often referred to as the ultimate carcinogens, within the target tissue [1–3]. These electrophiles bind covalently with nucleophilic centres on tissue macromolecules such as proteins and nucleic acids, the binding presumably initiating neoplastic transformation. The majority of carcinogens however, are not electrophilic as such, but require metabolic activation within the target tissue, often via the microsomal mixed function oxidase system [1–3]. In many instances, the precise chemical structures of the ultimate carcinogens are not known, due mainly to the high reactivities and low rates of production of these electrophiles, with consequent difficulties in extraction and identification.

The aromatic amide and aminoazo dye hepatocarcinogens, as exemplified by *N*-acetylaminofluorene (AAF) and 3′MeDAB respectively, appear to be metabolically activated by a common pathway [1–4]. Evidence indicates that these carcinogens undergo sequential *N*-hydroxylation and sulphate esterification, yielding unstable conjugates which decompose into electrophilic nitrenium ions. However, the majority of studies have been carried out using AAF, and recently a number of alternative pathways have been proposed for the activation of AAF [5–8]. These include *N*-*O*-transacetylation [5, 6], *O*-glucuronidation [7] and nitroxyl free radical formation [8]. Comparatively few studies have been undertaken with the aminoazo dyes, such as 3′MeDAB and its parent compound 4-dimethylaminoazobenzene (DAB), and further information is required concerning the precise mechanism(s) of metabolic activation of these carcinogens.

The present report describes an *in vitro* system using rat liver microsomes and cytosol, which catalyzes the covalent binding of [^{3}H]-3′MeDAB to yeast RNA and endogenous tissue protein, and is thus an indirect assay of carcinogen activation. The binding of [^{3}H]-3′MeDAB to RNA exhibits different enzyme and cofactor requirements and a different susceptibility to a number of inhibitors, as compared with the binding of [^{3}H]-3′MeDAB to protein. The results suggest the production of at least two electrophilic metabolites in this system, one binding predominantly to yeast RNA and the other to tissue protein.

*Abbreviations—3′MeDAB: 3′-methyl-4-dimethylaminoazobenzene; DAB: 4-dimethylaminoazobenzene; 3′MeMAB: 3′-methyl-4-methylaminoazobenzene; MAB: 4-methylaminoazobenzene; AAF: *N*-acetylaminofluorene; PAPS: 3′-phosphoadenosine-5′-phosphosulphate; G-6-P: glucose-6-phosphate; Bis-Tris: [bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane]; PMS: postmitochondrial supernatant; GSH: glutathione (reduced); α-NTU: α-naphthylthiourea.

MATERIALS AND METHODS

Reagents. The following reagents were purchased from Calbiochem (Australia): NAD^+ (free acid), $NADP^+$ (monosodium salt), glucose-6-phosphate (G-6-P, disodium salt) and G-6-P dehydrogenase (yeast, specific activity 471 I.U./mg). ATP (disodium salt), cysteine (free base) and [bis(2-hydroxyethyl)imino-

tris(hydroxymethyl)methane](Bis-Tris) were purchased from Sigma Chemical Co., St. Louis, MO. Yeast RNA and GSH were obtained from Boehringer Mannheim (Australia) and α-NTU from Koch–Light Laboratories Ltd., Colnbrook, U.K. [^{3}H]-3′MeDAB (specific activity 119 mCi/m-mole) was prepared by coupling [^{3}H]-*m*-methylbenzenediazonium hydrochloride with *N*,*N*-dimethylaniline and purified on alumina columns according to the method described by Hughes [9]. PAPS was prepared biosynthetically by the method of Irving *et al.* [10] except that phosphate buffer (0.03 M, pH 7.6) was used instead of Tris buffer. All other chemicals used were of analytical grade and solutions were prepared with metal-distilled water which had been passed through an Elgastat deionizer.

