Biochemical Pharmacology, Vol. 34, No. 16, pp. 2953–2959, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

ANALYSIS OF METABOLITES OF 2-ACETYLAMINOFLUORENE GENERATED IN AN EMBRYO CULTURE SYSTEM

RELATIONSHIP OF BIOTRANSFORMATION TO TERATOGENICITY *IN VITRO*

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(*Received* 3 *September* 1984; *accepted* 31 *January* 1985)

Abstract—2-Acetylaminofluorene (AAF) produced abnormal, open neural tubes in cultured whole rat embryos only in the presence of an added, NADPH-dependent monooxygenase system. Reactive intermediary metabolites, including N-hydroxy-AAF, N-hydroxy-2-aminofluorene, 2-nitrosofluorene and N-acetoxy-AAF, each elicited embryonic malformations under culture conditions, but a statistically significant increase in the incidence of abnormal neurulation was not observed. Using [^{14}C]AAF and high pressure liquid chromatography (HPLC) separation techniques, the biotransformation of AAF was studied under conditions in which embryos and the monooxygenase system were coincubated. The major metabolites produced cochromatographed with 5-hydroxy-AAF, 7-hydroxy-AAF, 9-hydroxy-AAF and 3-hydroxy-AAF. Other metabolic products also were detected. The embryonic effects of these major AAF metabolites were tested singly and in combination in the embryo culture system. Addition of 7-hydroxy-AAF to the embryo culture system resulted in open neural tubes in the absence of an added monooxygenase system. Other individual ring-hydroxylated metabolites produced retarded growth, but neurulation appeared normal. Ring-hydroxylated metabolites, added to the embryo culture system in combination in the same proportions as were formed during biotransformation in culture, also produced a marked increase in incidence of neural tube defects in the absence of an exogenous (added) biotransforming system. In combination with 3-, 5- and 9-hydroxy-AAF, 7-hydroxy-AAF exposure (86 μM) resulted in a 47% incidence of abnormal, open neural tubes. When tested individually, higher concentrations of 7-hydroxy-AAF (104 μM) produced a lower percentage of malformed embryos (13%). The results suggested that 7-hydroxy-AAF was principally responsible for the neural tube defects caused by AAF following monooxygenase-dependent bioactivation, but that other metabolites also appeared to contribute to the observed effect.

Previous investigations from this laboratory [1] have demonstrated that 2-acetylaminofluorene (AAF‖) will produce neural tube defects in cultured rat embryos only if a hepatic microsomal fraction (S-9) and cofactors for monooxygenation, NADPH and glucose-6-phosphate (G6P), are added to the embryo culture system. Additions to the culture system of reactive intermediary metabolites considered likely to be responsible for eliciting the defects, however, resulted in a qualitatively different spectrum of malformations than that produced by the bioactivated parent chemical [2, 3]. 2-Nitrosofluorene (NF) and N-hydroxy-2-aminofluorene (N-OH-AF) each caused abnormalities in axial rotation (flexure) as the primary observed defect [3]. N-Hydroxy-2-acetylaminofluorene (N-OH-AAF) and N-acetoxy-2-acetylaminofluorene (AAAF) each caused prosencephalic hypoplasia and microcephaly as the most characteristic abnormalities [1, 2]. These unexpected findings prompted us to investigate the biotransformation of AAF under the same culture conditions in which the arylamide was converted to metabolites that evoked neural tube abnormalities. In this paper, we report the results of those investigations. We also report evidence that 7-hydroxy-AAF may be one of the metabolites principally responsible for the characteristic neural tube defects. Other ring-hydroxylated metabolites also appeared to contribute to the elicitation of the defects.

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‖ Abbreviations: AAAF, N-acetoxy-2-acetylaminofluorene; AAF, 2-acetylaminofluorene; AF, 2-aminofluorene; DMSO, dimethyl sulfoxide; G6P, glucose-6-phosphate; HPLC, high pressure liquid chromatography; N-OH-AAF, N-hydroxy-2-acetylaminofluorene; N-OH-AF, N-hydroxy-2-aminofluorene; NF, 2-nitrosofluorene; and S-9, rat hepatic 9000 *g* (20 min) supernatant fraction.

