Journal of the American Association for Laboratory Animal Science
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Vol 57, No 6
November 2018
Pages 703–711

Acrylamide Production in Autoclaved Rodent Feed

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Sterilization of rodent feed by steam autoclaving is a common practice in many research institutions. Often we only consider the beneficial effects of this process—the reduction of microbial contamination—and forget that the high temperatures and pressures can have negative effects on diet quality. The purpose of our study was to assess both the physical and chemical changes to a standard rodent feed autoclaved at multiple sterilization temperatures and the effects of the treated diets on mice. Pelleted NIH31 rodent feed was autoclaved at 4 sterilization temperatures (230, 250, 260, and 270 °F). Feed pellet hardness and the acrylamide concentrations of the diets were tested and compared with irradiated NIH31 feed. Study diets were fed to mice for 28 d, after which tissue samples were collected for analysis of acrylamide, glycidamide (the active metabolite of acrylamide), and genotoxicity. Both feed pellet hardness and acrylamide concentration increased with increasing sterilization temperatures; however, neither affected feed intake or body weight gain. Plasma acrylamide and glycidamide were significantly elevated only in mice fed NIH31 diet autoclaved at 270 °F compared with the irradiated feed, whereas urine acrylamide and glycidamide metabolites were significantly elevated in most autoclaved diets. Liver DNA adducts, which correlate with genotoxicity, were significantly elevated in all autoclaved diets compared with the irradiated diet. Institutions that autoclave their animal diets should carefully consider the temperatures necessary to achieve feed sterilization and the type of studies in which these autoclaved diets are used.

Abbreviations: AAMA, *N*-acetyl-*S*-(2-carbamoylethyl)-L-cysteine; GAMA, *N*-acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-cysteine; LOD, limit of detection; NIEHS, National Institute of Environmental Health Sciences; PhSAA, acrylamide phenylthioether; PhSGA, glycidamide phenylthioether

DOI: 10.30802/AALAS-JAALAS-18-000011

Rodent feed is sterilized to eliminate the introduction of potentially pathogenic microorganisms that might result in clinical disease or subclinical infections that could affect physiologic responses. This practice became common as the demand for SPF animals increased. On rare occasions, we have isolated enteric pathogens, including *Salmonella* spp., *Escherichia coli*, and *Clostridium* spp., from unsterilized, natural ingredient rodent feeds (unpublished data). Recent publications regarding the isolation of contemporary, relevant rodent pathogens from rodent diets are unavailable; however, several recent reports implicated the persistence of mouse parvovirus in mouse colonies due to the use of unsterilized feed.[31,40]

Current feed sterilization methods are steam autoclaving and irradiation. The chosen method may depend on an institution's capabilities or budget. Steam autoclaves require a large initial capital investment, ongoing maintenance, and a lot of energy to operate. In contrast, few institutions have the capabilities to irradiate feed on a large scale, so most irradiation of feed is performed by or through the feed manufacturers and can add a substantial cost to the feed. Neither method will sterilize all loads 100% of the time. Sterilization depends on load size and ability for adequate quality steam or radiation penetration.

Both methods of sterilization can affect the quality of the feed. Many groups, including ours, have shown that autoclaving rodent feed increases feed pellet hardness.[16,35] Feed pellet hardness can affect rodents' ability to eat, especially young animals or strains with dental issues.[23,24] Autoclaving rodent feed can result in decreases in vitamins A, B1, and D; affect protein quality and availability; and alter isoflavone content.[9,15,36] To compensate for the vitamin loss, feed manufacturers often add additional vitamins to the feed mix for autoclavable formulations. Irradiation has little effect on the physical quality of feed but can alter its chemical composition. Feeding an irradiated, purified, AIN76 diet to germ-free mice resulted in high mortality (50%) that was ameliorated with vitamin K supplementation.[20] SPF cats fed an irradiated diet developed a progressive hindlimb ataxia suspected to be due to vitamin A deficiency.[7,13] More recently, irradiation has been shown to increase glycosinolates and the peroxidation of dietary lipids.[8,30]

In 2002, studies found that heating foods containing high levels of starches resulted in the production of acrylamide (C_3H_5NO; prop-2-enamide)[34] through the Maillard reaction between amino acids (primarily asparagine) and reducing sugars.[26,33,41] Soon after, acrylamide production was demonstrated in autoclaved rodent feed.[37] Acrylamide is a compound used widely in the chemical industry for numerous purposes (paper, grouting agents, water treatment, and cosmetics) and occurs in cigarette smoke. Acrylamide is a neurotoxin,[25] genotoxin,[4,10,12,17]

Received: 19 Jan 2018. Revision requested: 05 Mar 2018. Accepted: 09 Apr 2018.
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and carcinogen[27,39] in laboratory rodents and is considered a potential human carcinogen.[21]

In mice, the absolute bioavailability of acrylamide mixed in the diet is approximately 23%, as compared with 32% to 52% from aqueous gavage.[12] Acrylamide is metabolized primarily by epoxidation to glycidamide through the cytochrome P450 enzyme CYP2E1 (Figure 1) or glutathione conjugation.[14,18] Glycidamide is the primary active metabolite of acrylamide in vivo and reacts chemically with glutathione, hemoglobin,[14] and DNA.[10]

Our institution autoclaves our primary rodent feed (NIH31) at 250 °F and routinely tests feed sterility and pellet hardness as part of our feed sterilization quality assessment. We use feed pellet hardness as one mechanism to alert us to changes in feed quality and autoclave function. An increase in feed pellet hardness can indicate changes in autoclave function that require attention. In this study, we tested the effect of various autoclave sterilization temperatures on the physical and chemical quality of NIH31 rodent feed and the biologic sequalae to mice fed these diets.

Materials and Methods

Animals. The National Institute of Environmental Health Sciences (NIEHS) is fully AAALAC-accredited and is committed to the humane care and use of animals in research. All procedures using animals described were approved by the NIEHS Animal Care and Use Committee.

