

**NTP Technical Report
on Toxicity Studies of**

Glyphosate

(CAS No. 1071-83-6)

**Administered in Dosed Feed
to F344/N Rats and B6C3F₁ Mice**

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**NIH Publication 92-3135
July 1992**

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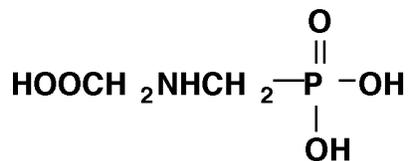
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Glyphosate



Molecular Formula: C₃H₈NO₅P

CAS Number: 1071-83-6

Molecular Weight: 169.1

Synonyms: Glyphosate, technical grade; Glycine, N-(phosphonomethyl); N-phosphonomethyl glycine; N-(phosphonomethyl)glycine; MON 0573; MON 2139.

ABSTRACT

Glyphosate is a systemic, broad-spectrum, post-emergence herbicide used for non-selective weed control. It was selected for study because of its widespread use, potential for human exposure, and the lack of published reports concerning comprehensive toxicity or carcinogenicity evaluations.

Chemical disposition, 13-week toxicity, and mutagenicity studies of glyphosate were conducted. In disposition studies, male F344/N rats were administered an oral dose (5.6 or 56 mg/kg) of ¹⁴C-glyphosate. Blood, urine, fecal, and tissue samples were collected and analyzed for radioactivity. Within 72 hours after glyphosate dosing, 20-30% of the administered radioactivity was eliminated via urine, 70-80% via feces, and about 1% of the radioactivity remained in the tissues. Studies following oral, intravenous, and intraperitoneal administration of glyphosate indicated that the urinary radioactivity represented the amount of glyphosate absorbed and that the fecal radioactivity represented the amount unabsorbed from the gastrointestinal tract.

In the 13-week toxicity studies, groups of 10 male and female F344/N rats and B6C3F₁ mice were administered glyphosate in feed at 0, 3125, 6250, 12500, 25000, or 50000 ppm. Glyphosate administration induced increases in serum bile acids, alkaline phosphatase, and alanine aminotransferase activities in rats, suggesting mild toxicity to the hepatobiliary system. Clinical pathology measurements were not performed with mice. No histopathologic lesions were observed in the livers of rats or mice. There was no evidence of adverse effects on the reproductive system of rats or mice. Cytoplasmic alteration was observed in the parotid and submandibular salivary glands of rats and parotid salivary glands in mice. The salivary gland

effects of glyphosate were demonstrated to be mediated through an adrenergic mechanism which could be blocked by the adrenergic antagonist, propranolol.

Glyphosate was not mutagenic in *Salmonella*, and did not induce micronuclei in mice. The no-observed-adverse-effect level (NOAEL) for the salivary gland lesions was 3125 ppm in the diet for mice. A NOAEL could not be determined from the rat study.

PEER REVIEW

Peer Review Panel

The members of the Peer Review Panel who evaluated the draft report on the toxicity studies on glyphosate on July 10, 1991, are listed below. Panel members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, panel members act to determine that the design and conditions of the NTP studies were appropriate and to ensure that the toxicity study report presents the experimental results and conclusions fully and clearly.

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Summary of Peer Review Comments

On July 9 and 10, 1991, the Technical Reports Review Subcommittee of the Board of Scientific Counselors for the National Toxicology Program met in Research Triangle Park, NC, to review the draft technical report on toxicity studies of glyphosate.

Dr. Po Chan, NIEHS, introduced the short-term toxicity studies of glyphosate by reviewing the uses and rationale for the study, findings from chemical disposition studies, experimental design, and results.

Dr. Garman, a principal reviewer, said that the report was thoroughly prepared and detailed, and that it did an excellent job reviewing the background for the study and the available literature on glyphosate. He added that the isoproterenol/propranolol study included in the report is quite interesting and helps establish the mechanism for salivary gland alteration.

Dr. Garman said that certain details of the salivary gland alteration study should be clarified, namely, which type of glandular acinus within the submandibular salivary gland was most affected by glyphosate, and whether, in Table 11, only the parotid salivary gland was assayed in measuring the severity of changes brought on by glyphosate treatment. Dr. J. Mahler, NIEHS, said the severity grades were based on the parotid glands only.

Dr. Goodman, another principal reviewer, said the report was well-written. He suggested that the the lack of of any reproductive toxicity attributable to glyphosate treatment was an important finding and should be included in the abstract of the report.

After further discussion of editorial matters, Dr. Longnecker accepted the report on behalf of the panel.

INTRODUCTION

Glyphosate is a nonvolatile white solid with a melting point of 200°C and a negligible vapor pressure. It is soluble to 1.2% in water at 25°C but is not soluble in organic solvents (Beste, 1983). Glyphosate has been available commercially since 1974. It is marketed as Roundup® (comprised of the isopropylamine salt of glyphosate (41.0%) and inert ingredients, including surfactants), and as Rodeo® (which contains the isopropylamine salt of glyphosate (53.5%) and inert ingredients). The surfactant in Roundup® facilitates foliage absorption. Roundup® is used as a nonselective, systemic, broad-spectrum, post-emergence herbicide for managing vegetation in agriculture and forestry; Rodeo® is used for aquatic weed control. Information on production volume, sales, and the identity of the "inert ingredients" is proprietary.