MATERIALS AND METHODS

Embryo culture system. A modification of the rat embryo explant culture system of Fantel *et al.* [4] was utilized in these studies. This system is based upon the explant systems described by New [5]. The modifications are similar to those detailed in

a previous publication [1]. Time-mated Sprague–Dawley rats were obtained from Tyler Laboratories, Bellevue, WA. The morning following mating (8:00 a.m.) was designated as day 0 of pregnancy if copulation plugs were present. On the morning of day 10 (9:00 a.m.), uteri were removed from rats under ether anesthesia.

A stereoscopic dissecting microscope was used to remove decidua, parietal yolk sac and Reichert's membrane. The visceral yolk sac, ectoplacental cone and amnion remained intact. The culture medium consisted of 7 ml of Waymouth's medium (752/1) supplemented with glutamine, 3.5 ml of rat serum, 3.5 ml of human serum, penicillin (100 I.U./ml) and streptomycin (50 μg/ml). Both human and rat serum were heat-inactivated at 56° for 20 min.

Prior to culturing, the medium was prewarmed and pregassed with 5% CO_2, 5% O_2 and 90% N_2 to maintain a pH of 7.3. After 20 hr of rotation on a roller apparatus, the medium was regassed with 95% O_2 and 5% CO_2.

The embryos were cultured at 37° for a total of 24 hr. At the end of the culture period the embryos were examined under a dissecting microscope for abnormalities as well as for indices of growth and development. Only viable embryos (as defined by the presence of active yolk sac circulation and heart beat) were assessed further. Measurements of head and crown-rump length, somite number and limb bud development were obtained. Developmental malformations were also recorded.

Protein determinations were made on embryos ultrasonically disrupted in 0.1 M sodium phosphate buffer (pH 7.4). The Bradford assay [6] was used to determine embryonic protein content. Embryonic DNA content was determined using an assay reported by Labarca and Paigen [7]. Covalent binding of [9-^{14}C]AAF to acid-insoluble macromolecules was assessed by the method described by Schwartz and Goodman [8].

Chemicals. [9-^{14}C]AAF (47.6 Ci/mole) was purchased from the New England Nuclear Corp., Boston, MA, and was purified with preparative high pressure liquid chromatography (HPLC) on a reverse phase column (Whatman Partisil 10, ODS-2) with a methanol–water, linear gradient (80–100%, 30 min). Final purity was approximately 99%. Reference standard chemicals were obtained from the Chemical Repository of the National Cancer Institute, Chicago, IL, and included AAF, N-hydroxy-AAF, 7-hydroxy-AAF, 5-hydroxy-AAF, 3-hydroxy-AAF, 1-hydroxy-AAF, 9-hydroxy-AAF, and 2-aminofluorene (AF). Several of these chemicals (suitable for embryotoxicity testing in culture, utilization as substrates, column conditioning, etc.) were synthesized in the laboratory. AAF, AF and 9-keto-AAF were synthesized as described by Fletcher and Namkung [9]; N-hydroxy-AF was synthesized according to methods described by Lotlikar *et al.* [10]; 9-hydroxy-AAF, 7-hydroxy-AAF and N-hydroxy-AAF were synthesized as described by Pan and Fletcher [11], Weisburger and Weisburger [12] and Poirier *et al.* [13] respectively. NF also was synthesized by the method described by Poirier *et al.* [13]. All nonradioactive chemicals were purified by recrystallization until constant melting points were obtained and also by subsequent preparative HPLC on a reverse phase column (Whatman Partisil 10, ODS-2) with a methanol–water linear gradient (80–100%, 30 min). All final purities were greater than 99%.