Animals and tissue preparation. Male Sprague–Dawley rats (200–250 g) were random bred from local departmental stock and housed in wire bottom cages with no bedding. The rats were starved overnight prior to sacrifice but were allowed continual access to tap water. They were killed by exsanguination under light ether anaesthesia between 08:00 and 09:00 hours. Livers were excised, rinsed in homogenising buffer at 4°, blotted dry and weighed, and all subsequent steps were carried out at 4°. Liver samples (4 g) were homogenized in 3 vol. of buffer containing 0.25 M sucrose, 0.05 M Bis-Tris, 0.025 M KCl and 0.005 M $MgCl_2$ (pH 7.4), with 10 passes of a Potter–Elvehjem homogenizer at 1150 r.p.m. The postmitochondrial supernatant (PMS) was prepared by centrifugation of the homogenate at 10,000 g_{max} for 15 min. Where specified, the PMS was fractionated into cytosol and microsomes by centrifugation at 100,000 g_{av} for 1 hr, the microsomes being resuspended in a volume of buffer equal to the volume of PMS centrifuged.

[^{3}H]-3′MeDAB binding to RNA and protein. The binding of [^{3}H]-3′MeDAB to yeast RNA and endogenous tissue protein was measured in an incubation mixture based on the system used by Kadlubar *et al.* [11], and contained in a final volume of 1.0 ml: Bis-Tris (100 μmoles, pH 7.0 at 37°), $MgCl_2$ (5 μmoles) and EDTA (0.5 μmole). Unless specified otherwise, the following were also included: $NADP^+$ (0.4 μmole), NAD^+ (0.3 μmole), G-6-P (5 μmoles), either ATP (10 μmoles) plus Na_2SO_4 (10 μmoles) or PAPS (0.32 μmole), yeast RNA (3.75 mg), absolute ethanol (20 μl), [^{3}H]-3′MeDAB (100 nmoles, sp. act. 119 mCi/m-mole, in 10 μl of the ethanol) and 0.1 ml PMS (equivalent to 25.0 mg wet wt of liver, containing approximately 3 mg protein and 0.1 mg RNA). In experiments using heat-denatured enzyme, the tissue preparation was heated on a boiling water-bath for 5 min prior to addition to the incubation mixture, and in experiments using microsomes only, 2 units of G-6-P dehydrogenase were added to each incubation. For the gassing experiments, the incubations were carried out in stoppered centrifuge tubes with the gasses being bubbled both into the mixture and above the mixture surface prior to incubation. Where specified, cysteine (1 μmole), GSH (1 or 10 μmoles) or α-NTU (0.17 μmole in 10 μl of the ethanol; a saturated solution) were added to the incubations. The incubations were carried out at 37° under air at 100 oscillations/min for 32 min unless stated otherwise. When samples were centrifuged at 100,000 g_{av} for 60 min following incubation, all of the yeast RNA fractionated with the supernatant and none was found in the microsomal fraction as determined by measuring the content of acid-insoluble RNA in each of the fractions (results not shown).

Following incubation, RNA and protein were extracted by a procedure based on the method of Muramatsu [12]. To each incubation, 5% (w/v) sodium dodecyl sulphate (0.2 ml) and water (1.0 ml) were added, and the solutions were deproteinized twice with 2 vol. of phenol reagent (phenol/*m*-cresol/H_2O:70/10/20 containing 0.1% (w/v) 8-hydroxyquinoline). The RNA was then precipitated overnight at 4° with 2 vol. ethanol/*m*-cresol (9/1) containing 2% (w/v) potassium acetate, washed successively with 2.0 ml of 75% ethanol containing 1% (w/v) potassium acetate, 95% ethanol containing 1% (w/v) potassium acetate and 3 times with 95% ethanol, the last 2 washes being carried out at 60° for 15 min. Negligible radioactivity was detected in the last 2 washings. The RNA was dissolved and hydrolyzed in 0.3 M KOH (1.0 ml), and aliquots were taken for ^{3}H-assay and for RNA and protein estimations. The average yield of RNA was 54 per cent of initial input, and the preparation was free of protein contamination. Protein from the first phenol extract following the removal of interfacial material was precipitated with 1.5 vol. of acetone overnight at 4°. The precipitate was washed successively 3 times with acetone (2.0 ml) at 20°, twice with 95% ethanol (2.0 ml) at 60° for 15 min and once more with acetone (2.0 ml). Negligible radioactivity was detected in the last 2 washings. The protein was dried at 60°, dissolved in 0.3 M KOH (1.0 ml) at 60° and aliquots were taken for ^{3}H-assay and for protein determination. The average yield of protein was 80 per cent of initial input. Although the text of this paper refers to the binding of [^{3}H]-3′MeDAB to yeast RNA, the extracted RNA contains both yeast RNA (3.75 mg/incubation) and endogenous tissue RNA (approximately 0.1 mg/0.1 ml PMS). However, since the levels of binding to endogenous RNA accounted for less than 10 per cent of the total RNA binding (results not shown), no corrections for endogenous RNA binding were made. Radioactivity was measured by acidifying aliquots of RNA or protein (0.8 ml) with concentrated HCl (0.1 ml) and adding toluene/Triton X-100 (2/1) containing 0.4% (w/v) 2,5-diphenyloxazole and 0.05% (w/v) 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene (8.0 ml). Samples were counted in a Packard Tri-Carb Liquid Scintillation Spectrophotometer model C2425 using automatic external standardization to convert c.p.m. to d.p.m. The mean counting efficiency was 20 per cent.

