Biochemical Pharmacology, Vol. 38, No. 16, pp. 2685–2692, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

INFLUENCE OF ELECTROPHILIC CHARACTER AND GLUTATHIONE DEPLETION ON CHEMICAL DYSMORPHOGENESIS IN CULTURED RAT EMBRYOS*

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(*Received* 3 *October* 1988; *accepted* 20 *December* 1988)

Abstract—To examine the importance of reduced intracellular glutathione (GSH) in the modulation of dysmorphogenesis and to gain insight into the electrophilic character of the embryotoxic intermediates generated in the rat embryo from *N*-acetoxy-2-acetylaminofluorene (AAAF) and acetaminophen (APAP) in cultured embryos, the effects of GSH depletion on the embryotoxicity, dysmorphogenesis and covalent binding of these agents were examined. Both AAAF (90 μM) and APAP (500 μM) produced concentration-dependent, statistically significant ($P \leq 0.05$) decreases in embryonic length as well as embryonic and visceral yolk sac protein content when rat embryos were exposed *in vitro* between days 10 and 11 of gestation. The predominant malformations observed upon exposure to AAAF and APAP were prosencephalic hypoplasia and abnormal neurulation respectively. Exposure of conceptuses to [^{3}H]APAP followed by separation and fractionation of the cellular RNA, DNA and protein via density gradient centrifugation resulted in detectable binding in fractions that contained protein, but not DNA or RNA. This suggested that the rat conceptus is capable of bioactivating APAP to a soft electrophile that selectively arylates protein. In contrast, conceptuses exposed to [^{3}H]AAAF exhibited detectable binding to RNA, DNA and protein, indicative of conversion to both hard and soft electrophiles. Depletion of GSH was accomplished by pretreating conceptuses with 500 μM L-buthionine-*S*,*R*-sulfoximine (BSO) from the start of the culture period (day 9.5) until the morning of day 10. When conceptuses were depleted previously of GSH by BSO, exposure to APAP resulted in significant potentiation (relative to APAP alone) of the observed embryotoxicity. These conceptuses displayed further decreases in both embryonic size and protein content of the embryo and yolk sac, as well as increased incidence of abnormally open anterior neuropores and increased binding (3-fold) of [^{3}H]APAP to protein. In contrast, pretreatment with BSO did not potentiate the AAAF-elicited decreases in embryonic size or protein content, nor the severity of prosencephalic hypoplasia, although a slight increase in binding of [^{3}H]AAAF to DNA was observed. Taken together, these data are consistent with the concept that abnormal neurulation elicited by APAP results from the generation of one or more soft electrophilic species, whereas elicitation of prosencephalic hypoplasia by AAAF appears to be a consequence of conversion to a relatively hard electrophile(s).

One of the principal mechanisms by which chemicals and reactive intermediates are thought to produce toxicity is via covalent binding to critical cellular macromolecules, principally DNA, RNA and protein. An important determinant of the specific cellular nucleophiles preferentially attacked by a given electrophilic metabolite is the physicochemical nature of the electrophilic center. Although attempts to rigorously quantify reactions between diverse classes of electrophiles and nucleophiles have not been successful [1], the selectivity of interactions between qualitatively similar classes of electrophiles and nucleophiles can, at least to a degree, be predicted and interpreted [2]. For example, the carcinogenic dye *N*-methyl-4-aminoazobenzene (MAB) can be biotransformed to two distinguishable intermediates, each displaying differing reactivities towards biological nucleophiles. Oxidation of the *N*-methyl moiety results in an electrophile that preferentially reacts with protein and reduced glutathione (GSH) [3], whereas formation of the *N*-sulfate generates an intermediate that selectively binds to DNA [4–6].

2-Acetylaminofluorene (AAF) is a potent and widely investigated model compound utilized in studies of carcinogenesis, mutagenesis, genotoxicity and cytotoxicity [7]. It is also teratogenic in rats, mice and chicks [8–10]. In adults, *N*-hydroxy-2-acetylaminofluorene is considered the proximate carcinogenic and mutagenic metabolite of AAF. This compound can then be further converted to a highly reactive nitrenium ion immediately after formation of the sulfate ester via heterolytic cleavage of the N–O bond [11]. An analogue, *N*-acetoxy-2-acetylaminofluorene (AAAF), which is less reactive and has a much longer half-life in aqueous solution than the sulfonyloxy derivative, forms a similar nitrenium ion in aqueous solution (Fig. 1) [12, 13] and, therefore, has been valuable as a model carcinogen and mutagen. When cultured rat embryos are exposed to AAAF, the predominant malformation is prosencephalic hypoplasia [14]. However, when exposed to *N*-*O*-sulfonyloxy-AAF, the most characteristic embryotoxic effect observed is

* This research was supported by NIH Grants ES-04041 and ES-03157.