Mouse colonies at NIEHS are routinely tested through serology or PCR analysis by commercial diagnostic laboratories and have consistently shown to be free of the following pathogens: enzootic diarrhea of infant mice virus, cilia-associated respiratory bacillus, ectromelia virus, *Encephalitozoon cuniculi*, Hantavirus, lactate dehydrogenase elevating virus, lymphocytic choriomeningitis virus, minute virus of mice, mouse adenovirus types 1 and 2, mouse cytomegalovirus, mouse hepatitis virus, mouse norovirus, mouse parvovirus types 1 through 5, mouse pneumotropic virus (K virus), mouse polyoma virus, mouse thymic virus, murine ectoparasites (*Myocoptes musculinis*, *Myobia musculi*, *Ramphotyphlops affinis*), murine *Helicobacter* spp., murine pinworms (*Syphacia muris*, *Syphacia obvelata*, *Aspiculuris tetraptera*), *Mycoplasma pulmonis*, *Pasteurella pneumotropica*, pneumonia virus of mice, reovirus 3, Sendai virus, and Theiler murine encephalomyelitis virus.

In this study, male C57BL/6NCrl mice ($n = 90$; age, 6 to 8 wk) were obtained from Charles River Laboratories (Wilmington, MA), where they were fed a natural-ingredient, irradiated diet (Lab Diet 5055, Purina Mills, Richmond, IN). Upon arrival at NIEHS, all mice were fed a natural-ingredient, irradiated, open-formula, NIH31 diet (7917, Envigo, Madison, WI) during their 7-d acclimation period. Mice were singly housed in ventilated cages (Tecniplast, Exton, PA) with hardwood bedding (Maple Sani-Chip, PJ Murphy Forest Products, Montville, NJ), Enviro-Dri enrichment (Shepherd Specialty Papers, Kalamazoo, MI) and reverse-osmosis-treated, deionized water without restriction. After the acclimation period, mice were fed the specific study diets (Table 1) for a period of 28 d. Study groups were randomized according to body weight. Group size was 10 animals for all groups except the irradiated and 270 °F pelleted (A270P) groups, which each had 15 animals due to the elimination of one group (NIH31, irradiated, ground) after the mice arrived but prior to study start. Specialized feeders (Lab Products, Seaford, DE) were used for all ground diets to minimize waste. Mice received daily health checks. Body weight and feed consumption were recorded biweekly. At study conclusion, all mice were euthanized through carbon dioxide inhalation and vital organ transection. Plasma, urine, and liver were collected, flash-frozen in liquid nitrogen, and stored at –80 °C for analysis.

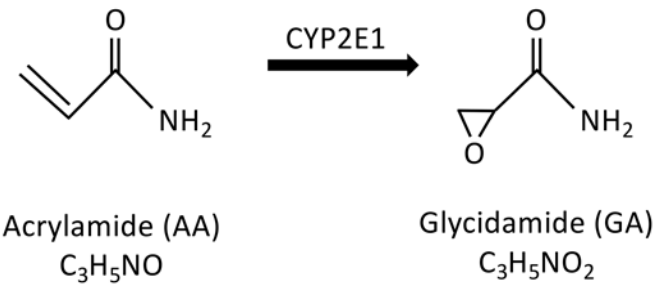

Figure 1. Chemical structures of acrylamide and glycidamide.

Table 1. Feed pellet hardness (kg; mean ± SEM) after irradiation or autoclaving

Diet	Feed pellet hardness (kg)	Increase (%) relative to irradiated diet
NIH31 irradiated (control)	28.73 ± 0.58	—
NIH31 autoclaved at 230 °F	43.43 ± 0.98[a]	51
NIH31 autoclaved at 250 °F	45.39 ± 1.02[a]	58
NIH31 autoclaved at 260 °F	55.30 ± 1.05[a]	93
NIH31 autoclaved at 270 °F	66.94 ± 1.30[a]	133

[a]Significantly ($P < 0.05$) different than NIH31 irradiated diet and all other autoclaved diets.

Study diets. NIEHS uses the NIH31, open-formula, maintenance diet as its standard rodent feed. Open-formula diets assure more consistency in diet constituents, compared with closed-formula diets.[1] NIH31 study diets sterilized by irradiation were obtained from the manufacturer (Envigo) and received an exposure of approximately 20 kGy. Autoclaved NIH31 diets were sterilized at NIEHS (Amsco Scientific Series, Stage 3 Autoclave, Steris Life Sciences, Mentor, OH) at 4 temperatures (230, 250, 260, and 270 °F). The sterilization exposure time was 20 min, the drying time was 5 min, and purge time was 1 min with 4 pulses for each of the temperature cycles. The feed was autoclaved in perforated paper bags that were stacked as single layers on an open shelf rack to allow for appropriate steam penetration and airflow. Biologic indicators were placed in multiple locations within bags and tested weekly. Bacteriologic testing was performed on all study diets to confirm sterility. Briefly, 25 g of feed were placed aseptically in thioglycolate enrichment broth, incubated at 37 ºC, subcultured after 48 h on 5% sheep blood agar plates, and incubated aerobically at 37 °C. Anaerobic subcultures were performed only when suspected in broth culture and were not performed as part of this study.

One group of mice was fed the NIH31 pelleted diet autoclaved at 270 °F and another with the same diet and autoclave temperature but ground (A270G) to see whether physical hardness played a role in feed intake and body weight gain during the study. We prepared 2 acrylamide-positive control diets by adding 200 ppb (AA-low) and 1000 ppb (AA-high) of acrylamide to 2 batches of ground, irradiated NIH31 diet. After the diet was thoroughly mixed by hand in a class II type A/B3 hood, samples of the spiked acrylamide diet were collected for analysis.

Feed hardness testing. We used a test stand (model TCM200, Chatillon, Greensboro, NC) to determine feed pellet hardness. The Chatillon hardness tester is typically used for measuring force (kg) that relates to the performance of finished products, and our group easily adapted it for testing rodent diet pellet

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hardness.[35] The upper exchangeable test jaw used was a round (diameter, 9.5 mm), flat, surface with a contact surface area of 71.0 mm^2. The force (kg) required to break the pellets was measured by using 90 randomly selected uniformly sized pellets from each test sample. Pellet hardness testing was performed at room temperature (25 °C).

Acrylamide and glycidamide analysis. ***Feed.*** Acrylamide and acrylamide-$^{13}C_3$ were purchased from Toronto Research Chemicals (New York, ON). Supported liquid extraction cartridges (SLE+, 1 mL) were purchased from Biotage (Charlotte, NC).