Analyses of AAF biotransformation. Radiolabeled AAF (0.76 μCi/ml of culture medium, 2.68 μCi/μmole) was incubated with day 10 rat embryos. The culture medium contained the following components: hepatic S-9 representing 0.4 mg protein/ml of medium from rats pretreated with a single intraperitoneal injection of 3-methylcholanthrene (40 mg/kg) 48 hr prior to being killed, six explanted rat embryos, 14 ml of embryo culture medium (as previously described), 0.5 mM NADPH and 5.0 mM G6P. Freshly redistilled dimethyl sulfoxide (DMSO) was employed as the vehicle for AAF; equivalent volumes of DMSO (35 mM, final concentration) were added to control flasks. Seven different culture conditions were utilized to account for all possible control permutations. Besides the complete incubation system, flasks were prepared in which embryos, S-9 and/or cofactors were omitted from the media. Reactions were initiated by addition of the S-9 fraction and were allowed to continue for 2 hr at 37° at pH 7.3 under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2 (conditions for culturing of day 10 embryos). Culture flasks were rotated constantly at 20–40 rpm. The reactions were terminated after 2 hr of incubation (24 hr in specific cases) by the addition of 1 vol. of an ice-cold solution (1 M) of sodium acetate to the reaction vessels, and the mixtures were placed on ice. Metabolites were extracted from the culture medium four times with 2 vol. each of ethyl ether. The ether layers were pooled and evaporated to dryness under a stream of N_2. Methanol, containing authentic standard metabolites of AAF, was added to the evaporated organic phase. Aliquots were injected into an HPLC column, and radioactivity associated with the various peaks was calculated and presented as a percentage of total dpm recovered from the HPLC column. An aliquot of the ether-extracted aqueous phase was also treated with *Escherichia coli* β-glucuronidase (Sigma Chemical Co., St. Louis, MO, 4500 units/ml). After incubation at 37° for 2 hr, this mixture was re-extracted from ether. Radioactivity associated with the aqueous phase was monitored before and after incubation with β-glucuronidase.

A modification of the HPLC system used by Smith and Thorgeirsson [14] was used to separate AAF and eight standard metabolites. The chromatographic instrumentation consisted of a Micrometrics liquid chromatograph, model 7000 with an ultraviolet absorbance detector and a Hewlett–Packard recorder. A Dupont Zorbax C-8 (4.6 mm × 15 cm) column was used to separate AAF and the metabolites. Initially, 25% isopropanol:75% 0.01 M acetic acid (pH 3.2 to 3.4) was used as the solvent, increasing to 28% isopropranol with a 10-min linear gradient. Both solutions contained 0.01% desferal mesylate to prevent chemisorption of N-OH-AAF. The solvent concentration was maintained at 28% isopropranol until AAF eluted (approximately 32 min) at which time the solvent was switched to 100% isopropanol. A solvent flow rate of 1.2 ml/min was

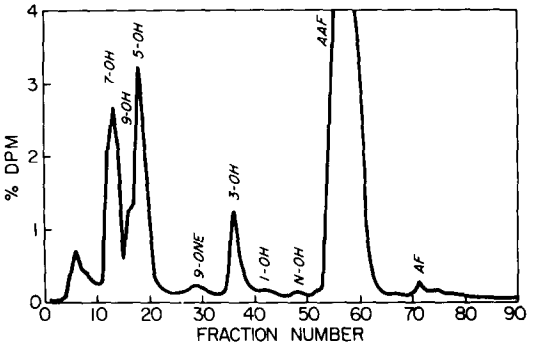

Fig. 1. HPLC elution profile of AAF metabolites coeluting with Chemical Repository standards and formed by incubating [9-^{14}C]AAF (0.76 μCi/ml of medium, 2.68 μCi/μmole) with rat embryos explanted on day 10. An hepatic S-9 fraction obtained from 3-MC-pretreated adult male rats and cofactors (NADPH and G6P) also were included in the culture medium. Incubation was at 37° for 2 hr with O_2:CO_2:N_2 (5:5:90) under conditions normally resulting in a high incidence of neural tube abnormalities. Seventy-three percent of the total radioactivity incubated in the culture medium could be accounted for in the metabolite profile (ether extractable radioactivity). Remaining radioactivity, not fully characterized, was probably associated primarily with serum proteins, embryos and aqueous medium. Radioactivity associated with each peak is expressed as a percentage of total dpm recovered from the column (704,820 dpm). Total radioactivity recovered was 98 ± 2%. A repeat experiment yielded very similar results.