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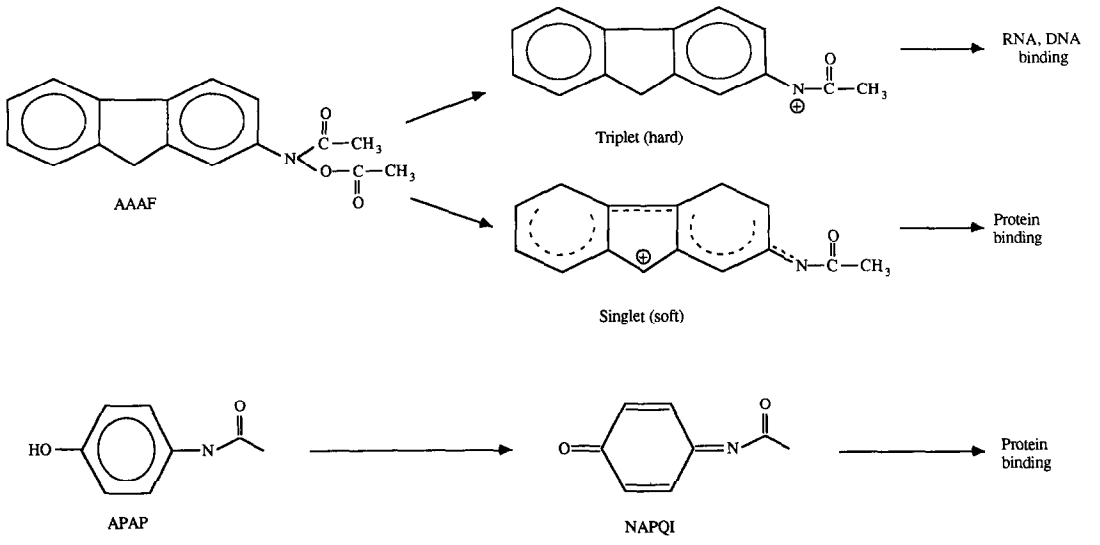

Fig. 1. Principal reactive pathways of AAAF and APAP. In aqueous solution, AAAF undergoes spontaneous heterolytic cleavage to form the highly reactive nitrenium ion. The nitrenium can exist in one of two states; the triplet state binds to RNA and DNA in addition to protein, while the singlet state preferentially attacks protein. Upon a 2-electron oxidation of APAP, the reactive intermediate NAPQI is formed, which subsequently binds almost exclusively to protein.

abnormal axial rotation. Addition of a cytochrome P-450-dependent bioactivating system with the parent compound (AAF) results in the formation of reactive metabolites that produce abnormal neurulation as the most characteristic defect. Concurrent addition of GSH to the culture medium has been shown to reduce the embryolethality as well as the severity (but not the incidence) of prosencephalic hypoplasia produced by AAAF [15].

Acetaminophen (APAP) is a widely used analgesic drug which, in excessive doses, causes hepatotoxicity and tissue necrosis in humans and adult experimental animals [16]. This toxicity has been attributed to the formation of the electrophilic intermediate *N*-acetyl-*p*-benzoquinoneimine (NAPQI) [17]. This intermediate selectively binds to, and oxidizes, both protein and GSH (Fig. 1) [18], although the exact mechanism of hepatotoxicity of NAPQI remains unknown. In addition to the elicitation of toxicity in adults, it has been demonstrated recently that APAP is capable of causing abnormal neurulation in cultured rat embryos exposed during the stage of organogenesis [19]. The defect produced is morphologically similar to that elicited by exposure to 7-hydroxy-2-acetylaminofluorene (7-OH-AAF) [20], but can be distinguished from the open neural tubes produced by cytochalasin D and valproic acid [21]. This specific dysmorphogenesis is also absent from rat conceptuses following exposure to the non-acetylated analogues *p*-aminophenol and 7-OH-AAF [20], suggesting that deacetylation does not play a significant role in the elicitation of abnormal neurulation by either 7-OH-AAF or APAP.