The extraction procedure recommended by the cartridge manufacturer for analysis of acrylamide from fried potato chips[5] was used for rodent feed analysis. The feed was ground and homogenized, and then 1.0 g was placed in a 15-mL, screw- capped centrifuge tube; 10 mL of water and 10 µL acrylamide-$^{13}C_3$ solution (7.5 ng/µL) were added to each tube. Samples were rotated for 1 h at room temperature and then centrifuged to remove particulates. Samples of the supernatant (0.65 mL each) were loaded onto the extraction cartridges. After 5 min for equilibration, analytes were eluted twice with 2.5 mL 1:1 (v/v) ethyl acetate:tetrahydrofuran. We then added 10 µL of 10% glycerol in methanol to each sample prior to solvent removal under vacuum. Samples were reconstituted in 50 µL 0.1% formic acid in water; 10 µL was injected for HPLC–MS/MS analysis.

For chromatographic separation (model 1100 Capillary HPLC System, Agilent, Santa Clara, CA), samples were injected onto a reversed-phase column (100 mm × 2 mm inner diameter, 2.5 µm, Synergi Hydro, Phenomenex, Torrance, CA) held at 40 °C in a column compartment (Phenomenex). Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in methanol. The initial condition of 0.5% B and a flow rate of 275 µL/min was held for 1.2 min. A linear gradient was applied to 95.5% B at 1.5 min. The column was flushed with 2.4 mL solvent before the solvent composition was returned to 0.5% B, and an additional 3 mL was passed through the column to equilibrate it prior to the next injection.

Acrylamide eluted at 1.87 min. The column flow between 0 and 4.5 min was directed to a triple quadrupole MS (API3000, AB Sciex, Framingham, MA) for tandem mass spectral detection. Positive electrospray ionization was performed with a spray voltage of 1750 V. Nebulizer gas, curtain gas, and collisionally activated dissociation gas were set to 6, 7, and 8 arbitrary units, respectively. The dry-gas temperature was 400 °C, and flow was 8 L/min. Declustering potential, focusing potential, and entrance potential were set to 25, 130, and 10 V, respectively. Both Q1 and Q3 were set to unit resolution. Acrylamide was monitored in Q1 at *m/z* 72.1 (75.1 for acrylamide-$^{13}C_3$) and Q3 at *m/z* 55.1 (58.1 for acrylamide-$^{13}C_3$) by using a collision energy of 16 V. The dwell time was 100 ms. The limit of detection (LOD) for acrylamide in feed was 5.0 parts per billion (ppb).

Plasma. Acrylamide, acrylamide-$^{13}C_3$, glycidamide, and glycidamide-$^{13}C_3$ were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Thiophenol was purchased from Millipore Sigma (St Louis, MO). Supported liquid extraction cartridges (SLE+, 400 µL) were purchased from Biotage.

Thioether derivatization of acrylamide in food products was established first[22] and then expanded to accommodate the simultaneous analysis of acrylamide and glycidamide.[19] We adapted the method for application to plasma samples. Briefly, 100 µL of plasma was diluted with 70 µL water, followed by the addition of 100 µL 0.1 N NaOH, 10 µL internal standard solution (100 pg/µL), and 1 µL thiophenol solution (5 mg in 1 mL methanol). Samples were incubated at 60 °C with shaking for 24 h, cooled to room temperature, and then loaded onto the supported liquid extraction cartridges. After a 5-min equilibration, analytes were eluted twice with 900 µL 95:5 (v/v) ethyl acetate:2-propanol. Samples were reconstituted in 100 µL 40% methanol, 0.05% formic acid, and 10 µL was injected for HPLC–MS–MS analysis (Ultimate 3000 UPLC System, Thermo Fisher Scientific, Waltham, MA). Samples were injected onto an C18–pentafluorophenyl column (150 × 2.1 mm [inner diameter], Excel 3, ACE, Aberdeen, Scotland, United Kingdom) held at 30 °C in a column compartment. Mobile phase A was 0.05% formic acid, and mobile phase B was methanol. The initial condition of 45% B and flow rate of 220 µL/min was held for 0.4 min. A linear gradient was applied to 70% B, 220 µL/min at 5 min and 97.5% B, 300 µL/min at 5.5 min. These conditions were maintained until 9.75 min to remove matrix components. The solvent composition was then returned to 45% B, and the flow rate was lowered to 220 µL /min at 14.25 min. Samples were measured in triplicate, and the LOD for plasma acrylamide was 0.2 ppb (pg/µL), or 2.81 nM.

Glycidamide phenylthioether (PhSGA) eluted at 3.5 min and acrylamide phenylthioether (PhSAA) eluted at 4.38 min. The column flow between 2.8 and 5 min was directed to a triple-quadrupole MS (Quantiva, Thermo Fisher) for tandem mass spectral detection. Positive electrospray ionization was performed with a spray voltage of 4000 V. Sheath gas, auxiliary gas, and sweep gas were set to 40, 10, and 1 arbitrary units, respectively. The vaporizer temperature was 350 °C, the ion transfer tube temperature was 325 °C; and Q1 and Q3 were both set to 0.7 full width at half maximum. The radiofrequency lens was set to 40 V, and the collision cell pressure was 2 mTorr. PhSGA was monitored in Q1 at *m/z* 198.1 (201.1 for PhSGA-$^{13}C_3$) and in Q3 at *m/z* 109.1 with a collision energy of 21 V. PhSAA values were: Q1, 182.1 (185.1 PhSAA-$^{13}C_3$); Q3, 137.1 (139.1 PhSAA-$^{13}C_3$); and collision energy, 12 V. The cycle time was 0.25 s and PhSGA was monitored from 2.9 to 4.2 min, whereas PhSAA was monitored from 3.9 to 4.9 min. Samples were measured in triplicate, and the LOD for plasma glycidamide was 0.1 ppb (pg/µL), or 1.14 nM.

Urine. Previously described methodology was adapted for analysis of the urine acrylamide metabolites, *N*-acetyl-*S*-(2-carbamoylethyl)-L-cysteine (AAMA) and *N*-acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-cysteine (GAMA).[5] AAMA, [methyl-d3]- *N*-acetyl-*S*-(2-carbamoylethyl)-L-cysteine (AAMA-D_3), GAMA dicyclohexylammonium salt, and [methyl-d3]- N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-cysteine (glycidamideMA-D_3) dicyclohexylammonium salt were purchased from Toronto Research Chemicals. Solid-phase extraction cartridges (Hypersep Retain PEP, 60 mg, 3 mL) were purchased from Thermo Fisher Scientific. Pooled urine samples (per group, 25 µL) were diluted with 150 µL 2% formic acid in water and 25 µL of internal standard solution (4 pg/µL glycidamideMA-D_3 and 8 pg/µL AAMA-D_3). Samples were loaded onto Hypersep Retain PEP cartridges preconditioned with 1 mL methanol and 1 mL 2% formic acid in water. Samples were washed with 200 µL 2% formic acid and then eluted with 500 µL 1% formic acid in 40% methanol. We added 10 µL 20% glycerol in methanol to the eluent prior to solvent removal by vacuum centrifugation. Samples were reconstituted in 50 µL 1% formic acid, and 5 µL was injected for HPLC-MS-MS analysis.