RNA and protein determinations. RNA was assayed by the method of Schmidt–Thannhauser as described by Blobel and Potter [13] and protein by the method of Lowry as described by Munro and Fleck [14].

Statistical analyses. Differences between values were evaluated using Student's two-tailed *t* test.

RESULTS

The basic incubation mixture used to catalyze the binding of [^{3}H]-3′MeDAB to yeast RNA and tissue protein contained rat liver PMS, an NADPH/NADH generating system ($NADP^+$, NAD^+, and G-6-P) and either PAPS or a PAPS generating system (ATP and Na_2SO_4). The extent of binding to both trapping agents (RNA and protein) under these standard conditions

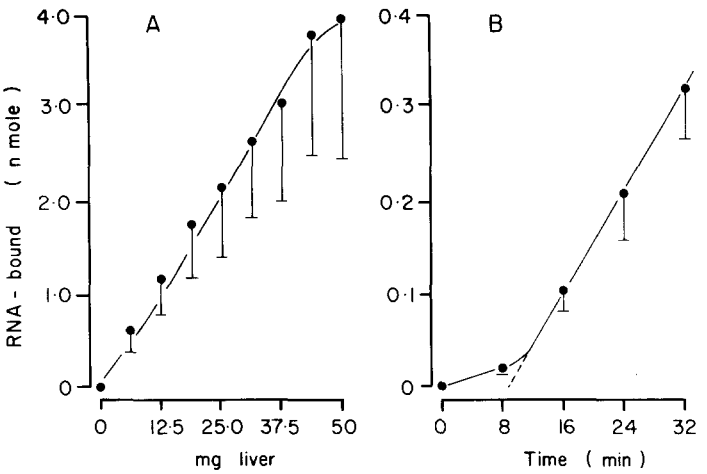

Fig. 1. The *in vitro* covalent binding of [^{3}H]-3′MeDAB to yeast RNA:enzyme concentration and time-dependency of the reaction. The *in vitro* covalent binding of [^{3}H]-3′MeDAB to yeast RNA was measured as described in Materials and Methods. A—Incubations were carried out for 32 min at 37° with increasing contents of PMS (0.1 ml equivalent to 25 mg liver). B—Incubations containing 0.1 ml PMS were carried out for increasing periods of time up to 32 min, at 37°. The results represent the mean ± S.E.M. values obtained from three separate experiments.

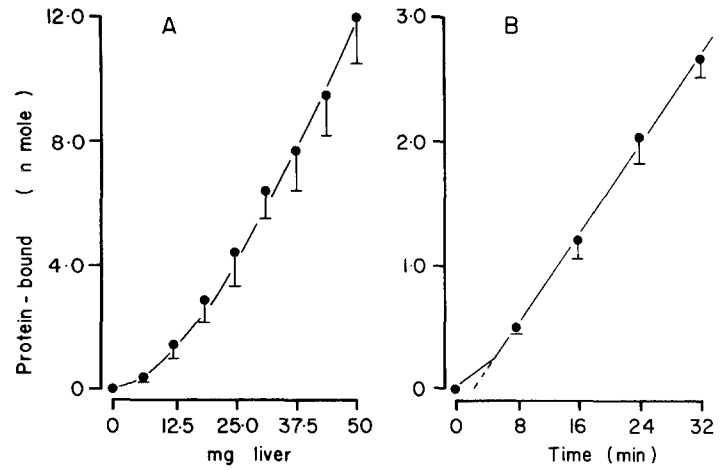

Fig. 2. The *in vitro* covalent binding of [^{3}H]-3′MeDAB to endogenous tissue protein:enzyme concentration and time-dependency of the reaction. The *in vitro* covalent binding of [^{3}H]-3′MeDAB to endogenous tissue protein was measured as described in Materials and Methods. A—Incubations were carried out for 32 min at 37° with increasing contents of PMS (0.1 ml equivalent to 25 mg liver). B—Incubations containing 0.1 ml PMS were carried out for increasing periods of time up to 32 min, at 37°. The results represent the mean ± S.E.M. values obtained from three separate experiments.