The tripeptide glutathione (GSH) is important in the regulation of diverse cellular processes such as proteolysis [22], intermediary metabolism and enzyme regulation [23], as well as serving a primary role in the biochemical defense against reactive intermediates. Accordingly, modulation of the intracellular concentration of reduced GSH in rat embryos has been described [24] and has been shown to be an important determinant in the protection of the embryo against reactive intermediates [25].

The purpose of this investigation was to: (a) determine whether the rat conceptus is capable of bioactivating APAP to reactive intermediates that bind to cellular macromolecules, (b) explore the electrophilic character of the intermediate generated by APAP and AAAF, and (c) determine the effect of GSH depletion on the dysmorphogenesis produced by APAP and AAAF.

METHODS

Animals and embryo culture. Time-mated, *primigravida*, Sprague–Dawley (Wistar-derived, 180–200 g) rats were obtained locally (Tyler Laboratories, Bellevue, WA). Animals were housed as described [26] and provided with food and water given *ad lib.* The morning following copulation (as evidenced by seminal plugs) was designated as day 0 of gestation.

Dams were anesthetized with ether on the afternoon of gestational day 9, and the uteri were removed. Conceptuses were explanted from the uterus as previously described [27], and cultured overnight in medium saturated with 5% O_2:5% CO_2:90% N_2. To accomplish GSH depletion, conceptuses were exposed to 0.5 mM L-buthionine-*S*,*R*-sulfoximine (BSO) from the beginning of the culture period until the morning of day 10. At 8:30 a.m. on day 10 of gestation, the conceptuses were staged according to somite number, yolk sac diameter, and elevation of the neural folds. Appropriately developed embryos were then cultured for an additional 24-hr period in roller bottles containing fresh medium (saturated with 20% O_2:5% CO_2:75% N_2)

and AAAF or APAP dissolved in either dimethyl sulfoxide (DMSO) or Hanks' Balanced Salt Solution (HBSS), respectively.

At the end of the 24-hr culture period, conceptuses were evaluated for viability as defined by the presence of a heartbeat and active vitelline circulation; non-viable conceptuses were not considered further. Viable embryos were then examined for embryonic length, degree of axial rotation, and neural tube morphology. In addition, two measurements were conducted to assess the severity of prosencephalic hypoplasia. The maximum distance (×2) in mm from the tip of the prosencephalon to the center of the optic cup was designated as the "a" measurement, while the maximum distance from the tip of the prosencephalon to the dorsal edge of the rhombencephalon was designated as the "b" measurement. All morphological assessments were conducted without the investigator having prior knowledge of the treatment. Following assessment, embryos and yolk sacs were separated and placed in 0.5 ml of potassium phosphate (0.1 M)-EDTA (5 mM) buffer (pH 8.0) and stored at −75°. Samples were thawed and ultrasonically disrupted immediately prior to analysis.

Assays. Protein content was determined according to Bradford [28] as modified for a microplate reader. Aliquots of sample homogenates (15 μl) were mixed with diluted protein reagent (200 μl, Bio-Rad Protein Reagent). Bovine plasma γ-globulin was used as the standard (Bio-Rad Standard I). DNA was quantified by the method of Labarca and Paigen [29].

Chemicals. AAAF was synthesized according to Lotlikar *et al.* [30], and recrystallized until a constant melting point (111–112°) in agreement with the published literature values was obtained. The purity was verified to be greater than 99% by reverse phase HPLC on a DuPont Zorbax C-8 column. AAAF (ring-G-^{3}H, 1.1 Ci/mmol) was purchased from the National Cancer Institute repository. Radioactive APAP (ring-G-^{3}H, 4.8 Ci/mmol) was purchased from New England Nuclear (Wilmington, DE) and was greater than 99% radiochemically pure as determined by HPLC on a Zorbax ODS column using 94% water: 6% acetonitrile.