For chromatographic separation (Ultimate 3000 UPLC System, Thermo Fisher), samples were injected onto a C18–pentafluorophenyl column (150 × 2.1 mm [inner diameter], Excel 3, ACE) cooled to 20 °C in a column compartment. Mobile phase A was 0.05% formic acid, and mobile phase B was methanol. The initial condition of 5% B and a flow rate of 220 µL/min was

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held for 6 min. GAMA eluted at 3.5 min, and AAMA eluted at 5.2 min. From 6 to 7 min, the %B was increased to 95%. These conditions were maintained until 13 min to remove matrix components. Between 13 min and 13.5 min, the %B was decreased back to 5%, and the column was reequilibrated until 21 min.

The column flow between 2 and 6.3 min was directed to a triple-quadrupole MS (Quantiva, Thermo Fisher) for tandem mass spectral detection. Negative electrospray ionization was performed with a spray voltage of –3250 V. Sheath gas, auxiliary gas, and sweep gas were set to 40, 15, and 1 arbitrary units, respectively. The vaporizer temperature was 350 °C, and the ion transfer tube temperature was 325 °C; Q1 and Q3 were both set to 0.7 full width half maximum. The radiofrequency lens was set to 52 V. The collision cell pressure was 2 mTorr. GAMA was monitored in Q1 at *m/z* 249.1 (252.1 for glycidamideMA-D_3) and in Q3 at *m/z* 120.1, with a collision energy of 12 V; AAMA values were 233.1 (236.1 AAMA-D_3), 104.1, and 10 V, respectively. The cycle time was 0.6 s, and GAMA was monitored from 2.48 to 4.48 min, whereas AAMA was monitored from 4.2 to 6.2 min. The LOD for AAMA and GAMA were both 10.0 ppb (pg/µL), or 0.043 µM (AAMA) and 0.040 µM (GAMA).

Quantification of hepatic DNA adducts. Glycidamide–DNA adducts (N7-glycidamide–guanine and N3-glycidamide–adenine) were quantified by using HPLC–MS–MS with stable isotope dilution as described previously.[17] Briefly, DNA was isolated from liver, and the adducts were released by neutral thermal hydrolysis. In this case, a Thermomixer R (Eppendorf North America, Hauppage, NY) set at 99 °C for 15 min was used for the hydrolysis. After hydrolysis, an aliquot of the [^{15}N]-labeled adduct internal standards was added to each sample. The samples were filtered (Amicon 3K Molecular Weight Cutoff Filters, Merck Millipore, Tullagreen, Ireland) to separate the adducts from the intact DNA. The filters were prewashed with water before the samples were eluted. The HPLC-MS-MS system used for quantification consisted of an Acquity UPLC (Waters, Milford MA) and a triple-quadrupole mass spectrometer (Xevo TQ-S, Waters). The same multiple reaction monitoring transitions as previously described were monitored by using a cone voltage of 50 V and collision energy of 20 eV for each adduct transition and its corresponding isotopically labeled internal standard transition. The limit of detection for both adducts was 0.16 per 10^8 nucleotides.

Statistical analysis. Statistical analysis was performed by using the D'Agostino and Pearson normality test, one-way ANOVA, and Dunnett multiple comparison tests for all comparisons except plasma glycidamide. For Dunnett tests, the irradiated diet was considered the control. Several plasma samples had undetectable glycidamide concentrations, and we used a 1-sample *t* test with a hypothetical mean set at the LOD of 1.1 nM. All analyses were performed by using Prism 7.02 (GraphPad Software, San Diego, CA). Significance was set at a *P* value of less than 0.05.

Results

Study diets. All study diets were confirmed free of bacteria and fungi through aerobic cultures in our laboratory (data not shown). Feed pellet hardness of the study diets increased with increasing sterilization temperatures as compared with the irradiated control (Table 1).

Measurable levels of acrylamide were detected in all study diets, including the irradiated diet (Table 2). Each feed sample was measured in quadruplicate, and data are reported as mean ± SEM. The concentration of acrylamide in the diets increased with increasing sterilization temperatures. The acrylamide concentrations of the positive control, AA-spiked diets (low, 200 ppb; high, 1000 ppb) were slightly higher than expected (237.75 and 1355.00 ppb, respectively); however, the acrylamide concentrations used in these positive control diets were well below the reported Lowest Observed Effect Level of 18,500 ppb in mice after a 2-wk exposure in feed.[27] An approximate acrylamide intake for each study group is listed in Table 2 and is based on an overall average daily feed intake of 3.67 g and average body weight of 23.96 g.

Feed intake and body weight. Feed intake and body weight were measured in all groups twice weekly. Feed intake (in g/d) is reported as the mean ± SEM for the 26-d measurement period (Figure 2). The average feed intake for the control group (irradiated diet) was 3.76 ± 0.08 g/d. The A260 group had the lowest intake (3.51 ± 0.06 g/day). Feed intake did not differ among groups except for the AA-high group, which had a higher ($P < 0.05$) feed intake (4.50 ± 0.35 g/d) than the irradiated group. However, the AA-high group showed increased variability as well, with 3 of the 10 mice consuming an average of more than 5.6 g /d without a significant difference in body weight over the course of the study. We noted this increased variability in feed intake for all ground diets (Figure 2). One mouse in the A270G group with an average intake of 7.3 g/d was eliminated from the analysis as an outlier because its body weight did not differ from others in the same group (data not shown). We attributed this increased variability in the groups fed ground diets (A270G, AA-low, and AA-high) to greater feed waste than for the pelleted diets. Regardless, all groups gained weight during the study period, and no differences in body weight gain were noted for any groups during the study period (Figure 3).

Plasma acrylamide and glycidamide analysis. Measurable plasma levels of acrylamide occurred in all study groups, including those fed the irradiated diet (Figure 4). Of the 15 mice in the irradiated control group, only 10 plasma samples were collected and tested. No differences were observed in plasma acrylamide levels between the mice fed the irradiated control diet and those diets autoclaved at 230, 250, or 260 °F, whereas significantly elevated levels of acrylamide were present in the A270P, A270G, and both AA-spiked groups compared with the control group.