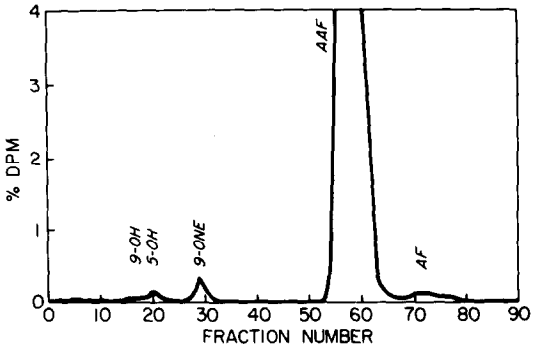

Fig. 2. HPLC elution profile obtained under the same conditions as described in the legend of Fig. 1 except that NADPH and G6P were not added to the culture medium. Under these conditions, neural tube defects failed to occur. A total of 816,530 dpm were recovered from the column. Total radioactivity recovered was 98 ± 2%. A repeat experiment yielded very similar results.

used throughout the elution. The Dupont Zorbax C-8 column was conditioned by recirculating a solution of 0.03% N-OH-AAF in methanol overnight. This pretreatment was necessary since N-OH-AAF adsorbed to the new column even when desferal mesylate was present. Eluate fractions were collected every 0.5 min and radioactivity in the fractions was assessed with a Beckman LS 800 liquid scintillation spectrometer and New England Nuclear Aquasol scintillation fluids.

The metabolites of AAF detected in these studies were tested individually and in combination for their capacity to produce embryotoxicity *in vitro*. These were the 3-, 5-, 7- and 9-hydroxy derivatives as well as AF. The metabolites were also tested in combination by adding these metabolites to the embryo culture system in the same proportions as appeared in the HPLC profiles (5-hydroxy-AAF:7-hydroxy-AAF:3-hydroxy-AAF:9-hydroxy-AAF, 25:20:10:7 respectively).

RESULTS

A typical HPLC profile of AAF metabolites produced under embryo culture conditions is illustrated in Fig. 1. Major peaks of radioactivity coeluted with 7-hydroxy-AAF and 5-hydroxy-AAF. Minor peaks coeluted with 9-hydroxy-AAF, 9-keto-AAF, 3-hydroxy-AAF, 1-hydroxy-AAF, AF and N-hydroxy-AAF. With this system, N-hydroxy-AF coeluted with N-hydroxy-AAF and NF coeluted with AF. Omission of NADPH and G6P resulted in profiles typical of that illustrated in Fig. 2. Under those conditions, only very small quantities of radioactivity eluted with the standard metabolites. This radioactivity eluted with retention times similar to those of 9-hydroxy-AAF, 5-hydroxy-AAF, 9-keto-AAF and AF. As indicated above (see beginning of paper), inclusion of NADPH and G6P in the embryo culture system resulted in the appearance of neural tube abnormalities in rat embryos; these abnormalities were not observed if the two monooxygenase cofactors were omitted. Ninety-nine to 100% of radioactivity remained in the aqueous phase following β-glucuronidase treatment and re-extraction into ether. Thus, under these culture conditions, glucuronide conjugation did not appear to be a significant biotransformational pathway.

The ring-hydroxylated metabolites that appeared most prominently in the HPLC profiles were tested individually and in combination for their capacity to produce embryotoxicity *in vitro*. These were the 3-, 5-, 7- and 9-hydroxy derivatives. Experiments also were performed with AF. N-Hydroxy-AAF, N-hydroxy-AF, AAAF and NF were tested previously [1–3] and were not included in these studies. The results of the experiments designed to test the embryotoxicity of individual metabolites are presented in Table 1. The 3-, 5- and 9-hydroxy derivatives each produced significant decreases in protein and DNA content, but viability and detectable malformations did not differ significantly from the controls except at the highest concentration of 5-hydroxy-AAF. No open neural tubes were observed after treatment of embryos with any of these three agents. AF did not produce a significant change in viability or malformations but did decrease embryonic protein and DNA content at high test concentrations. Treatment with 7-hydroxy-AAF, in contrast, resulted not only in concentration-dependent decreases in protein and DNA content but also in increases in detectable embryonic malformations. A large proportion of the malformations observed were abnormally open neural tubes. At the highest concentration tested, 67% of the malformed embryos exhibited abnormal neurulation. Typical embryos exposed to 7-hydroxy-AAF are illustrated in Fig. 3.

