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www.toxsci.oxfordjournals.org

TOXICOLOGICAL SCIENCES, 146(2), 2015, 386–394

doi: 10.1093/toxsci/kfv104
Advance Access Publication Date: July 3, 2015
Research Article

Dosimetry of Acrylamide and Glycidamide Over the Lifespan in a 2-Year Bioassay of Acrylamide in Wistar Han Rats

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ABSTRACT

Acrylamide is an industrial chemical used to manufacture polymers, and is produced in foods during cooking at high heat. Hemoglobin adducts provide a long-lived dosimeter for acrylamide and glycidamide. This study determined acrylamide and glycidamide hemoglobin adducts (AAVal and GAVal) during a lifetime carcinogenesis bioassay. Exposure to acrylamide in drinking water began *in utero* in pregnant rats on gestation day 6. Dams were administered acrylamide until weaning, and male and female F1 rats were exposed for a further 104 weeks. Acrylamide concentration in drinking water was adjusted to provide a constant dose of 0.5, 1.5, and 3 mg/kg/day. Blood was collected from animals euthanized at 2, 60, 90, and 120 days and 53, 79, and 104 weeks after weaning. Low levels of AAVal and GAVal at postnatal day 24 suggested that little exposure to acrylamide occurred by placental or lactational transfer, and extensive metabolism to glycidamide occurred with a GAVal:AAVal ratio of 4. Adduct levels varied somewhat from 60 days to 2 years, with a GAVal:AAVal ratio of approximately 1. Adduct formation/day estimated at each timepoint at 3 mg/kg/day for AAVal was 1293 ± 220 and 1096 ± 338 fmol/mg/day for male and female rats, respectively. Adduct formation per day estimated at each timepoint at 3 mg/kg/day for GAVal was 827 ± 78 fmol/mg/day for male rats, and 982 ± 222 fmol/mg/day for female rats. The study has provided estimates of linearity for dose response, and variability in internal dose throughout an entire 2-year bioassay, including the early phases of pregnancy and lactation.

Key words: acrylamide; glycidamide; hemoglobin adducts

Acrylamide is an industrial chemical used in the production of a variety of polymers which are used in waste water treatment and recovery, oil processing, and paper manufacture. Acrylamide is neurotoxic in animals and humans, it is genotoxic and caused heritable translocations in exposed rodents, and it is carcinogenic in rats and mice (IARC, 1994). Acrylamide is produced during the cooking of a variety of foods, including French fries, potato chips, and baked goods (Friedman, 2005; Tareke *et al.*, 2000, 2002). The discovery of acrylamide in foods raised concern about the potential health effects of everyday exposure through the diet (Tareke *et al.*, 2002; WHO, 2002).

Acrylamide is reactive and is a Michael acceptor, and reacts primarily with glutathione and sulfhydryl groups (Edwards, 1975; Friedman, 2005). It is metabolized by 2 main pathways—conjugation with glutathione and excretion as mercapturic acids (Dixit *et al.*, 1982), and by oxidation to glycidamide, a reactive epoxide (Calleman *et al.*, 1990; Fennell *et al.*, 2005; Sumner *et al.*, 1992). Glycidamide undergoes further metabolism by

conjugation with glutathione, to form several mercapturic acids, and by hydrolysis (Sumner *et al.*, 1992).

Both acrylamide and glycidamide react with proteins, and form adducts with hemoglobin (Bailey *et al.*, 1987; Bergmark, 1997; Calleman *et al.*, 1990, 1994; Perez *et al.*, 1999; Sumner *et al.*, 2003). Glycidamide also reacts with DNA, causing adducts that may lead to mutagenicity (Gamboa da Costa *et al.*, 2003; Segerbäck *et al.*, 1995). Adducts in hemoglobin form a convenient means of estimating the extent of exposure to reactive chemicals, and in the case of acrylamide and glycidamide, both form adducts with the N-terminal valine residue of hemoglobin (AAVal and GAVal). The determination of AAVal and GAVal together indicate the extent of exposure and the extent of metabolism of acrylamide to glycidamide (Fennell *et al.*, 2005; Sumner *et al.*, 2003). Hemoglobin adducts are formed at low levels, and with constant exposure, will accumulate to reach a steady state where the rate of formation equals the rate of removal (Ehrenberg and Osterman-Golkar, 1980; Ehrenberg *et al.*, 1986; Osterman-Golkar *et al.*, 1976). This will be achieved when the exposure has continued for the lifespan of the erythrocyte, which in the rat is approximately 60–65 days. The determination of hemoglobin adducts will provide a long term integrated dosimeter over the lifespan of the red blood cell.

In recent years, concern about the potential health effects of exposure to chemicals during development *in utero* and in children has led to changes in risk assessment and in the design of carcinogenesis bioassays. Inclusion of exposure *in utero* and of mothers during lactation provides a means of evaluating the effect of early life exposures to chemicals.

This study was designed to combine examination of the effect of exposure to acrylamide over the entire lifespan in the rat, beginning *in utero* at implantation, continuing through lactation, into adulthood with the determination of hemoglobin adducts from acrylamide and glycidamide.

The objective of this study was to evaluate the formation of AAVal and GAVal as indicators of internal dose of acrylamide and metabolism to glycidamide over the course of a 2-year oncogenicity study.