Cesium trifluoroacetate gradients. The simultaneous separation of RNA, DNA and protein in the homogenates of conceptuses was determined by the method of Mirkes [31], with the exception that all glassware and plasticware was treated previously with diethyl pyrocarbonate (Sigma) or baked (450° for 4 hr) to inhibit RNase. Twenty conceptuses (day 10, 10 ± 2 somites) were exposed to 5 μCi of radiolabeled AAAF or APAP in 15 ml of Waymouth's medium (37°, 20% O_2) for 6 hr, rinsed three times in ice-cold HBSS (Gibco) and frozen at −75° in a minimal volume of HBSS until ready for gradient separation. The conceptuses were then centrifuged at 16,000 *g* for 5 sec and processed for the gradient as described [31]. This procedure results in a gradient that contains a visible RNA band at the lowest portion of the tube, followed by an intermediate DNA band, with proteins remaining on the top of the gradient. Following centrifugation, 10-drop fractions were collected from the bottom of the tube. Since the gradient does not separate unbound radioactivity from the protein band at the top of the gradient, 100-μl aliquots of the fractions were precipitated with trichloroacetic acid (TCA, 10% final concentration) after addition of 15 μg carrier protein (bovine serum albumin) and washed with 5% TCA until the radioactivity in the supernatant fraction equaled the background level. The TCA-precipitable macromolecules were redissolved in 1 ml Solulyte (37°, overnight), neutralized with 140 μl glacial acetic acid (final pH 6.0), and counted with 3 ml Ready-Safe (Beckman Instruments, Fullerton, CA) in a liquid scintillation counter. The protein and DNA concentrations in each fraction were determined as described above.

Statistics. Analysis of variance (one-way, two-tailed) followed by the Student–Newman–Keuls' multiple range test [32] were used to evaluate differences among sample means for protein content, somite number and embryonic length. Malformation incidence and viability were evaluated using a chi-square test. The level of significance chosen was 95% ($P \leq 0.05$).

RESULTS

Bioactivation and binding of APAP and AAAF. To investigate simultaneously whether conceptuses were capable of bioactivating APAP to a reactive intermediate(s) that binds to cellular macromolecules, as well as to determine which cellular nucleophiles were preferentially attacked by APAP and AAAF, the RNA, DNA and protein from rat conceptuses exposed to radiolabeled APAP and AAAF were separated by cesium trifluoroacetate density centrifugation. Upon exposure of conceptuses to 5 μCi of [^{3}H]APAP for 6 hr, TCA-precipitable radioactivity was present only in gradient fractions containing protein (fractions 25–32), while no radioactivity was present in fractions that contained RNA (determined visually to be approximately fractions 10–12) or DNA (fractions 14–19). A representative gradient fractionation is shown in Fig. 2A. These data indicate that organogenesis-stage rat conceptuses possess the capacity to bioactivate APAP to a reactive intermediate(s) that selectively binds to cellular protein. In contrast, exposure of rat conceptuses to 5 μCi of [^{3}H]AAAF for 6 hr resulted in much higher levels of binding in protein-containing fractions (26–31), and also resulted in readily detectable binding to cellular RNA (fractions 8–10) and DNA (fractions 17–22), as shown in Fig. 2B. Since AAAF breaks down spontaneously in aqueous solution to yield the nitrenium ion, embryonic bioactivation enzymes are not necessarily involved in the binding of this chemical to cellular macromolecules.

To determine the effects of GSH depletion on the binding of APAP and AAAF to embryonic macromolecules, conceptuses were pretreated with BSO for 16 hr prior to exposure and subjected to gradient separation. When the quantities of radioactivity bound to DNA and protein were averaged for several gradients, the data in Table 1 were obtained. Prior depletion of GSH resulted in significant increases in covalent binding of both APAP and AAAF, although each compound displayed differing trends. Relative to exposure to APAP alone, GSH depletion