Table 1. Toxic effects of AAF metabolites on whole rat embryos in culture

Chemical	Conc (μM)	N*	% Viable†	% Malformed†	Protein content‡ (% of control)	DNA content§ (% of control)
3-Hydroxy-AAF	318	6	100	0	42 ± 15‖	76 ± 25‖
	209	7	86	0	60 ± 15‖	83 ± 25‖
	25	6	100	17	105 ± 16	105 ± 23
5-Hydroxy-AAF	318	12	92	27‖	57 ± 17‖	76 ± 14‖
	209	10	100	0	85 ± 15‖	87 ± 19‖
	104	11	100	9	101 ± 14	93 ± 17
9-Hydroxy-AAF	318	15	100	7	53 ± 11‖	71 ± 16‖
	251	16	100	6	73 ± 18‖	74 ± 16‖
	104	12	100	0	79 ± 15‖	89 ± 14‖
7-Hydroxy-AAF	318	18	33‖	60‖	19 ± 2‖	56 ± 8‖
	260	25	80‖	40‖	46 ± 20‖	74 ± 20‖
	209	16	81‖	31‖	51 ± 11‖	75 ± 16‖
	104	26	88‖	13	74 ± 19‖	97 ± 29
	60	11	100	9	76 ± 16‖	96 ± 29
Aminofluorene	385	14	100	14	56 ± 13‖	71 ± 18‖
	275	12	100	0	80 ± 9‖	82 ± 13‖
	138	12	100	0	89 ± 20	92 ± 25

* Number of embryos assessed. Only embryos with active yolk sac circulation and heart beat were scored as viable. Malformations and macromolecular content were assessed only in viable embryos.

† Control embryos were exposed to 35 mM DMSO and were incubated concurrently in each experiment. The pooled control embryos had a background malformation rate of 6.5% and 100% viability (N = 77).

‡ Control embryos were exposed to 35 mM DMSO (solvent) only. Protein content of treated embryos were expressed as a mean percentage (± S.D.) of the protein content of individual exposed embryos as compared to the pooled mean value obtained from embryos cultured concurrently under control conditions. The combined protein value of control embryos was 286 ± 51 μg protein per embryo (N = 77).

§ Embryonic DNA content is expressed as a mean percentage (± S.D.) of the DNA content of individual exposed embryos as compared to the pooled mean value obtained from embryos cultured concurrently under control conditions (35 mM DMSO). The combined DNA value of all control embryos was 18 ± 3.4 μg DNA per embryo (N = 77).

‖ Statistically different from controls at $P < 0.05$. For viability and malformations, the X^2 criteria was utilized. For protein and DNA content, Student's *t*-test was employed. All statistical methods are described in Steel and Torrie [15].

Four ring-hydroxylated metabolites (3-, 5-, 7- and 9-hydroxy AAF) were also tested in combination. They were added to the embryo culture system in the same proportions as they appeared in the HPLC profiles and in four different total concentrations (Table 2). Concentration-dependent decreases in protein and DNA content again were observed but embryonic viability was only decreased significantly at the highest concentration. Malformations of the same type as those elicited by 7-hydroxy-AAF were produced by the combination of ring-hydroxylated derivatives. The concentration of 7-hydroxy-AAF in the mixture that produced 47% abnormal neurulation was only 86 μM. When tested individually,