MATERIALS AND METHODS

Animal Exposures

The in-life phase of this study was conducted under GLP guidelines enacted in Germany in the "Chemikaliengesetz", current edition and "OECD Principles of Good Laboratory Practice" Document Nos. 1, 8, and 13 ENV/MC/CHEM (98) 17, ENV/JM/MON0(99) 24, and ENV/JM/MONO (2002).

Test Material. Acrylamide (C_3H_3NO, CAS no 79-06-1, 1,2-propenamide) >99.9 % pure was purchased from Sigma Aldrich (Buchs, Switzerland) and stored at room temperature. In a separate study, solutions of acrylamide were prepared in tap water and evaluated for stability by liquid chromatography at 6, 13, 20, 27, 41, 55, or 90 test days after preparation and recovery ranged from 96.9 to 102.6%. Acrylamide solutions were prepared weekly after adjustment for body weight and water consumption. The concentration was adjusted to provide doses of 0, 0.5, 1.5, and 3 mg/kg/day through the study. Water bottles were changed weekly. Aliquots for analysis were taken at the beginning of exposure and at the end of exposure (to verify stability). Acrylamide concentration in the drinking water was determined at test week 4, 10, 16, 22, 28, 34, 40, 46, 52, 65, 78, and 91.

Animals. Sperm positive female Wistar Han/RccHan:WIST rats were obtained from Harlan Laboratories GmbH, Serumweg 48, 27324 Eystrup, Germany in multiple deliveries. At gestation day (GD) 6, pregnant dams were provided acrylamide in their drinking water. Exposure of the dams continued through pregnancy, and the dams with their offspring following birth through weaning at postnatal day (PND) 21. Exposure of the pups continued through PND 722. Dams were housed individually, and then with their pups until weaning at PND 21. Thereafter, the offspring were housed 1 per cage in MACROLON cages with granulated wood bedding (Brandenburg, 49424 Goldenstedt/Arkeburg). Rats were fed commercial ssniff R/Z V1324 feed (ssniff Spezialdiäten GmbH, 59494 Soest, Germany) ad libitum. Only batches with an acrylamide content <30 μg/kg diet were used in this study. This food was offered daily. Food residue was removed and weighed on a weekly basis. Feed and tap water were available ad libitum. The animal rooms were alternately lit (about 150 lux at approximately 1.50-m room height) and darkened in a 12 h dark/12 h light cycle. Cage side observations were conducted twice per day during the week and once per day on weekends. On day 4 after birth, the weights of the pups were determined. The size of each litter was adjusted by eliminating extra pups to yield, as nearly as possible, 5 males and 5 females per litter. The remaining animals were allowed to remain with the dams until PND 21 (weaning), at which time the F1 animals were randomized using a computer randomization program to assign the animals to the subsets within each group. No blood samples were collected from the dams. Five male and 5 female rats were euthanized for terminal blood sample collection and hemoglobin adduct analysis at each timepoint (see Table 1). These animals were selected to be devoid of gross lesions.

At least 2 ml EDTA preserved blood was obtained from each animal from the retrobulbar venous plexus under light ether anesthesia. The whole blood sample was cooled using an IsoTherm-Rack until centrifugation for 10 min at 800×g. Plasma was removed and the pellet resuspended in saline and recentrifuged. This washing procedure was repeated 3 times. Samples were then frozen at −20°C.

Hemoglobin Adduct Analysis

Samples were analyzed at RTI International for the presence of AAVal and GAVal using previously described methods (Fennell *et al.* 2005), based on the modifed Edman degradation method for N-terminal valine adducts reported by Törnqvist *et al.* (1986). Globin isolation was conducted from washed red blood cells using the method of Mowrer *et al.* (1986). Lysed red blood cells were treated with 50 mM HCl in 2-propanol and centrifuged to remove heme, and the resulting supernatant was mixed with ethyl acetate to precipitate globin, which was collected by centrifugation. The globin was then washed with n-pentane and dried under vacuum. Globin samples were derivatized with phenylisothiocyanate in formamide to yield adduct phenylthiohydantoin derivatives. AAValPTH-$^{13}C_5$ and GAValPTH-$^{13}C_5$ were added as internal standards, and the samples were extracted using a Waters Oasis HLB 3 cc (60 mg) extraction cartridge (Milford, Massachusetts). The samples were eluted with methanol, dried, and reconstituted in 100 μl of 50:50 MeOH:H_2O (containing 0.1% formic acid). Analysis was conducted with an Agilent 1200 HPLC system coupled to a PE Sciex API 5000 LC-MS/MS with a Turboionspray interface. Separation was conducted on a Phenomenex Luna Phenyl-Hexyl Column (50 × 2 mm, 3 μM) eluted with 0.1% acetic acid in water and methanol at a flow rate of 400 μl/min, with a gradient of 40–70% methanol in 3 min. AAVal and GAVal were quantitated using the ratio of analyte to internal standard peak area, with a calibration curve generated

TABLE 1. Exposure Duration for Each Timepoint, Indicating the Duration of *In Utero*, Lactational, and Postlactational Exposure

Timepoint[a]	*In Utero* Exposure	Lactational Exposure	Postlactational Exposure
2 days	GD 6—Birth	Birth – PND 21	2 days
60 days	GD 6–Birth	Birth–PND 21	60 days
90 days	GD 6–Birth	Birth–PND 21	90 days
120 days	GD 6–Birth	Birth–PND 21	120 days
1 year	GD 6–Birth	Birth–PND 21	366 days
18 months	GD 6–Birth	Birth–PND 21	549 days
2 years	GD 6–Birth	Birth–PND 21	729–731 days

[a]Timepoint designated as the duration of the time following weaning.

using AAVal-leu-anilide, or GAVal-leu-anilide (Bachem Americas, Torrance California).