was linear with increasing PMS concentration up to 0.175 ml PMS (equivalent to 44 mg wet wt liver) per incubation (Figs. 1A and 2A). The large variation in values represents the variation between individual animals and not experimental error, since duplicate assays agreed to within 10 per cent. The binding was also linear with time for at least 32 min following an initial lag period of 9 min for RNA binding (Fig. 1B) and 3 min for protein binding (Fig. 2B) when using 0.1 ml PMS per incubation. The lag period was determined by extrapolating the linear portion of each graph towards the time axis (abscissa). This lag period possibly represents a finite period of time required for production of the electrophilic metabolites rather than being a temperature equilibrium period, since no such lag period was obtained when measuring the reaction rates of five xenobiotic metabolising enzymes reported in a previous study*. Increasing the quantity of [^{3}H]-3′MeDAB per incubation did not alter the levels of binding (results not shown).

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Table 1. The *in vitro* covalent binding of [^{3}H]-3′MeDAB to yeast RNA and endogenous protein:Enzyme and cofactor requirements

Addition	RNA binding (%)	Protein binding (%)
Experiment 1		
Microsomes, cytosol and NADPH/NADH and PAPS generating systems	100	100
Heated microsomes and cytosol*	0.2 ± 0.2‡	2.0 ± 1.2‡
Minus microsomes	6.2 ± 3.0‡	1.3 ± 0.9‡
Minus cytosol+	25.8 ± 5.0‡	170.0 ± 18.0‡
Minus NADPH/NADH generating system	6.5 ± 2.5‡	1.2 ± 1.2‡
Experiment 2		
Microsomes, cytosol and NADPH/NADH generating system	14.2 ± 0.9‡	105.0 ± 23.0
Plus PAPS	100	100
Plus ATP/Na_2SO_4	112.0 ± 9.2	38.9 ± 4.9‡
Plus ATP	41.7 ± 2.3‡	34.3 ± 3.8‡
Plus Na_2SO_4	19.3 ± 0.5‡	117.0 ± 20.0

The *in vitro* covalent binding of [^{3}H]-3′MeDAB to yeast RNA and endogenous tissue protein was measured as described in Materials and Methods. In experiment 1, 100 per cent values represent binding levels of 0.495 ± 0.086 nmole of [^{3}H]-3′MeDAB/incubation to RNA and 1.63 ± 0.25 nmoles of [^{3}H]-3′MeDAB/incubation to protein respectively. In experiment 2, 100 per cent values represent binding levels of 0.852 ± 0.054 nmole of [^{3}H]-3′MeDAB/incubation to RNA and 6.92 ± 0.50 nmoles of [^{3}H]-3′MeDAB/incubation to protein respectively. Incubations containing microsomes and/or cytosol equivalent to 25 mg liver, were carried out at 37° for 32 min. The results represent the mean ± S.E.M. values obtained from four rats for each experiment.

* Microsomes and cytosol were pre-heated on a boiling water bath for 5 min.

+ Supplemented with 2 units of G-6-P dehydrogenase per incubation.

‡ Significantly different from 100 per cent value, $P < 0.05$.

Studies on the tissue and cofactor requirements for the covalent binding showed that the binding of [^{3}H]-3′MeDAB to yeast RNA and tissue protein required both microsomes and the NADPH/NADH generating system (Table 1, experiment 1). Heating the tissue preparation on a boiling water bath before adding it to the incubation mixture prevented the binding (Table 1, experiment 1), suggesting an enzyme-mediated reaction. The binding to yeast RNA required the presence of cytosol as well as microsomes and an NADPH/NADH generating system (Table 1, experiment 1); PAPS or the PAPS generating system containing ATP and Na_2SO_4 was also necessary for binding to RNA (Table 1, experiment 2). ATP alone however, could support the binding to the extent of approximately 40 per cent of that seen in the presence of both ATP and Na_2SO_4, whereas Na_2SO_4 alone had no significant effect on binding. Gel filtration of the cytosol through Sephadex G-25, a method previously used to remove endogenous sulphate [15], did not alter the pattern of results presented in Table 1, experiment 2 (data not shown). In contrast, the binding of [^{3}H]-3′MeDAB to tissue protein did not require the additional presence of cytosol, PAPS or the PAPS generating system, and was actually inhibited by cytosol and ATP (Table 1).