Measurable plasma levels of glycidamide were detected in all study groups, but the groups fed the irradiated or any autoclaved diet all had mice with undetectable levels. The LOD for plasma glycidamide was 0.1 ppb (1.1 nM). For statistical analysis, any samples below the LOD were assigned a value of LOD/√2 (0.8 nM), and significance was determined by using a 1-sample *t* test with a hypothetical mean of the LOD (1.1 nM). By using this approach, the A260, A270P, A270G, and both AA-spiked groups had significantly ($P < 0.05$) elevated plasma levels of glycidamide (Figure 5).

Urine acrylamide and glycidamide metabolite analysis. Due to the small volumes of urine collected from individual mice, urine samples from each group were pooled and analyzed as single samples, except for the A270P group, for which 2 separate pooled urine samples were collected. Urine AAMA and GAMA analysis was performed in duplicate for each sample. The small sample size per group precluded normality testing, but a significant ($P < 0.05$) increase in urine AAMA was present in all groups (Figure 6) compared with the control group. A significant ($P < 0.05$) increase in urine GAMA was present in all the groups compared with the control group (Figure 7). Given the limitations in the data available, the urine results should be viewed as preliminary.

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Table 2. Acrylamide levels (ppb; mean ± SEM) in feed after irradiation or autoclaving

Diet	Diet form	Acrylamide (ppb)	Approximate daily acrylamide intake (µg/kg)[a]
NIH31 irradiated (control)	Pellet	6.41 ± 0.39	0.10
NIH31 autoclaved at 230 °F	Pellet	58.18 ± 2.01[b]	0.90
NIH31 autoclaved at 250 °F	Pellet	103.03 ± 3.01[b]	1.58
NIH31 autoclaved at 260 °F	Pellet	126.25 ± 2.53[b]	1.93
NIH31 autoclaved at 270 °F	Pellet	186.50 ± 5.92[b]	2.86
NIH31 irradiated (control)	Ground	186.50 ± 5.92[b]	2.86
NIH31 irradiated + 200 ppb acrylamide	Ground	237.75 ± 3.20[b]	3.64
NIH31 irradiated + 1000 ppb acrylamide	Ground	1355.00 ± 25.98[b]	20.76

[a]Based on average daily feed intake of 3.67 g/d over all groups fed pelleted feed and an average body weight of 23.96 g over all groups and time points.
[b]Significantly ($P < 0.005$) greater than NIH31 irradiated feed and all other autoclaved diets, except for 230 °F compared with 250 °F and 250 °F compared with 260 °F, which did not differ from each other.

Liver DNA adducts. Epoxides such as glycidamide are highly reactive chemicals that are mutagens, reacting with DNA to form adducts.[18] Eight different glycidamide–DNA adducts have been described with N7-(2-carbamoyl-2-hydroxyethyl)-guanine (or N7-glycidamide-guanine) being the most prominent, followed by N3-(2-carbamoyl-2-hydroxyethyl)-adenine (that is, N3-glycidamide-adenine), among those identified in the liver.[4,18] In our study, the mean number of N7-glycidamide–guanine adducts in the irradiated control group was 1.63 per 10^8 nucleotides. All groups that received autoclaved and AA-spiked diets showed a significant increase in the number of these N7-glycidamide–guanine adducts compared with the control group, ranging from a 7.6-fold increase in the A230 group to a 118-fold increase in the AA-high group (Figure 8). The concentrations of N3-glycidamide–adenine adducts were much lower, with several groups having samples below the limit of detection of 0.16 adducts per 10^8 nucleotides (Figure 9). Only the A270P and AA-high groups had significantly ($P < 0.05$) increased numbers of N3-glycidamide–adenine adducts.

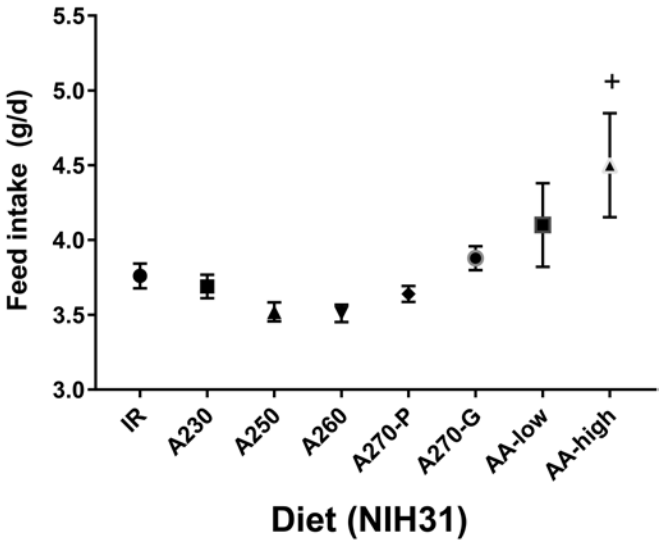

Figure 2. Daily feed intake (g; mean ± SEM) in mice fed NIH31 study diets through day 26. IR, irradiated pelleted feed (control); A230, pelleted feed autoclaved at 230 °F; A250, pelleted feed autoclaved at 250 °F; A260, pelleted feed autoclaved at 260 °F; A270P, pelleted feed autoclaved at 270 °F; A270G, ground feed autoclaved at 270 °F; AA-low, irradiated ground feed spiked with 200 ppb acrylamide; AA-high, irradiated ground fee spiked with 1000 ppb acrylamide. All groups contained 10 mice except irradiated ($n = 15$), A270P ($n = 15$), and A270G ($n = 9$).[+], $P < 0.005$ compared with irradiated control group. Note that A270G, AA-low, and AA-high were fed as ground diets, whereas all others were pelleted. Ground feed waste likely resulted in the increased variability within the acrylamide groups.

Discussion

This study is the first to report physical and chemical changes in autoclaved rodent feed sterilized at different temperatures. An increase in sterilization temperature resulted in a parallel increase in the pellet hardness of the feed. One of our initial study goals was to determine a measurable pellet hardness that might result in reduced intake or body weight gain in adult mice. Essentially, we wanted to determine how hard was too hard. Previous studies have shown a detrimental effect to growth in neonatal mice fed pellets that were too hard.[23,24] According to our body weight data, none of the study diets tested were too hard for the mice to maintain normal growth (Table 1).