Adduct Simulation

Hemoglobin adduct levels were simulated with an Excel (Microsoft Excel 2013) encoded version of the model reported by Fennell *et al.* (1992) for the formation and removal of hemoglobin adducts based on exposure and erythrocyte synthesis and removal by a zero-order process. The input parameters were duration of exposure, daily adduct formation, the erythrocyte lifespan (65 days for rats), and a first-order removal term ($k = 0.005$ day^{-1}) (Fennell *et al.*, 1992). Correction for body weight changes was made as described by Walker *et al.* (1992), using the mean change in body weight sampled weekly or biweekly, with calculation of a daily body weight change. Simulation of adduct formation was conducted with fitting each timepoint mean value for GAVal and AAVal individually, to estimate the daily adduct formation. Linear regression analysis was conducted with Prism 5 (GraphPad Software, Inc., La Jolla California).

RESULTS

Animal Exposures

Animal exposure was conducted to span the entire development and life of male and female rats, with administration of acrylamide beginning *in utero* on GD 6, and continuing to 107 weeks of age (Table 1). The administration of acrylamide was adjusted to be a constant dose (on a mg/kg/day basis), rather than using a constant concentration. Drinking water consumption data were used to adjust the concentration of acrylamide at weekly intervals of the first 13 weeks, and then at intervals of 6 weeks, to achieve the target dose. For the first week, the concentration was targeted based on the expected water consumption to achieve the dose. However, in the first week after weaning, water consumption was less than expected, and the calculated dose of acrylamide for that week was approximately half of that expected. By the second week, the estimated dose achieved the targeted dose levels, and continued throughout the 2 years. At the end of 2 years, mammary gland fibroadenomas in females and thyroid follicular cell tumors in both sexes were the only tumors increased in acrylamide-treated rats (see Maronpot *et al.*, 2015 for details).

Hemoglobin Adduct Determination

The main study design included the terminal sampling of blood for adduct measurement at various times over the course of 2 years. The initial timepoint collected immediately after weaning in male pups on PND 24 indicated that the extent of AAVal formation was extremely low (Table 2). At the lowest dose, AAVal was 100 fmol/mg increasing to 240 and 515 fmol/mg at the middle and high dose. GAVal was substantially higher than AAVal, with ratios of 3.6–4.2 at this time point. The low levels of AAVal are consistent with the estimated dose based on drinking water consumption only for a short duration.

AAVal values determined in male and female adult rats for the timepoints from 60 days to 2 years are shown in Figure 1. In general within each dose group, the mean level of adducts showed an upward trend but the differences were not substantial at 60, 90, and 120 days. This trend was consistent with an increase in adduct level reaching steady state when the exposure duration exceeded the lifespan of the erythrocyte, which in the rat is approximately 60–65 days. The adduct levels formed in the exposed animals were substantially higher than those found at PND 24. Very low levels of adducts were detected in the control group, consistent with low levels of AA in feed (mean values $\pm$ SD at all timepoints from 60 days to 2 years were 21 ± 11 and 27 ± 11 fmol/mg globin for AAVal in male and female rats, respectively, and 22 ± 11 and 30 ± 16 fmol/mg globin for GAVal in male and female rats, respectively).

In the 1-year samples, there was a decrease in mean values for AAVal that was apparent in both the 1.5 and 3 mg/kg/day groups in male rats. At 18 months and 2 years, the mean values for AAVal increased to levels similar to those observed at the early timepoints. In female rats, a similar pattern was observed with the early timepoints showing approximately the same mean values (27 079–32 295 fmol/mg globin at 3 mg/kg/day). By 1 year, AAVal decreased to 18 187 fmol/mg at the high dose, and then substantially rebounded by 18 months to 46 522 fmol/mg. It should be noted that for this timepoint (18 months), blood samples were only available from 3 rats. By 2 years, AAVal was 28 655 fmol/mg. With the exception of 1-year timepoint, the dose response curves overlapped (Fig. 2). GAVal values in general did not substantially change over the course of the study in male rats (Fig. 3). In female rats, GAVal and AAVal showed a similar pattern of change with dose (Fig. 4) and time.

In both male and female rat control animals, there was variability in the ratio of GAVal:AAVal, with mean values of approximately 1 (Fig. 5). Control mean values ranged from 0.79 to 1.45 in females and 0.88–1.33 in males. In male rats administered acrylamide, there was a general decrease in the ratio compared with controls, with a range of values from 0.53 to 0.92 (Fig. 5). There did not appear to be a change associated with age or dose. In female rats, the range of values in control rats (0.79–1.45) overlapped the range observed in acrylamide dosed rats (0.76–1.2). There did not appear to be an age- or dose- dependent change in this ratio in female rats.