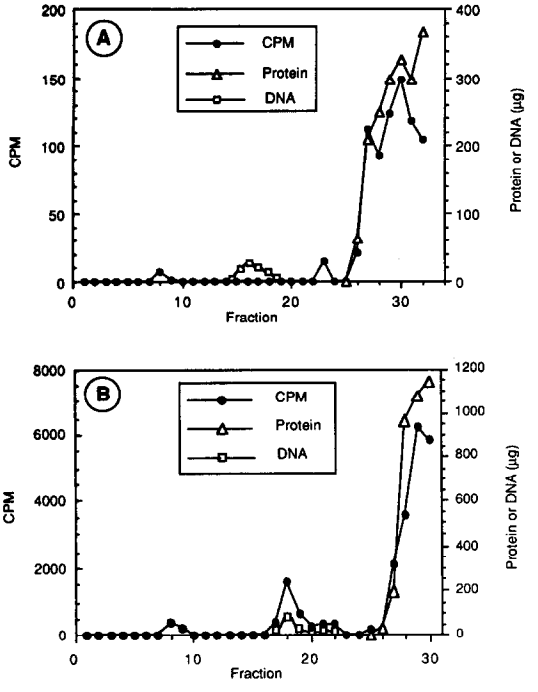

Fig. 2. Covalent binding of [^{3}H]APAP (A) and [^{3}H]AAAF (B) to RNA, DNA and protein of rat conceptuses. Rat conceptuses were exposed for 6 hr on the morning of day 10 to 5 μCi of radiolabeled chemical. Cellular macromolecules were separated by cesium trifluoroacetate gradient centrifugation. The gradients were fractionated, unbound counts were removed by TCA precipitation, and the amounts of radioactivity, DNA, and protein were determined for each fraction.

Table 1. Binding of radiolabeled AAAF and APAP to DNA and protein of the rat conceptus

Treatment	Protein (cpm/μg)	DNA (cpm/μg)
APAP	0.437 ± 0.05	ND*
APAP + BSO	1.44 ± 0.18†	ND
AAAF	6.19 ± 1.3	18.5 ± 0.41
AAAF + BSS	8.76 ± 0.83	26.8 ± 0.89†

Data are the means (± SE) of three gradients.
* Not detected.
† Significantly different from conceptuses not pre-exposed to BSO ($P \leq 0.05$, Student's t-test).

resulted in a more than 3-fold increase in binding to protein, while binding to DNA remained undetectable. In contrast, GSH depletion resulted in significant increases in the binding of AAAF to DNA, but not to protein.

Embryonic growth parameters. After exposure for 24 hr, both AAAF and APAP produced significant embryotoxicity and malformations, as assessed by measurements of embryonic growth parameters (Table 2). Significant decreases in embryonic size were observed after exposure to initial concentrations greater than 90 μM (AAAF) or 500 μM (APAP). Based upon the observed effects on the gross appearance of the embryos and yolk sacs, as well as the embryonic size, AAAF was more toxic to the embryos than was APAP. At higher concentrations of AAAF (250 μM), the extent of yolk sac vasculature was decreased markedly, with only the larger vitelline vessels visible.

When GSH depletion was desired, conceptuses were pre-exposed to BSO (0.5 mM, dissolved in the culture medium) from the start of the culture period on day 9.5 until the morning of day 10. Pretreatment with BSO resulted in significantly ($P < 0.05$) decreased embryonic size and increased incidence of abnormal axial rotation after exposure to 500 μM APAP.

As previously described [20], exposure to APAP produced abnormally open anterior neuropores as the most characteristic malformation, with the neural folds remaining elevated but separated by approximately 45 degrees. The predominant malformation observed upon exposure to AAAF was prosencephalic hypoplasia accompanied by decreased yolk sac vasculature and decreased embryonic size.

Embryonic and yolk sac protein content. When conceptuses exposed to APAP were assayed for protein content, the results depicted in Fig. 3 were obtained. A significant decrease in protein, relative to control, was observed only for embryos exposed to 500 μM APAP. This is consistent with the observation that APAP produced a relatively specific defect with a minimum of generalized embryotoxicity, necrosis, and decreased size. However, when the GSH was depleted previously by BSO pretreatment, exposure to APAP resulted in decreased protein content in both the embryo and yolk sac, relative to the effects observed with APAP alone. Pretreatment with BSO alone did not alter significantly the protein concentration in either the embryo or the yolk sac relative to untreated controls.

In contrast to the effects of exposure to APAP, exposure to AAAF produced marked decreases in both embryonic and yolk sac protein in the absence of GSH depletion (Fig. 4). For example, the maximum decrease in embryonic protein associated with APAP exposure was 22% compared with 55% for AAAF-exposed embryos. In further contrast to APAP, when AAAF exposure was superimposed upon GSH depletion, no significant differences relative to AAAF alone were observed.