The binding of [^{3}H]-3′MeDAB to RNA and protein showed differential sensitivity to a number of inhibitors (Table 2). Although gassing the incubation mixtures with N_2 or CO inhibited the binding to both RNA and protein, RNA binding of [^{3}H]-3′MeDAB metabolites was much more susceptible to inhibition. Similarly, the presence of 1 mM cysteine in the incubation medium inhibited binding to RNA to approximately 50 per cent of the control value, but had virtually no effect on the binding of [^{3}H]-3′MeDAB to protein. In contrast, the presence of 10 mM GSH or 0.17 mM α-naphthylthiourea (α-NTU) each equally inhibited the binding of [^{3}H]-3′MeDAB to RNA and protein by approximately 50 per cent. α-NTU is a compound reported to be a specific inhibitor of the flavoprotein amine oxidase (as distinct from the cytochrome P-450 system) [16]. The addition of 1 mM GSH to the system had very little effect on the binding to either RNA or protein.

DISCUSSION

The metabolic activation of aminoazo dyes such as DAB to ultimate carcinogenic metabolites in rat liver is considered to require three sequential enzyme-catalyzed reactions. The first step is mono-*N*-demethylation to 4-methylaminoazobenzene (MAB) which takes place within the microsomal cytochrome P-450 system [17, 18], and requires NADPH and O_2 as cofactors and is inhibited by CO [18]. The secondary amine is then *N*-hydroxylated by a microsomal flavoprotein amine oxidase [19] which is distinct from the cytochrome P-

Table 2. The *in vitro* covalent binding of [^{3}H]-3′MeDAB to yeast RNA and endogenous protein: Effects of several inhibitors

Addition	RNA binding (%)	Protein binding (%)
Experiment 1		
Control	100	100
N_2	14.3 ± 3.4*	58.4 ± 9.9*
CO	13.8 ± 7.3*	51.4 ± 20.9*
GSH (1 mM)	95.5 ± 3.5	86.1 ± 3.5*
GSH (10 mM)	52.1 ± 5.0*	51.9 ± 3.8*
Cysteine (1 mM)	53.0 ± 4.9*	87.9 ± 13.2
Experiment 2		
Control	100	100
α-NTU (0.17 mM)	55.9 ± 1.0*	50.7 ± 0.3*

The *in vitro* covalent binding of [^{3}H]-3′MeDAB to yeast RNA and endogenous tissue protein was measured as described in Materials and Methods. Control incubations contained cytosol and microsomes equivalent to 25 mg liver and the NADPH/NADH and PAPS generating systems, and were incubated for 32 min at 37°. In experiment 1, 100 per cent values represent binding levels of 4.62 ± 3.05 (pmoles/mg RNA)/mg protein/min and 9.34 ± 2.65 (pmoles/mg protein)/mg protein/min to RNA and protein respectively. In experiment 2, 100 per cent values represent binding levels of 6.59 ± 2.44 (pmoles/mg RNA)/mg protein/min and 11.03 ± 3.33 (pmoles/mg protein)/mg protein/min to RNA and protein respectively. The results represent the mean ± S.E.M. values obtained from three rats for each experiment.

* Significantly different from 100 per cent value, $P < 0.05$.

450 system. This amine oxidase, which is also involved in the *N*-oxidation of *N,N*-dimethylaniline [19, 20], requires NADPH and O_2 as cofactors, but is not inhibited by CO [19]. Although both the amine oxidase and cytochrome P-450 catalyze the *N*-oxidation of *N,N*-dimethylaniline in rabbit liver [21], it appears that only the amine oxidase is involved in the *N*-oxidation of this substrate in rat liver [19]. The product of the *N*-oxidation, *N*-hydroxy-MAB, then undergoes sulphotransferase-catalyzed sulphate conjugation within the cytosol [11], a reaction requiring an activated sulphate donor in the form of either PAPS or a PAPS generating system such as ATP and Na_2SO_4 [15, 22]. The *N*-sulphate conjugate is thought to then rapidly decompose, yielding an electrophilic nitrenium ion, which is capable of binding covalently with tissue nucleophiles and presumably initiates neoplastic transformation [1–4, 11]. A similar mechanism of activation is probable for the other aminoazo dye analogues, including 3′MeDAB.