Simulation of the adduct formation (Fennell *et al.*, 1992), with estimation of the amount of adduct formed per day, was conducted for each timepoint, assuming an erythrocyte lifespan of 65 days. The simulation covered the duration from birth to termination at 2 years. Exposure was simulated starting at weaning for the 60-day and subsequent timepoints. From the data in Table 2 for the PND 24 rats, it appeared that exposure to AA was very limited during lactation, and simulation of exposure was conducted starting at weaning. AAVal after 2 days of exposure appeared to be approximately 50% of that for a single day estimated for adult rats. Because there is substantial growth during the course of the study, correction for adduct dilution from growth was included. With the zero-order turnover of red blood cells, adduct levels accumulate with repeated exposure and reach a steady state when the duration of exposure exceeds the erythrocyte lifespan. The simulations required input of the

TABLE 2. Adducts Formed in Male Rat Pups on PND 24 Administered Acrylamide in Drinking Water

Group (mg/kg/day)	AAVal (fmol/mg globin)	GAVal (fmol/mg globin)	GAVal:AAVal
0	17.0 ± 3.1	27.5 ± 1.89	1.62
0.5	100.0 ± 23.3	364 ± 39.7	3.64
1.5	240.2 ± 52.7	1015 ± 200	4.23
3	515.4 ± 97.8	2071 ± 291	4.02

Dams were administered acrylamide in drinking water from GD6–PND 21.

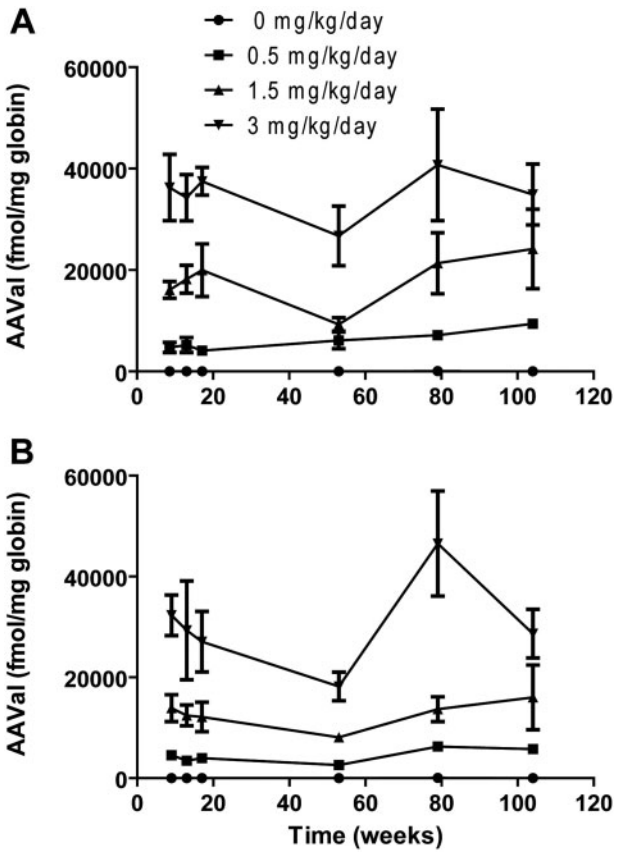

FIG. 1. AAVal in male (A) and female (B) rats administered acrylamide in drinking water from GD6 through PND 722. Animals (5 per group) were euthanized at 9, 13, 17, 53, 79, and 104 weeks after weaning. Values represent mean ± SD.

daily adduct increment from exposure to achieve a simulated value that matched the measured adduct concentration. The estimated values of adduct formed per day of exposure are shown in Table 3, as the mean ± SD of the calculated values across the 2-year period for each dose group. In male rats, the average adduct increment per day of exposure at the high dose was 1293 ± 220 fmol/mg globin/day for AAVal and for GAVal was 827 ± 78 fmol/mg globin/day. The extent of adduct formation per day estimated for female rats at the high dose was 1096 ± 338 fmol/mg globin/day for AAVal and 982 ± 222 fmol/mg globin/day for GAVal. Similarly the values were estimated for the low and mid doses. Determination of the slopes of the curves gives a response normalized per mg acrylamide/kg body weight. Several drinking water studies with measured acrylamide hemoglobin adducts provides a comparison. Tareke *et al.* (2006) conducted exposure of male and female F344 rats to approximately 1 mg acrylamide/kg daily for 50 days in drinking

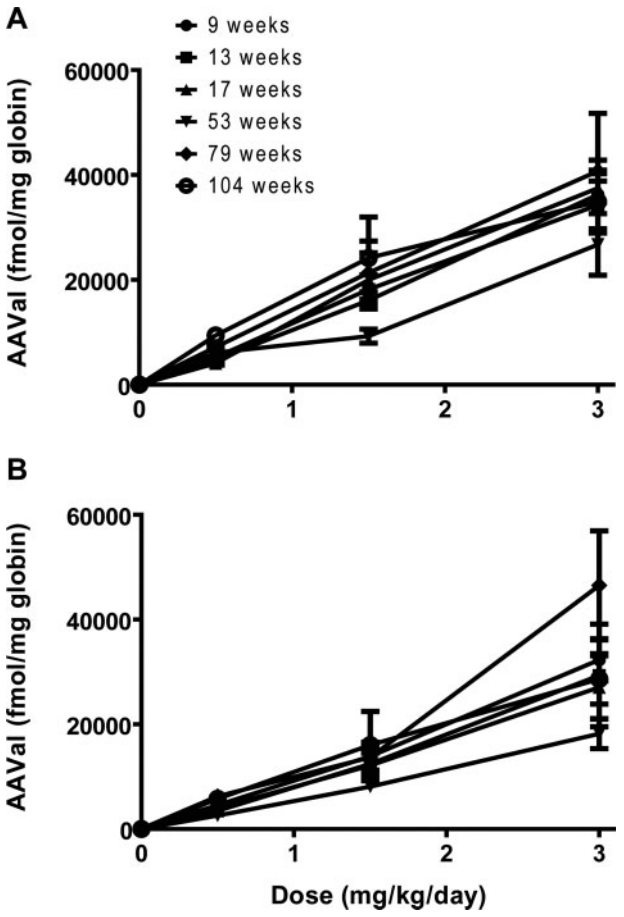

FIG. 2. AAVal Dose response in male (A) and female (B) rats administered acrylamide in drinking water at 9, 13, 17, 53, 79, and 104 weeks after weaning. Values represent mean ± SD (5 rats per group).