Chemical dysmorphogenesis. To further determine the relative response of conceptuses to these agents, it was of interest to compare the effect of GSH depletion on the characteristic dysmorphogenesis produced by each of these agents in addition to the more non-specific growth parameters and biochemical end-points discussed above. Therefore, the incidence of abnormally open anterior neural tubes was determined for embryos exposed to APAP, while the degree of prosencephalic hypoplasia produced by AAAF was quantified by measuring the maximum distance from the edge of the prosencephalon to the center of the optic cup ("a" measurement) as well as to the dorsal edge of the rhombencephalon ("b" measurement).

As shown in Fig. 5, exposure to APAP (250–500 μM) resulted in concentration-dependent and

Table 2. Growth parameters of embryos exposed for 24 hr to AAAF or APAP in conjunction with GSH depletion

Treatment group	% Viability	Embryonic length (mm)	% Abnormal axial rotation	N
Medium control	100	3.11 ± 0.05*	3	62
BSO control	100	3.10 ± 0.06	7	31
AAAF (60 μM)	97	3.02 ± 0.05	7	38
AAAF (60 μM) + BSO	90	2.90 ± 0.06	13	30
AAAF (90 μM)	100	2.59 ± 0.05†	6	16
AAAF (90 μM) + BSO	94	2.51 ± 0.04	6	16
AAAF (120 μM)	100	2.57 ± 0.03†	7	24
AAAF (120 μM) + BSO	100	2.64 ± 0.05	8	28
APAP (250 μM)	100	3.20 ± 0.05	4	24
APAP (250 μM) + BSO	100	3.13 ± 0.06	5	19
APAP (500 μM)	100	2.93 ± 0.05†	0	26
APAP (500 μM) + BSO	100	2.79 ± 0.08‡	28	16

* Mean ± SE.
† Statistically significant ($P \leq 0.05$) compared to corresponding control.
‡ Statistically significant ($P \leq 0.05$) from exposure to APAP alone.

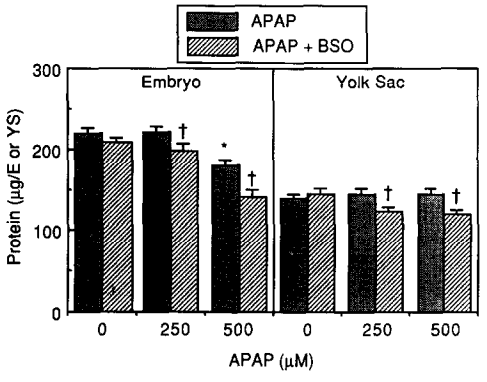

Fig. 3. Protein content of embryos and yolk sacs following 24-hr exposure of the conceptus to various concentrations of APAP alone and in combination with GSH depletion. Values are the means (± SE) of at least three experiments and are expressed as μg protein/embryo or yolk sac. BSO denotes pre-exposure of conceptuses to 0.5 mM BSO for 16 hr prior to addition of APAP. Key: (*) significantly ($P \leq 0.05$) different from control, and (†) significantly ($P \leq 0.05$) different from APAP exposure alone. Pretreatment with BSO alone resulted in protein values that did not differ significantly ($P > 0.05$) from the corresponding controls.

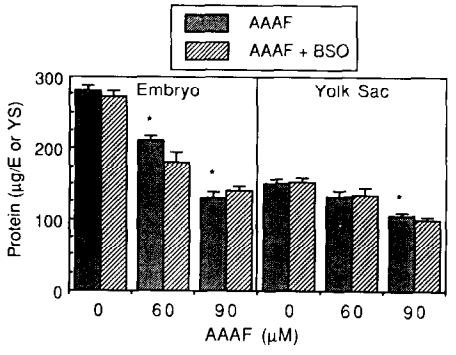

Fig. 4. Protein content of embryos and yolk sacs following 24-hr exposure of the conceptus to various concentrations of AAAF alone and in combination with GSH depletion. Values are the means (± SE) of at least three experiments and are expressed as μg protein/embryo or yolk sac. BSO denotes pre-exposure of the conceptus to 0.5 mM BSO for 16 hr prior to addition of AAAF. Key: (*) Significantly ($P \leq 0.05$) different from control. Pretreatment with BSO alone resulted in protein values that did not differ significantly ($P > 0.05$) from the corresponding controls.

statistically significant increases ($P \leq 0.05$) in the incidence of abnormally open neural tubes relative to non-exposed conceptuses. Pretreatment of the conceptuses with BSO to deplete GSH resulted in further increases relative to embryos exposed to only APAP.