As previously reported, this study demonstrates that heating rodent feed results in the production of measurable levels of acrylamide. The increase in sterilization temperature results in a parallel increase in the levels of acrylamide produced, which has not been demonstrated previously. The initial report of acrylamide produced in an autoclaved rodent diet used a single sterilization temperature (275 °F for 5 min) and reported acrylamide concentrations of 240 ppb for the NIH31 diet.[37] In that same study, mice were fed the diet for a period of 30 min, with peak serum levels of 27 nM acrylamide and 35 nM glycidamide at 60 min after feeding. The authors reported an estimated average acrylamide intake of 7 to 32 µg/kg daily and a 7-fold increase in glycidamide–DNA adducts compared with an irradiated diet.[37]

In the current study, we found similar levels of acrylamide in the diet and plasma (186.5 ppb in feed and 42 nM in plasma in mice fed the feed sterilized at 270 °F) to those in the previous report.[37] However, the plasma levels of glycidamide in our mice were much lower (3.5 nM) than previous levels (35 nM).[37] The difference in glycidamide concentrations might be due to the differences in the time course of the 2 studies (30 min compared with 28 d). Urinary excretion of the mercapturic acids (AAMA and GAMA) was increased in mice fed the diet autoclaved at 270 °F or spiked with acrylamide (both low and high) compared with the irradiated diet controls. In addition, we observed a much higher concentration of hepatic glycidamide–DNA adducts in our study. Although the irradiated feed controls had comparable levels in the 2 studies (1.62 ± 0.46 per 10^8 nucleotides in our study compared with 1 ± 0.2 per 10^8 nucleotides previously),[37] we observed a 7.6-fold increase in

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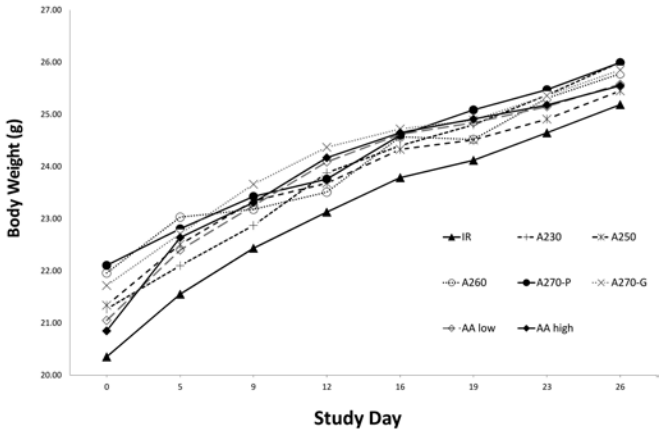

Figure 3. Mean body weight (g) in mice fed NIH31 study diets through day 26. Error bars indicating the SEM (range, 0.233 – 0.7089 g) were removed for clarity. All groups contained 10 mice except irradiated and A270P (n = 15 in each group). Body weight did not differ between groups at any time point. IR, irradiated pelleted feed (control); A230, pelleted feed autoclaved at 230 °F; A250, pelleted feed autoclaved at 250 °F; A260, pelleted feed autoclaved at 260 °F; A270P, pelleted feed autoclaved at 270 °F; A270G, ground feed autoclaved at 270 °F; AA-low, irradiated ground feed spiked with 200 ppb acrylamide; AA-high, irradiated ground fee spiked with 1000 ppb acrylamide.

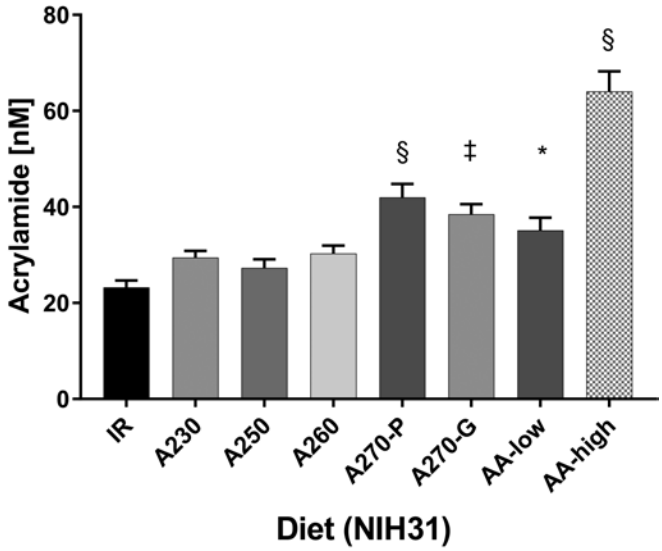

Figure 4. Plasma acrylamide concentration (nM) from mice fed NIH31 study diets. Data are given as mean ± SEM (n = 10) for all groups except A270P (n = 15). IR, irradiated pelleted feed (control); A230, pelleted feed autoclaved at 230 °F; A250, pelleted feed autoclaved at 250 °F; A260, pelleted feed autoclaved at 260 °F; A270P, pelleted feed autoclaved at 270 °F; A270G, ground feed autoclaved at 270 °F; AA-low, irradiated ground feed spiked with 200 ppb acrylamide; AA-high, irradiated ground fee spiked with 1000 ppb acrylamide. *, $P < 0.05$; ‡, $P < 0.001$; and §, $P < 0.0001$ compared with irradiated control group.

N7-glycidamide–guanine adducts in the livers of mice fed the feed sterilized at 230 °F (12.3 ± 1.0 adducts per 10^8 nucleotides) and as much as a 22.6-fold increase in the liver of mice fed the feed sterilized at 270 °F (35.6 ± 3.1 adducts per 10^8 nucleotides). Once again, the differences observed could be related to the differences in the study time courses and the accumulation of these glycidamide–DNA adducts in the livers of the mice over time in our study. A previous report of mice exposed to acrylamide in their drinking water (approximately 1 mg/kg daily) demonstrated N7-glycidamide–guanine adduct concentrations of approximately 400 per 10^8 nucleotides by 15 d of exposure; these