water, and measured AAVal and GAVal at various times. Simulation of the adduct data, without correction for body weight change, yielded values for the daily adduct increment (Table 3). The slopes for the data reported here plotted against dose (the amount of adduct formed per day per mg/kg dose, Table 3) were compared with values for 1 mg/kg from Tareke *et al.* (2006). The AAVal values in males and females here were approximately 2.8- and 2.4-fold higher than those reported by Tareke *et al.* (2006). However, the GAVal values were slightly lower for males (0.9-fold) and substantially lower for females (0.6-fold). Törnqvist *et al.* (2008) exposed Fischer 344 rats to acrylamide in drinking water for 7 days, to doses of 0.5 and 2 mg/kg/day (males) and 0.1, 0.5, and 2 mg/kg/day (females). The reported adduct levels were used to estimate adduct increment values, as well as an estimate of the amount of adduct formed per mg/kg/day (Table 3). Comparison with the results of this study indicates higher AAVal adduct formation in both male and female rats, but similar GAVal formation in both genders.

DISCUSSION

This adduct study was conducted as part of an animal bioassay to assess the carcinogenic activity of acrylamide in a strain of rats that had not been investigated previously (Maronpot *et al.*, 2015). Johnson *et al.* (1986) conducted a study in Fischer 344 rats administered 0, 0.01, 0.1, 0.5, and 2 mg/kg/day in drinking water for 2 years. Friedman *et al.* (1995) administered acrylamide in

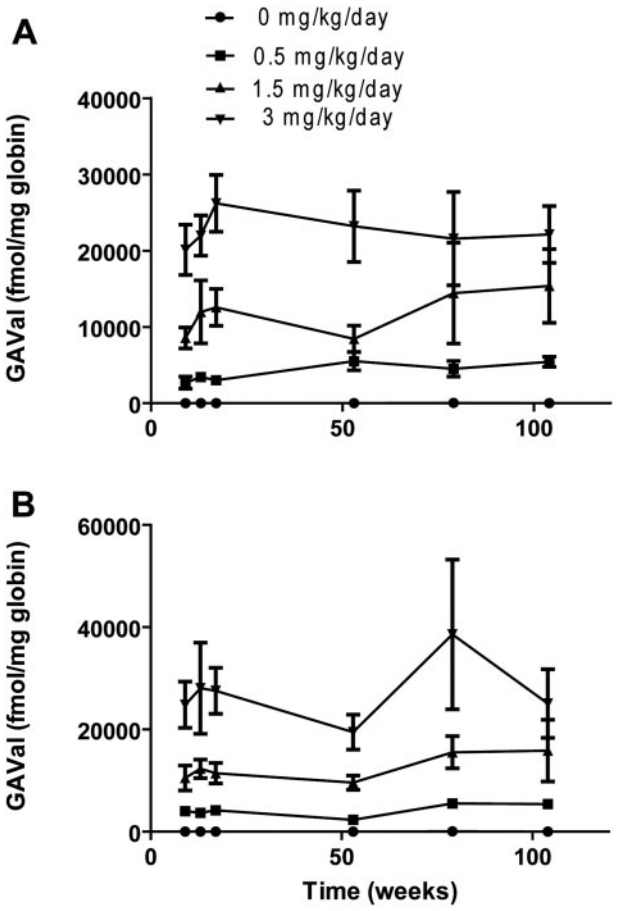

FIG. 3. GAVal in male (A) and female (B) rats administered acrylamide in drinking water from GD6 through PND 722. Animals 5 per group were euthanized at 9, 13, 17, 53, 79, and 104 weeks after weaning. Values represent mean ± SD.