Exposure to AAAF resulted in decreased prosencephalic length (as measured by both the "a" and "b" measurements) which was significantly different ($P \leq 0.05$) from that of the corresponding controls at the 90 μM concentration (Fig. 6). Since conceptuses exposed to 120 μM AAAF typically were anophthalmic, these embryos were not examined for prosencephalic length. In contrast to the effects of GSH depletion on APAP-elicited dysmorphogenesis, prior depletion of GSH did not result in additional decreases in either the "a" or "b" measurement relative to AAAF exposure alone.

DISCUSSION

The data presented here indicate that both GSH status as well as the character of the electrophilic intermediate can have marked effects on chemical dysmorphogenesis produced in rat embryos. Prior depletion of GSH potentiated the binding of APAP to protein, the APAP-elicited decreases in embryonic length and protein, as well as increases in the incidence of abnormal axial rotation and abnormally open neural tubes. In contrast, neither the incidence

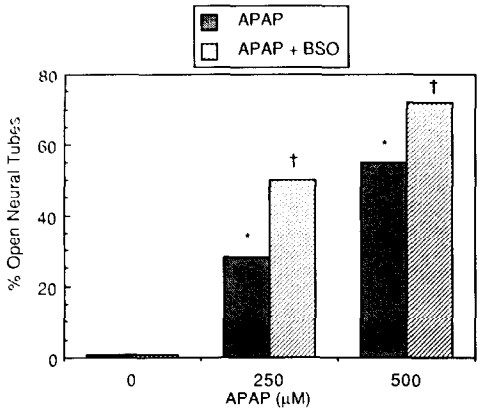

Fig. 5. Incidence of abnormally open anterior neural tubes following 24-hr exposure to APAP alone or in combination with GSH depletion. BSO denotes pre-exposure to the conceptus to 0.5 mM BSO for 16 hr prior to addition of APAP. Key: (*) significantly ($P \leq 0.05$) different from control; and (†) significantly ($P \leq 0.05$) different from APAP exposure alone. Pretreatment with BSO alone had no significant effect ($P > 0.05$) on the incidence of abnormal neurulation.

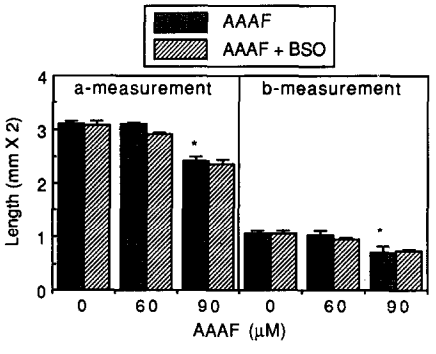

Fig. 6. Severity of prosencephalic hypoplasia following exposure for 24 hr to AAAF alone and in combination with GSH depletion. The "a" measurement was determined by measuring the maximum distance (in mm, ×2) from the edge of the prosencephalon to the center of the optic cup; the "b" measurement was determined by measuring the maximum distance (in mm, ×2) from the edge of the prosencephalon to the dorsal edge of the rhombencephalon. BSO denotes pre-exposure of the conceptus to 0.5 mM BSO for 16 hr prior to addition of AAAF. Key: (*) significantly ($P \leq 0.05$) different from control. BSO pretreatment alone had no significant ($P > 0.05$) effect on the severity of prosencephalic hypoplasia. Values are the means ± SE of at least three experiments.

nor the severity of malformations produced by AAAF was altered by GSH depletion.

As mentioned previously, the interaction of electrophiles with biological nucleophiles can be interpreted based upon the character of the electrophile, i.e. whether the intermediate is chemically "hard" or "soft" [2, 33]. Previous investigations have demonstrated that the nitrenium ion produced upon heterolytic cleavage of AAAF is a relatively hard electrophile [34]. While the nitrenium ion can exist as a ground state singlet or triplet ion, MNDO calculations reveal that the singlet state is more stable [35, 36]. In the singlet state, the electrophilic center is delocalized throughout the aromatic ring system, while the positive charge is highly localized on the nitrogen in the triplet state [34]. Thus, the soft singlet ion will preferentially react with soft nucleophiles (such as GSH), and the hard triplet ion will preferentially react with hard nucleophiles (DNA, RNA). In support of this hypothesis is the observation that RNA does not compete with GSH for binding, supporting the presence of two different reactive intermediates [37]. Studies of the interactions between cellular nucleophiles and AAAF have shown the order of nucleophilicity to be tRNA = polyguanylic acid > *N*-acetyl-L-methionine, indicating that AAAF can preferentially react with hard nucleophiles [37, 38].