The evidence on which the above-mentioned metabolic pathway is based however, is largely indirect. Synthetic *N*-benzoyloxy-MAB, an analogue considered to exhibit the same electrophilic properties as MAB-*N*-sulphate, was found to induce sarcomas at the site of s.c. injection [23, 24], and when incubated with proteins and nucleic acids *in vitro* in the absence of enzymes, yielded identical products to those obtained after MAB treatment of rats *in vivo* [23, 25–27]. *N*-Benzoyloxy-MAB and its *N*-acetoxy analogue are also direct mutagens, unlike *N*-hydroxy-MAB and MAB, which require the presence of activating enzymes for mutagenic activity [28–30]. These results implicate a reactive ester of *N*-hydroxy-MAB as being involved in tumour induction. The possible involvement of a reactive sulphate ester in the metabolic activation of azo dyes to ultimate carcinogens is also suggested from similar studies with AAF and *N*-hydroxy-AAF (reviewed in Ref. 4), by the observation that the addition of Na_2SO_4 to diets containing 3′MeDAB enhances the carcinogenic potency of the dye [31], and by the finding that the covalent binding of MAB to guanosine in the presence of rat liver PMS, required PAPS as cofactor [11].

The results of the present study show that the metabolite involved in the *in vitro* covalent binding of [^{3}H]-3′MeDAB to RNA exhibits the same enzyme and cofactor requirements for its production as would the presumed 3′-MeMAB-*N*-sulphate in that binding is dependent on the presence of both microsomes and cytosol as source of enzymes, and NADPH/NADH, PAPS and O_2 as cofactors. Furthermore, the binding to RNA is inhibited by CO, an established inhibitor of the cytochrome P-450 pathway, and by α-NTU, a reputedly specific inhibitor of the microsomal flavoprotein amine oxidase [16]. The results thus support the notion that activation of 3′-MeDAB requires sequential *N*-demethylation, *N*-hydroxylation and sulphate conjugation. The electrophilic nature of the activated metabolite is shown by the inhibition of binding to RNA following *in vitro* addition of either GSH or cysteine. These two nucleophiles are normal constituents of rat liver, one of their presumed functions being the detoxification of activated carcinogen and toxin metabolites. GSH and cysteine inhibit the *in vitro* covalent binding to tissue macromolecules [32, 33], the mutagenic activity [34] and the *in vivo* tissue necrotizing activity [35, 36] of a number of chemical carcinogens and toxins.

One unexpected observation in the present study was the finding that ATP, in the absence of sulphate ions, supported the binding of [^{3}H]-3′MeDAB to RNA at approximately 40 per cent of the rate of binding in the presence of both ATP and sulphate. This finding was obtained irrespective of whether or not the cytosol was first chromatographed through Sephadex G-25. Filtration of cytosol through either Sephadex G-10 or G-25 has been previously used to remove endogenous sulphate from the cytosol [15, 22]. Previous studies have shown that the apparent ATP-dependent binding of *N*-hydroxy-AAF to trapping agents such as methionine when using unchromatographed cytosol was due to the *in situ* formation of PAPS from endogenous sulphate and added ATP, since no significant binding occurred in the presence of ATP when using Sephadex G-10 chromatographed cytosol [22]. The effectiveness of sulphate removal from sulphate (10^{-1} M)-supplemented cytosol by Sephadex G-25 filtration was found to be adequate in the present study, by adding $BaCl_2$ (1.0 M) to fractions of the eluant and observing visible precipitation of $BaSO_4$ (limit of sulphate detection, 10^{-3} M SO_4^{2-}, results not shown). The apparent ATP-dependency of binding of [^{3}H]-3′MeDAB metabolites to RNA in the present study may reflect either a true ATP-dependent activation pathway, and hence possibly implicate phosphorylation of *N*-hydroxy-3′MeMAB in the activation process, or alternatively may indicate that an adequate sulphate supply is being

generated from endogenous cysteine by the microsomal fraction [37]. In the previous study by De Baun *et al.* [22], as *N*-hydroxy-AAF was used as substrate, the presence of microsomes was not required [22], and therefore ATP-dependent binding in the presence of microsomes would not have been observed.