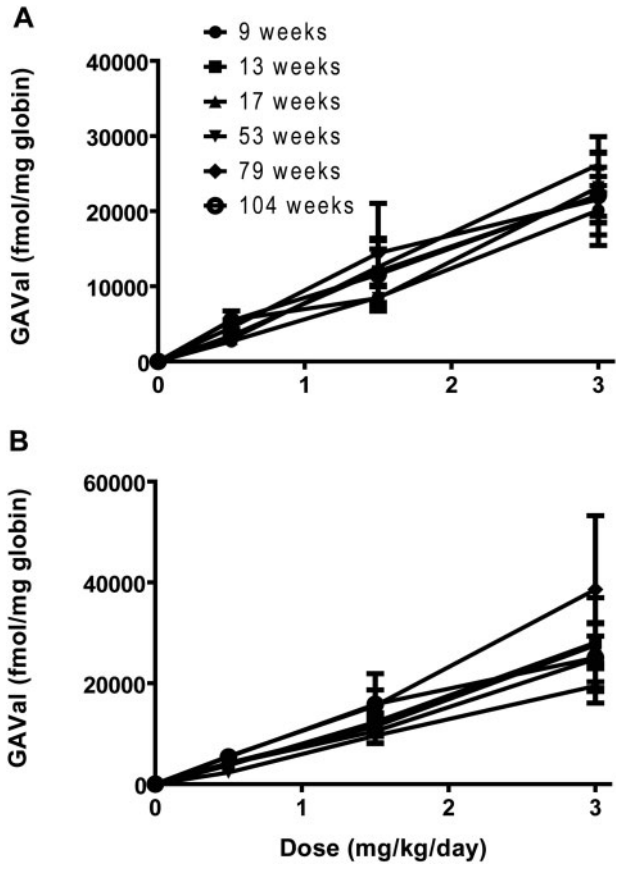

FIG. 4. GAVal Dose response in male (A) and female (B) rats administered acrylamide in drinking water at 9, 13, 17, 53, 79, and 104 weeks after weaning. Values represent mean ± SD (5 rats per group).

drinking water at doses of 0, 0.1, 0.5, and 2 mg/kg/day to male Fischer 344 rats for 2 years, and at 0, 1, and 3 mg/kg/day in female Fischer 344 rats. In both of these studies, a sub-linear increase in the incidence of tunica vaginalis tumors was observed in male rats. It has been suggested that the induction of tunica vaginalis tumors are specific to the aging Fischer 344 rats. This bioassay was conducted in the Wistar Han rat to investigate the consistency of tumor response between strains, and the results of the bioassay are reported elsewhere (Maronpot *et al.*, 2015). There were no treatment related changes in mortality in the bioassay. Mammary fibroadenomas, adenomas and carcinomas were statistically significantly increased at 3 mg/kg/day in female rats. Thyroid follicular cell carcnomas and adenomas were increased in a dose dependent manner at 1.5 and 3 mg/kg/day in female rats and at 0.5, 1.5, and 3 mg/kg/day in male rats.

Glycidamide, the reactive metabolite of acrylamide, forms adducts on reaction with DNA and is thought to be involved in the genotoxicity of acrylamide, and may lead to the carcinogenicity of acrylamide (Dearfield *et al.*, 1988, 1995; Doerge *et al.*, 2005; Ghanayem *et al.*, 2005; Segerbäck *et al.*, 1995; Von Tungeln *et al.*, 2012; Zeiger *et al.*, 2009). The extent of AAVal adduct formation is associated with the area under the curve for acrylamide in blood, which is dependent on the dose administered, and the extent of metabolism (Calleman *et al.*, 1993; Fennell *et al.*, 2005). GAVal reflects the AUC of glycidamide in blood. The ratio of GAVal/AAVal reflects the relative metabolism of acrylamide to glycidamide. The extent of metabolism of AA to GA is known to be species dependent, with higher oxidation in mice compared with rats, is dose-dependent with higher oxidation at lower doses reflecting saturation of oxidation, and depends on the oxidation of acrylamide by CYP 2E1 (Calleman *et al.*, 1993; Sumner *et al.*, 1992, 1999, 2003).

Assessment of hemoglobin adducts at the initial timepoint investigated (PND 24) indicated extremely low protein adduct concentrations for AAVal and for GAVal. This occurred after the exposure of the dams to acrylamide during pregnancy from GD6 through PND 21. Fetal forms of rat hemoglobin disappear from circulation by GD 18 (Iwahara *et al.*, 1996). The adult α and β subunits entirely replace the fetal forms by GD 18. Therefore, hemoglobin sampled on PND 24 may potentially contain information from acrylamide exposure *in utero* but from only a small period of *in utero* exposure. In pregnant Fischer 344 rats administered AA by gavage from GD 6 through birth, AA and GA determined in maternal and fetal serum on GD 20 demonstrated similar levels, indicating transfer across the placenta (Ferguson *et al.*, 2010). Von Stedingk *et al.* (2011) demonstrated a correlation between AAVal and GAVal in maternal and umbilical cord blood at birth in Danish mothers and babies. They concluded that hemoglobin adducts in cord blood reflected exposure during the third trimester, that the maternal and fetal doses were similar and that the placenta provided "negligible protection" against the 3 compounds investigated: ethylene oxide, glycidamide and acrylamide. In addition, with sampling on PND 24 (2 days following weaning), adducts formed *in utero* and early after birth