Although AAAF is capable of being converted to either hard or soft electrophiles, the results presented in this study are consistent with the concept that AAAF-elicited prosencephalic hypoplasia observed in rat embryos results from the generation of a hard electrophile(s). The most direct evidence for generation of a hard electrophilic species comes from the observation that AAAF is capable of binding to hard (DNA, RNA) cellular nucleophiles in addition to softer nucleophiles (protein). Since the interaction of the nitrenium ion with a soft nucleophile such as GSH would not be favored, the depletion of GSH in the rat conceptus would not be expected to modulate significantly the incidence or severity of AAAF-elicited dysmorphogenesis. The inability of GSH depletion to potentiate either the decrease in embryonic protein or the prosencephalic hypoplasia produced by AAAF supports this conclusion, although GSH depletion resulted in a slight (1.4-fold) increase in binding to DNA.

An important but largely unanswered question pertains to the presence, activity and substrate specificity of glutathione-*S*-transferase isozymes (GST) in the early rat conceptus. Since GSTs catalyze the conjugation of GSH with a variety of electrophilic substrates, the interaction between hard electrophiles and GSH can thereby become relatively efficient. The best example of this may be the role of GST and GSH in detoxifying the 2,3-oxide intermediate of aflatoxin B_1 (AFB_1), a hard electrophile. This conjugation reaction occurs only if GST is present [39], and the species susceptibility to AFB_1 correlates with the presence and activity of GSTs [40]. It is conceivable that the presence of such transferases could potentially influence the interpratation of the results presented here. In addition, physico-chemical factors must also be taken into consideration prior to drawing any final conclusions.

In contrast to AAAF, the embryotoxic electrophilic species generated from APAP (presumably NAPQI) appears to behave almost exclusively as a soft electrophile. The order of nucleophilic attack by several sulfhydryl compounds was found to be GSH = *N*-acetyl-cysteine > cysteine > methionine (soft to hard) [41]. In addition, protein sulfhydryls and GSH have long been regarded as the primary sites of electrophilic attack following APAP

exposure [42], and a thiol-protein adduct of APAP has been identified [43]. Although the exact mechanism(s) of toxicity is unknown, evidence suggests that NAPQI depletion of cellular thiols and the subsequent oxidation of cellular proteins may be critical [44].

Since APAP requires enzymatic bioactivation to exert toxicity in hepatocytes, the extent and capacity of rat conceptuses to convert APAP to embryotoxic metabolites would expectedly be an important biochemical determinant for embryonic susceptibility. The data presented here suggest that the rat conceptus, as is the case in the adult, possesses the capacity to bioactivate APAP to a soft electrophile which interacts almost exclusively with protein and GSH. APAP also was found to produce dysmorphogenesis in the same concentration range as that required to produce toxicity in isolated hepatocytes. The critical role of GSH in modulating APAP-elicited embryotoxicity is supported by the increase in binding to protein, and the potentiation of embryotoxicity and dysmorphogenesis upon GSH depletion.

In summary, the data presented here indicate that the interaction of two factors—the electrophilic character of the responsible reactive intermediates as well as the quantitative presence of GSH (and, by extension, GST) in target cells—can exert marked effects on the observed embryotoxicity. Considerations of electrophilic character may help explain previous observations of chemical embryotoxicity, such as the report that GSH depletion enhances the embryotoxicity of acrolein, but not phosphoramide mustard [45], the influence of embryonic age upon the toxicity of 7-OH-AAF following GSH depletion [21], and the role of extracellular GSH in modulating the embryotoxicity of AAAF [15]. Future investigations of the presence of GST isozymes during embryonic development can be expected to provide further valuable insight into the capacity of embryos to mount a biochemical defense against reactive intermediates.

Acknowledgements—The technical assistance of Hseuh Ying-Yang and the generous assistance with the density gradients by Dick Grace are gratefully acknowledged.

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