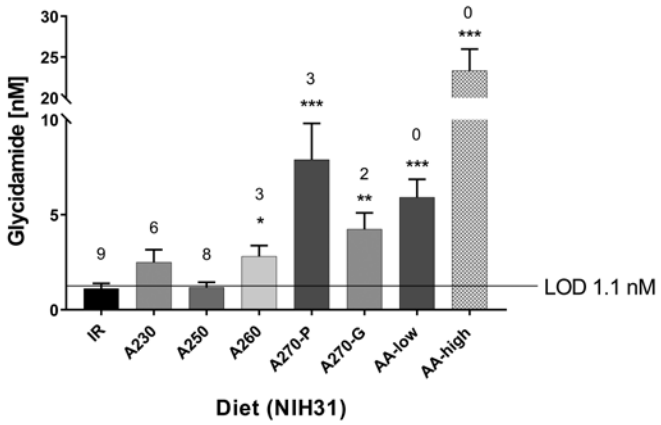

Figure 5. Plasma glycidamide concentration (nM) from mice fed NIH31 study diets. Data represents mean ± SEM; n = 10 for all groups except A270P (n = 15). Several groups had samples in which some or all sample replicates were below the limit of detection (LOD) of 1.1 nM. Those replicates were assigned a value of LOD/√2 (0.8 nM). The number above each column indicates the number of samples with 1 to 3 replicates below the LOD. IR, irradiated pelleted feed (control); A230, pelleted feed autoclaved at 230 °F; A250, pelleted feed autoclaved at 250 °F; A260, pelleted feed autoclaved at 260 °F; A270P, pelleted feed autoclaved at 270 °F; A270G, ground feed autoclaved at 270 °F; AA-low, irradiated ground feed spiked with 200 ppb acrylamide; AA-high, irradiated ground fee spiked with 1000 ppb acrylamide. *$P < 0.05$, **$P < 0.01$, *$P < 0.001$.

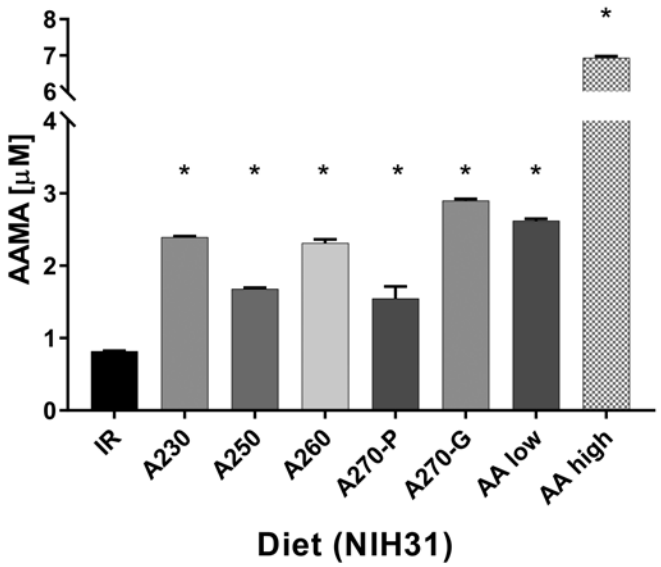

Figure 6. Urine N-acetyl-S-(2-carbamoylethyl)-cysteine concentration (AAMA, μM) from mice fed NIH31 study diets. All groups represent a single pooled sample except A270P which had 2 pooled samples. Data represents mean of duplicates ± SEM. IR, irradiated pelleted feed (control); A230, pelleted feed autoclaved at 230 °F; A250, pelleted feed autoclaved at 250 °F; A260, pelleted feed autoclaved at 260 °F; A270P, pelleted feed autoclaved at 270 °F; A270G, ground feed autoclaved at 270 °F; AA-low, irradiated ground feed spiked with 200 ppb acrylamide; AA-high, irradiated ground fee spiked with 1000 ppb acrylamide. *$P < 0.0001$ compared with irradiated control group.

concentrations remained level through day 50 of the study.[10] Most studies reporting the toxicokinetics and genotoxicity of acrylamide and glycidamide in rodents used much higher doses (1 to 50 mg/kg daily) than were produced in our autoclaved feeds.[10-12,17,18] The acrylamide levels produced by autoclaving our diets resulted in an intake comparable to the estimated

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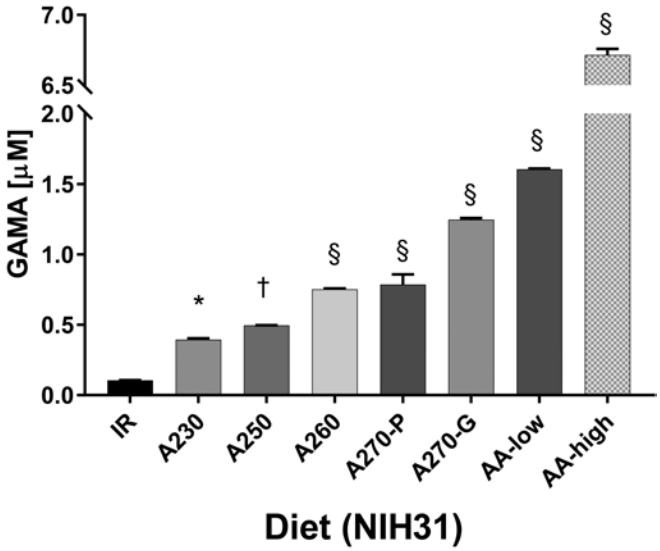

Figure 7. Urine N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-cysteine concentration (GAMA, μM) from mice fed NIH31 study diets. All groups represent a single pooled sample except A270P which had 2 pooled samples. All samples run in duplicate. Data are presented as the mean ± SEM of duplicate samples. IR, irradiated pelleted feed (control); A230, pelleted feed autoclaved at 230 °F; A250, pelleted feed autoclaved at 250 °F; A260, pelleted feed autoclaved at 260 °F; A270P, pelleted feed autoclaved at 270 °F; A270G, ground feed autoclaved at 270 °F; AA-low, irradiated ground feed spiked with 0.2 ppm acrylamide; AA-high, irradiated ground fee spiked with 1.0 ppm acrylamide. *, $P < 0.05$; †, $P < 0.01$; and §, $P < 0.0001$ compared with the irradiated control group.