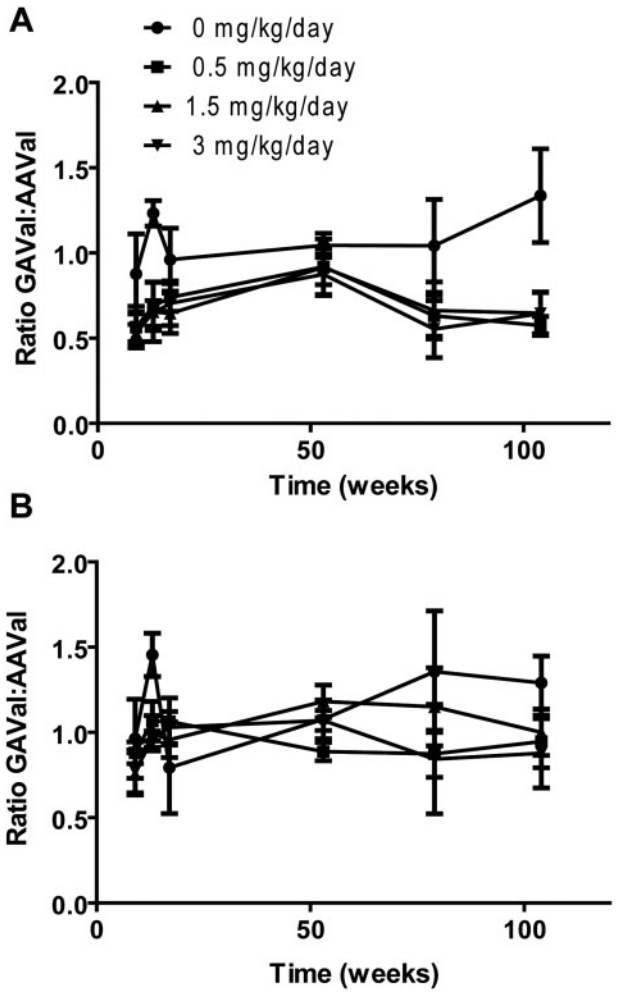

FIG. 5. Ratio of GAVal to AAVal in male (A) and female (B) rats administered acrylamide in drinking water. Animals (5 per group) were euthanized at 9, 13, 17, 53, 79, and 104 weeks after weaning. Values represent mean ± SD.

will undergo substantial dilution as a result of growth with the change in body weight from approximately 6 g at birth to approximately 55 g at PND 24. However, exposure of pups via lactation has been reported to be low. Takahashi *et al.* (2009) determined AAVal in globin in rat dams and offspring on PND 21, following exposure of the dams and offspring to 25, 50 or 100 ppm acrylamide in the drinking water. AAVal in the pup globin was approximately 6% of that formed in the dams. GAVal was not determined. No reports of AA determination in rat milk during lactation have been found. The transfer of AA to human milk has been reported in mothers consuming potato chips (Sorgel *et al.*, 2002). With the high levels of GAVal in rats at this timepoint, it is not possible to distinguish between extensive oxidative metabolism to GA at this timepoint, and high exposure to GA via lactation. No reports of GA determination in rat milk have been located. However, Ferguson *et al.* (2010) investigated AA and GA in serum from pregnant F344 dams and pups administered AA by gavage (from GD 6 to birth administered to the dams, and from PND 1 to 22 administered to the pups). Low levels of GA in serum at PND 1 indicated little conversion of AA to GA, but at PND 22, GA serum concentrations were approximately 2-fold higher than in female adult rats. This suggests that exposure to AA around weaning would result in higher conversion to GA and reaction to form GAVal.

In this study, the formation of AAVal and GAVal were similar in adult male rats over the period from 9 to 104 weeks. AAVal and GAVal levels were also similar across the extent of the study in adult female rats. The decrease in adducts at 53 weeks and the rebound at 72 weeks are unexplained and perhaps need replication. The ratio of GAVal:AAVal was higher in female rats compared with male rats. In rats at weaning, the GAVal:AAVal ratio was substantially higher than in the adult rats. This is consistent with a high activity of CYP 2E1 reported in weaning rats, that rises between PND 3 and 14, peaks at PND 15–18, and falls gradually to adult levels (Saghir *et al.*, 2012). In gpt delta transgenic F344 rats administered acrylamide at 3 and 11 weeks of age, the extent of formation of N7-(2-carbamoyl-2-hydroxyethyl)-guanine(N7-GA-Gua),the major DNA adduct induced by GA, in the liver, testis and mammary gland was substantially higher in the 3-week-old animals compared with the 11-week-old rats (Koyama *et al.*, 2011). Glutathione (GSH) in liver and other organs changes with age. In male Wistar rats, hepatic GSH and CYP 2E1 expression were highest at 8 months compared with 3, and 11, 18 months (Wauthier *et al.*, 2004). The evaluation of changes in GSH with age in rats has produced a range of effects from no change to decreases with aging, depending on the tissues, strain, and timepoints examined (Maher, 2005).

The variability in the hemoglobin adduct measurements over the duration of the study was unexpected. The outbred nature of the Wistar Han rat used may have contributed to this variability. However, this illustrates the potential utility of the determination of hemoglobin adducts, because of the variability of the internal dose, even with the attempt to control the dose administered by regular adjustments of drinking water concentration. This variability of adduct levels over the lifetime of the animals serves as a reminder of the utility of hemoglobin adducts as an indicator of internal dose, and not simply of acrylamide intake. While hemoglobin adduct levels are proportional to dose defined as concentration × time in blood (Ehrenberg and Osterman-Golkar, 1980), there are many variables which can impact the values measured. This indicates that the potential impact of change in gene expression for multiple enzyme systems involved in the metabolism of acrylamide over the lifespan of the animal may be substantial.

In a 50-day study, Tareke *et al.* (2006) administered acrylamide via drinking water to male and female F344 rats and assayed adducts in DNA (N7-GA-Gua) and in hemoglobin (AAVal and GAVal) after 1, 3, 7, 14, 28, 42, and 50 days. In male rats N7-GA-Gua rose to peak at 14 days, and dropped at subsequent timepoints. In female rats, N7-GA-Gua reached a steady state at 14 days. AAVal and GAVal in this study rose through the study, appearing to reach a plateau at the end of the study. The formation of GAVal was substantially higher than AAVal in both male and female rats, with approximately 2-fold higher levels in male rats, and 3-fold higher levels in female rats. Although we observed higher ratios of GAVal:AAVal in female rats compared with males, the ratios were approximately 1 in females and 0.6 in males, contrasting with those reported (Tareke *et al.*, 2006). AAVal levels were approximately 2-fold higher in this study with Wistar rats compared with the F344 rats in Tareke *et al.* (2006). The observations of Törnqvist *et al.* (2008) in F344 rats align with this study, with respect to GAVal, but are lower for AAVal. These data suggest that there are substantial strain differences in the metabolism of AA to GA, which are manifested by the differences in the relative ratios of AAVal and GAVal.