The metabolite(s) involved in the covalent binding of [^{3}H]-3′MeDAB to tissue protein in the cell-free system described in this report appear to be of different nature to that involved in the binding to RNA. The binding to protein is solely dependent on the presence of microsomes, NADPH and O_2 and does not require cytosol or PAPS. However, as in the instance of the RNA-binding metabolite, the binding to protein is inhibited by CO and α-NTU, thus implicating both cytochrome P-450 and the flavoprotein amine oxidase, and by the nucleophile GSH indicating that an electrophile is involved. A number of possible metabolites exhibiting these characteristics could be involved in the protein binding. One such possibility is 3′MeDAB-*N*-oxide, since DAB-*N*-oxide has been reported to be produced from DAB by a purified porcine microsomal flavoprotein amine oxidase [20] and has also been shown to decompose non-enzymatically in the presence of porphyrin compounds, yielding a product which binds covalently to proteins [38, 39]. Secondly, *N*-hydroxy-MAB (and presumably *N*-hydroxy-3′-MeMAB) has been shown to undergo further non-enzymatic oxidation to the corresponding nitrone and nitroso derivatives, both of which exhibit electrophilic properties [19], and could therefore be involved in the protein-binding in the present study. Previous studies have shown that *N*-hydroxy-AAF can undergo cytosol-dependent *N*-*O*-transacetylation [5, 6] and microsome-dependent *O*-glucuronidation [7] and nitroxyl free radical formation [8] as well as cytosol-dependent *N*-sulphation, all four products being electrophilic and capable of binding to tissue molecules. Similar studies using *N*-hydroxy-MAB however, have failed to detect *O*-acetylation [11] or *O*-glucuronidation [40], but no studies have apparently been carried out testing for nitroxyl free radical production from the aminoazo dyes. Finally, since the binding to protein in the present study was less susceptible to inhibition by N_2 than that to RNA, it is probable that products of the microsomal NADPH-dependent azo reductase pathway, an anaerobic reaction [41], are also involved in the protein binding. Aniline, a reduction product of DAB, has been shown to bind covalently to tissue molecules both *in vitro* and *in vivo* [42, 43].

The finding that one electrophilic metabolite of 3′MeDAB binds preferentially to yeast RNA and the other(s) to tissue protein, may be explained in the following two ways. Firstly, this preferential binding may be due to different affinities for RNA and protein by the various [^{3}H]-3′MeDAB metabolites, a similar phenomenon being previously observed with other carcinogens [33, 44, 45]. Alternatively, since the yeast RNA is retained within the supernatant during the incubation and not sedimented with the microsomes, the different specificities may also reflect different availabilities of the RNA and protein to the various metabolites.

The results of a number of previous studies indicate that the electrophilic metabolites involved in the protein binding in the present study are probably not involved in tumour induction by the azo dyes. Aniline for example, is non-carcinogenic when fed to rats for prolonged periods [46, 47], and DAB-*N*-oxide is only weakly carcinogenic when repeatedly injected subcutaneously [23]. Although DAB-*N*-oxide is a potent hepatocarcinogen when administered orally to rats for more than 8 months [48], it is unstable in the presence of porphyrin compounds [39] and would therefore have probably decomposed to DAB and MAB prior to reaching the liver. 4-Aminoazobenzene and its 3′-methyl analogue, non-carcinogenic metabolites of MAB and 3′MeMAB respectively, were both found to bind covalently to tissue molecules when incubated with rat liver fractions supplemented with only NADP(H)/NAD(H), at rates similar to those obtained with the respective parent carcinogens [32, 42, 49]. Furthermore, the physical properties of the dye–protein complexes obtained following the *in vitro* incubation of 3′MeDAB with rat liver fractions supplemented with NADP(H)/NAD(H) only, differed markedly from the properties of similar complexes obtained following *in vivo* administration of 3′MeDAB [49]. From these previous studies, it appears that the *in vitro* binding of 3′MeDAB metabolites to protein in the present study is probably not a relevant model for studying activation of the aminoazo dyes to ultimate carcinogens. In contrast, the binding of 3′MeDAB metabolites to yeast RNA, which appears to involve the *N*-sulphate ester, does seem a relevant system for studying the metabolic activation of aminoazo dye carcinogens.

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