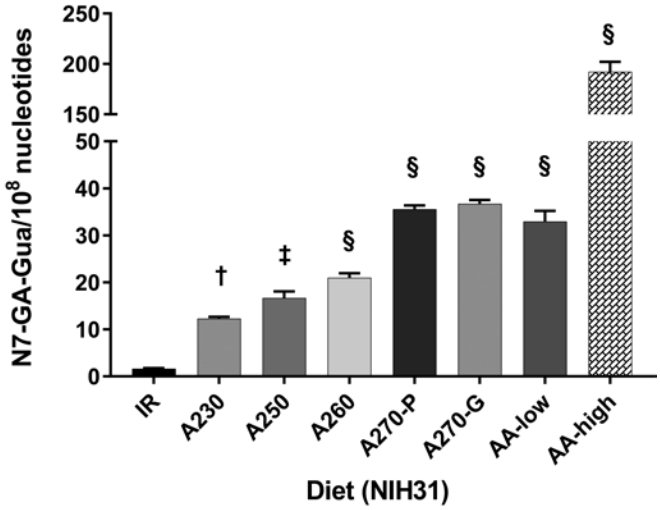

Figure 8. The number of hepatic glycidamide–guanine DNA adducts (N7-[2-carbamoyl-2-hydroxyethyl]-guanine or N7-glycidamide-guanine) per 10^8 total nucleotides in mice fed NIH31 irradiated or autoclaved diet. Data are given as mean ± SEM (n = 10) for all goups except A230 (n = 8) and A270P (n = 14). IR, irradiated pelleted feed (control); A230, pelleted feed autoclaved at 230 °F; A250, pelleted feed autoclaved at 250 °F; A260, pelleted feed autoclaved at 260 °F; A270P, pelleted feed autoclaved at 270 °F; A270G, ground feed autoclaved at 270 °F; AA-low, irradiated ground feed spiked with 200 ppb acrylamide; AA-high, irradiated ground fee spiked with 1000 ppb acrylamide. †, $P < 0.01$; ‡, $P < 0.001$; and §, $P < 0.0001$ compared with the irradiated control group.

human dietary intake of acrylamide from heated foods (0.8 μg/kg daily in nonsmokers).[38]

Several studies have demonstrated the carcinogenicity of acrylamide and glycidamide in rodents;[27,28,32,39] however, their

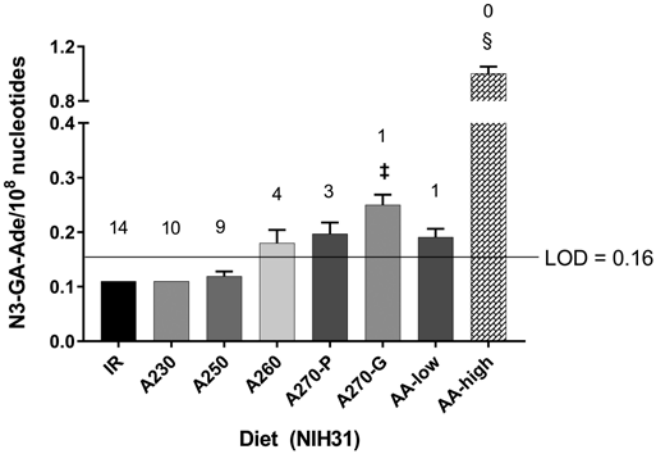

Figure 9. The number of hepatic glycidamide–adenine DNA adducts (N3-[2-carbamoyl-2-hydroxyethyl]-adenine or N3-glycidamide-adenine] per 10^8 total nucleotides in mice fed NIH31 irradiated or autoclaved diet. Several groups had samples in which some or all measurements were below the limit of detection (LOD) of 0.16 adducts per 10^8 nucleotides. Those samples were assigned a value of LOD/√2 (that is, 0.11 adducts/10^8 nucleotides). Data are given as mean ± SEM (n = 10) for all groups except IR (n = 14) and A270P (n = 15). The number above each column indicates the number of samples per group that were below the LOD. IR, irradiated pelleted feed (control); A230, pelleted feed autoclaved at 230 °F; A250, pelleted feed autoclaved at 250 °F; A260, pelleted feed autoclaved at 260 °F; A270P, pelleted feed autoclaved at 270 °F; A270G, ground feed autoclaved at 270 °F; AA-low, irradiated ground feed spiked with 200 ppb acrylamide; AA-high, irradiated ground fee spiked with 1000 ppb acrylamide. Statistical analysis used a 1-sample t test with hypothetical mean of 0.16. ‡, $P < 0.001$; §, $P < 0.0001$ compared with irradiated control group.

carcinogenic potential in humans is unclear. Neonatal mice lack sufficient cytochrome P450 activity including Cyp2E1, and neonatal mice dosed with glycidamide—but not acrylamide—have an increased incidence of liver tumors compared with controls.[39] In contrast, the tumor profiles observed in several 2-y rodent bioassays were similar when animals were dosed with either acrylamide or glycidamide in feed or drinking water.[2,27,28] These findings likely indicate that glycidamide is the active metabolite that results in DNA adducts and tumor formation and requires the full complement of cytochrome P450 enzymes. DNA adduct formation is considered an early and presumably required event in chemical carcinogenesis.[29] Our study found increasing levels of hepatic DNA adduct formation in mice fed the diets autoclaved at the higher temperatures compared with the irradiated control; however, the number of adducts formed were 50-fold less than those found in mice treated with intraperitoneal doses of glycidamide (0.70 mmol [approximately 50 mg/kg]) linked to an increase in the formation of hepatocellular adenomas.[39] However, mice exposed to acrylamide[2,27] or glycidamide[3,28] through feed or drinking water for 2 y had significant increases in hardarian gland and lung or bronchiolar adenomas at concentrations (87,500 to 175,000 ppb) comparable to those measured in our study diets (Table 2). Our study duration was too short to determine whether the increase in DNA adduct formation would have resulted in an increase in tumor formation.

This study demonstrates the production of acrylamide when rodent diets are autoclaved, and the levels produced correlate with the sterilization temperature of the feed. Because there are no standards regarding the autoclaving parameters used to sterilize rodent diets, institutions are feeding autoclaved NIH31 (or other natural-ingredient diets) to rodents that may have different levels of AA. The increasing levels of acrylamide

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produced in rodent diets as the sterilization temperature increases is an important reason to assure standard autoclave function. Given the results of this study, investigators should consider the method of feed sterilization used routinely at their institutions and the potential effects on their particular studies. Irradiation appears to result in the least detrimental effects to the feed compared with autoclaving, but it is not without its own issues. For studies of acrylamide toxicity or chemical carcinogenesis, we strongly recommend the use of irradiated feeds over autoclaved. Institutions which cannot convert to irradiated feed should use sterilization temperatures at or below 250 °F and assure consistent autoclave function, to minimize the potential effects of acrylamide in autoclaved rodent feed.

Acknowledgments

This work was supported by the Intramural Research Program, National Institutes of Health, National Institute of Environmental Health Sciences, including the Summer Internship Program. We would like to thank the Alpha Omega/Priority One Services Animal Care Staff for their exemplary care of the mice during this study. The views presented in this article do not necessarily reflect those of the US Food and Drug Administration.

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