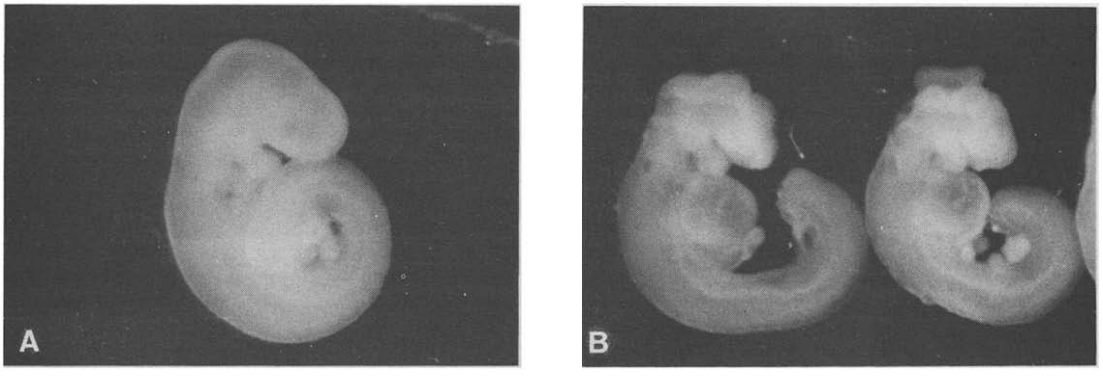

Fig. 3. Day 11 rat embryos after 24 hr in culture. These embryos at the right (B) were exposed to 7-hydroxy-AAF *in vitro*. Each of the exposed embryos exhibited abnormal open neural tubes and significant decreases in DNA and protein content. The embryos were exposed to 318 μM 7-hydroxy-AAF (final concentration). The embryo on the left (A) represents a typical unexposed embryo.

Table 2. Toxic effects of a mixture* of ring-hydroxylated metabolites on cultured whole rat embryos

Total Conc (μM)	N†	% Viable	% Malformed	Protein content‡ (% of control)	DNA content§ (% of control)
267	17	88‖	47‖	57 ± 15‖	69 ± 23‖
133	17	100	24‖	72 ± 18‖	90 ± 18‖
67	16	94	13	71 ± 23‖	91 ± 17
33	17	100	6	88 ± 22‖	105 ± 19

* The mixture consisted of 5-hydroxy-AAF, 7-hydroxy-AAF, 3-hydroxy-AAF and 9-hydroxy-AAF added to the culture medium in the same ratio (25:20:10:7) as was observed in the HPLC profiles.

† Number of embryos assessed. For further details, see footnote to Table 1.

‡ See footnote ‡, Table 1.

§ See footnote §, Table 1.

‖ See footnote ‖, Table 1.

higher concentrations (e.g. 104 μM, Table 1) produced a lower percentage of malformed embryos (13%).

Under the embryo culture conditions used in these experiments, comparatively small quantities of metabolite (or metabolites) that cochromatographed with AF and NF were observed. As Fig. 2 indicates, when cofactors (NADPH and G6P) for monooxygenation were excluded from the system, amounts of radioactivity eluting with retention times identical to those of AF and NF were not altered markedly. Since deacetylation of AAF to AF does not require the monooxygenase cofactors, the metabolite(s) generated would appear to be primarily AF. Incubations conducted with higher S9 concentrations or for 24 hr resulted in much larger quantities of metabolite(s) that cochromatographed with AF and NF and the quantities detected again were not appreciably altered by addition of cofactors. Addition of NaF (a deacetylase inhibitor) to the reaction mixture markedly decreased the quantity of AF-cochromatographing metabolite(s) formed. In previous work [3], we also demonstrated that less than 10% of NF could be recovered from the culture immediately after addition of the chemical to the medium. Together, these results strongly suggested that AF was the principal metabolite detected under the peak cochromatographing with AF and NF. The results presented in Table 1 suggest that AF plays a minor if any role in the elicitation of neural tube defects. In some experiments, AF was added to the mixture of ring-hydroxylated metabolites in an attempt to determine whether this deacetylated metabolite might contribute to the embryotoxic effect. The amine (AF) was added to the embryo culture system in combination with the four previously tested phenolic metabolites. Total final concentrations of AF in these cultures ranged from 67 to 267 μM. When compared with the data shown in Table 2, the results indicate that the primary aromatic amine contributed little if any effect in terms of embryotoxicity.