One additional study that was considered for comparison was conducted in Sprague Dawley rats with administration of

TABLE 3. Estimates of Adduct Formation Per Day of Exposure From Timepoints at 60 Days to 2 Years, and Comparison With Literature Values

	Dose (mg/kg/day)	AAVal (fmol/mg globin)[a]	Daily AAVal Formation (fmol/mg globin /day)[b]	GAVal (fmol/mg globin)[a]	Daily GAVal Formation (fmol/mg globin/day)[b]
Male rats	0.5	6120	222 ± 58	4099	148 ± 35
	1.5	18 167	667 ± 176	11 254	412 ± 73
	3	35 066	1293 ± 220	22 534	827 ± 78
	Slope (1)[c]		427 ± 8		272 ± 4
Female rats	0.5	4441	161 ± 48	4192	151 ± 40
	1.5	12 720	460 ± 100	12 541	451 ± 80
	3	30 344	1096 ± 338	27 267	982 ± 222
	Slope (1)[c]		378 ± 34		334 ± 15
Male rats[d]	1	340–2900	150	520–9000	310
Female rats[d]	1	163–4230	160	608–14 800	600
Male rats[e]	0.5	540	75.9	790	111
Male rats[e]	2.0	1890	266	3300	464
	Slope (1)[c]		127		235
Female rats[e]	0.1	160	22.2	320	44.4
Female rats[e]	0.5	680	94.3	1710	237
Female rats[e]	2.0	2640	366	5430	758
	Slope (1)[c]		181		368

[a]Values represent mean of all timepoints from 60 days to 2 years.
[b]Values represent mean ± SD of daily adduct increment from each timepoint from 60 days to 2 years, estimated from simulation using model of Fennell *et al.* (1992).
[c]fmol/mg globin per mg acrylamide/kg/day
[d]Values are time course data for up to 50 days exposure in Fischer 344 rats from Tareke *et al.* (2006), and are simulated without body weight correction.
[e]Values from Törnqvist *et al.* (2008) for Fischer 344 rats administered acrylamide for 7 days in drinking water, and are simulated with body weight correction.

AA via feed or via gavage, with gavage doses of 100 μg/kg/day administered under isoflurane anesthesia (Berger *et al.*, 2011). AAVal was found to increase with repeated dosing and with increasing dose. However, GAVal did not appear to increase, and an increase was only apparent with a substantial increase in AA dose to 10 mg/kg for 1 day. The authors concluded that at the lowest AA dose of 100 μg/kg/day, GA was effectively coupled to GSH in the liver, and did not escape to the vascular system. Given the potential for inhibition of CYP 2E1 by isoflurane during dosing to influence the formation of hemoglobin adducts, and that the dose was substantially below those used here, this study was not included for comparison in Table 3.

Few studies have investigated hemoglobin adducts throughout a long term bioassay. Osterman-Golkar *et al.* (1983) determined hydroxyethylhistidine hemoglobin adducts in a bioassay of ethylene oxide in male Fischer 344 rats exposed to 0, 3, 10, 33, and 100 ppm, 6 h/day, 5 days/week for 2 years. Swenberg *et al.* (2000) have reported the formation of butadiene adducts in hemoglobin to distinguish various reactive metabolites of butadiene, 1,2-epoxy-3-butene (BDO), 1,2,3,4-diepoxybutane (BDO2), and 1,2-dihydroxy-3,4-epoxybutane (BDO-diol) in rats exposed to butadiene, 1000 ppm for 6 h/day, 5 days/week for 13 weeks. Walker *et al* (1992) evaluated the time course and dose response of hydroxyethylvaline between 1 and 4 weeks of inhalation exposure of rats and mice to ethylene oxide. The formation of adducts from acrylonitrile in rats administered 0.3–300 ppm acrylonitrile in drinking water for up to 105 days was determined (Osterman-Golkar *et al.*, 1994). This study has provided dose estimation throughout the course of 2 years of exposure to acrylamide and provided a means to estimate internal dose of acrylamide and its reactive metabolite glycidamide and the variability of internal dose over the 2-year study.

A relatively new concept in the assessment of exposure is that of the "exposome" (Wild, 2005), which represents the totality of exposure from conception onward. The evaluation of all sources at all times has been described as a bottom up approach. An alternative top down approach is to sample at strategically chosen times and to evaluate components in the blood rather than in all sources of exposure (Rappaport and Smith, 2010). In this study, we have investigated the exposure to an electrophilic chemical and its carcinogenic metabolite glycidamide over the lifespan of the rat, with an integrated dose measure in the long lived hemoglobin adduct. Timepoints were chosen to cover the time of birth, lactation, weaning, and development through adulthood. Development of a dataset of this type together with a detailed understanding of the challenges in its collection and interpretation will aid in the refinement of study designs investigating the exposome.

FUNDING

This work was supported by SNF SAS, ZAC de Milieux, Andrezieux, France through contracts with LPT Laboratory of Pharmacology & Toxicology, Hamburg, Germany, and RTI International, Research Triangle Park, North Carolina

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