Results of experiments designed to measure the effect of the added monooxygenase system on irreversible binding of [9-^{14}C]AAF to media and embryonic macromolecules are presented in Table 3. When an exogenous biotransformation system was added, more radioactivity per unit of protein was bound to embryonic acid-precipitable material. Addition of the rat hepatic monooxygenase system resulted in profound increases in binding to proteins present in the medium, but only minor increases in quantities bound to embryonic proteins.

DISCUSSION

The biotransformation of AAF is a crucial determinant of its known biologic effects and has been studied intensively in relationship to the known, potent carcinogenic and mutagenic properties of this arylamide [16, 17]. Little is known about the relationship of the biotransformation of AAF to its teratogenic properties. For both the mutagenic and carcinogenic effects, N-hydroxylation is regarded as a necessary first step in the metabolic activation of AAF; however, the immediate product of the reaction, N-hydroxy-AAF, will not covalently bind to macromolecular nucleophiles in the absence of still further biotransformation. Several N-O esters (N-O sulfate and N-O-acetate) are demonstrably high in reactivity, and studies [18–20] have suggested that the nitrenium and carbonium ions generated from these esters form covalent adducts with DNA. Thus, these reactive intermediates are often regarded as ultimate carcinogenic species. In terms of mutagenicity, however, it has been reported by several investigators that inclusion of a sulfation system in the reaction mixture actually inhibits the conversion of AAF to a mutagenic intermediate [21–24]. The authors have suggested that deacetylation is of critical importance in the mutagenic bioactivation of AAF, presumably because deacetylated derivatives are readily converted to highly mutagenic N-hydroxy-AF and NF [25, 26]. It is of considerable interest that the relative embryotoxic effects of non-acetylated versus acetylated reactive intermediates contrasted qualitatively [2, 3]. The deacetylated derivatives elicited characteristic defects in axial rotation (flexure), and the acetylated derivatives each produced prosencephalic hypoplasia and microcephaly. Speculation that somatic cell mutations may

Table 3. Covalently bound radioactivity

Incubation conditions*	Total acid-insoluble radioactivity† (pmoles/mg protein) Bound to media proteins‡	Bound to embryonic macromolecules§
[^{14}C]AAF + embryos	8 ± 5	158 ± 43
[^{14}C]AAF + embryos + S-9	12 ± 5	187 ± 57
[^{14}C]AAF + embryos + S-9 + cofactors	458 ± 89‖	236 ± 76

* Day 10 rat embryos were incubated at 37° with [9-^{14}C]AAF (0.76 μCi/ml medium, 2.68 μCi/μmole) for 2 hr under O_2:CO_2:N_2 (5:5:90) under culture conditions described in Materials and Methods. Values in the table are means with standard deviations from three separate experiments.

† Total acid-insoluble radioactivity was determined after four extractions with 2 vol. of ethyl ether and four extractions with 5 vol. of ice-cold 5% trichloroacetic acid [8]. Bovine serum albumin was added as a carrier protein when embryonic proteins were analyzed.

‡ Serum proteins include human and rat serum proteins added to the Waymouth's culture medium to support embryonic growth. Total incubation volume was 5.0 ml, 1.25 ml rat serum, 1.25 ml human serum plus 2.5 ml Waymouth's medium. An average of 27 mg of serum proteins was added per ml of medium.

§ Embryonic macromolecules include those of the visceral yolk sac, ectoplacental cone region, amnion and embryo *per se*. An average of 156 μg of protein was observed per embryonic unit. Approximately six embryos were used per incubation.

‖ Statistically different from control conditions at $P < 0.05$.

be responsible for the effects of NF and N-hydroxy-AF, however, is premature at this stage. Nevertheless, the studies described by Thorgeirsson *et al.* [17], in which a definitive separation between the macromolecular covalent binding and mutagenicity of reactive intermediates has been demonstrated, lead to the hope that these particular effects may be definitely related to specific embryonic malformations and provide important insights into mechanisms of chemical teratogenesis.

Studies *in vivo* have indicated that AAF produces teratogenic effects in mice [27, 28], rats [29] and chicks [30]. When AAF was administered on days 8–11 of gestation, skeletal abnormalities were the principal malformations observed in mice and hydrocephaly was observed in rats. In those experiments, large doses (300 mg/kg) of the arylamide were required to produce the embryotoxic effects *in vivo*. In view of its potent carcinogenic and mutagenic effects, a rationale for its apparently weak teratogenic effects remains to be elucidated.

Previous studies *in vitro* [1–3] demonstrated that unmetabolized AAF did not produce malformations in cultured embryos at concentrations up to 75 μg/ml (336 μM). The arylamide did, however, produce numerous abnormally open neural tubes if rat hepatic S-9 and cofactors for monooxygenation were coincubated in the embryo culture system. One hundred percent of the embryos were malformed at 282 μM if an exogenous hepatic monooxygenase system was added. It was considered likely that one or more of the metabolites known to be mutagenic and/or carcinogenic was responsible for the evoked malformations. Thus, we were quite surprised to find [2, 3] that several established reactive metabolites including N-hydroxy-AAF, N-acetoxy-AAF, N-hydroxy-AF and NF did not produce the characteristic neural tube defects observed in the embryo-culture system (although other types of defects were observed). It was the purpose of this study to identify other AAF metabolites generated under teratogenic embryo culture conditions and to investigate the nature of the malformations elicited by these metabolites. The present studies suggest that 7-hydroxy-AAF may be the metabolite most responsible for the neural tube defects. This is also surprising because the 7-hydroxy metabolite is generally regarded as an inactive breakdown product—particularly in terms of chemical carcinogenesis, covalent binding to macromolecules and mutagenesis [31–34]. Studies of the mechanism whereby 7-hydroxy-AAF produces neural tube defects should prove to be extremely interesting. The structure–activity relationships presented in Table 1 suggest that the neural tube defects that were observed were not related to a non-specific toxic effect; however, the exact mechanism of abnormal neurulation has not been determined.

It should be borne in mind that, when tested as an individual agent, 7-hydroxy-AAF produced malformations only at relatively high concentrations. In combination with other metabolites, much lower concentrations were required but only the ring-hydroxylated metabolites and AF were tested. It is possible that combinations with other metabolites could be even more effective, especially combinations of ring- and N-hydroxylated metabolites. These are currently under investigation. It also seems possible that various metabolites not yet investigated may play an important role in the evocation of neural tube abnormalities *in vitro*. In view of their reputation as carcinogenic chemicals, the N-O esters may be of special interest. N-Acetoxy-2-AAF is often employed as a prototype of these agents but the N-O-sulfate ester is more heavily implicated as an ultimate hepatocarcinogenic metabolite of AAF. The acetoxy derivative elicited primarily prosencephalic abnormalities when tested as a single agent *in vitro* [2]; comparisons with the corresponding

sulfate or other N-O esters also will be of high interest.

The possibility that the embryos themselves may play a role in the bioactivation process is one that deserves careful investigation. Various reports [35–38] have suggested that mammalian embryos may be capable of bioactivating certain chemicals. A dramatic increase in the amount of radioactivity covalently bound to serum proteins was seen when a complete monooxygenase system was included in the incubation mixtures. This suggests that metabolic activation was required for AAF to covalently bind to macromolecules. Covalently bound radioactivity to embryonic proteins was not as dependent on the presence of such a monooxygenase system. These results are consistent with the possibility that the day 10 embryo may be capable of some metabolic activation of 2-AAF. Further studies on the possibilities of embryonic bioactivation are currently in progress.

Acknowledgements—The authors wish to acknowledge the expert technical assistance of Steve Moss and Elizabeth Walker. We also thank Cathy Manning for typing the manuscript. This research was supported by NIH Grants HD-04839, HD-12717, HD-00836, EH-07032, and OH-01281.

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