The mechanism of phytotoxic action of glyphosate is inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EC 2.5.1.19) activity, thus blocking aromatic amino acid synthesis (Amrhein *et al.*, 1980, 1981). The resulting reduction in protein synthesis causes cessation of growth and, eventually, cellular disruption and death. Glyphosate has nonspecific, metal-chelating properties (Glass, 1984); it inhibits enzymes which require transitional metal cations for activity such as the 3-deoxy-2-oxo-D-arabino-heptulosonate-7-phosphate synthase and 5-dehydroquinate synthase (Ghassemi *et al.*, 1982; Hoagland and Duke, 1982). Glyphosate's effectiveness as a phytotoxin is due in part to its low molecular weight and high water solubility, which aid its rapid absorption and translocation by plant tissues; it is not metabolized to any significant degree in plant tissues (Ghassemi *et al.*, 1982).

Glyphosate is strongly adsorbed to soils and is not readily leached. The mobility of glyphosate in the soil is affected by soil type, phosphate level, and pH. Adsorption of glyphosate is higher in soils containing clay and organic matter than in sandy loam soils, but lower in high-phosphate or high-pH soils. It is susceptible to degradation, possibly by microbial co-metabolism (Sprankle *et al.*, 1975), and thus relatively nonpersistent in soils. Information provided by Monsanto to the U.S. Environmental Protection Agency reportedly showed the half-life of glyphosate in soil normally was less than 60 days (U.S. EPA, 1979). The half-life was 17 to 19 weeks in sandy soil and 3 weeks in silt loam (Ghassemi *et al.*, 1982). Newton *et al.* (1984) reported the half-lives of glyphosate in a forest-brush field ecosystem in Oregon after aerial spray were 10.4 to 26.6 days in the foliage and litter, 40.2 days for exposed soil, and 29.2 days for litter-covered soil. However, Stark (1983) reported that residues still may be found in the soil for 2 years or longer. The major degradation product of glyphosate in soil is (aminomethyl)phosphonic acid. Minor metabolites include N-methylaminomethylphosphonic acid, glycine, N-dimethylaminomethylphosphonic acid, and hydroxy-methylphosphonic acid (Ghassemi *et al.*, 1982; Rueppel *et al.*, 1977; Sprankle *et al.*, 1975).

No information is available on the absorption of glyphosate after oral administration to mammals. Wester *et al.* (1991) reported poor absorption of glyphosate, as Roundup®, after dermal application to rhesus monkeys. It has been reported that glyphosate does not bioaccumulate in living cells (Ghassemi *et al.*, 1982) because of its high water solubility and the absence of any active processes which concentrate or conserve glyphosate.

Fifty-six cases of unspecified toxicities associated with exposure to Roundup® were reported in Japan between June, 1984, and March, 1986 (Sawada *et al.*, 1988). Analyses showed that the surfactants used in the formulation, rather than glyphosate *per se*, were the main cause of toxicity. A similar conclusion was reached by Folmar *et al.* (1979) in evaluating the toxicity of a technical-grade glyphosate (MON 0573), the isopropylamine salt of glyphosate (MON 0139), Roundup® (MON 2139), and the Roundup® surfactant (MON 0818) in aquatic species. Wan *et al.* (1989) confirmed that the surfactant MON 0818 is a more potent toxicant to salmonids than glyphosate, MON 8709, or Roundup®. The authors further demonstrated that the toxicity of glyphosate to salmonids is affected by the hardness and pH of the water; glyphosate is more toxic to juvenile salmonids in soft water than in hard water (Wan *et al.*, 1989).

The acute lethal oral dose (LD₅₀) of glyphosate without surfactants is 4873 mg/kg for rats and 1568 mg/kg for mice (Bababunmi *et al.*, 1978); the acute lethal dose by intraperitoneal injection is 235 mg/kg for rats and 130 mg/kg for mice (Olorunsogo and Bababunmi, 1980). Glyphosate administered intragastrically to rats at 1 mMol/kg daily for 2 weeks had no effect on kidney and intestinal drug-metabolizing enzymes, including aryl hydrocarbon hydroxylase, ethoxycoumarin-O-deethylase, epoxide hydrolase, or UDP-glucuronosyltransferase (with 4-nitrophenol or 4-methylumbelliferone as the aglycone) (Ahotupa *et al.*, 1983). In rats administered glyphosate intragastrically at 500 mg/kg (3 mMol/kg) for 4 days followed by 300 mg/kg (1.8 mMol/kg) for 10 days, there were significant decreases in the activities of hepatic cytochrome c reductase, cytochrome P-450 mediated diphenyloxazole hydroxylase, ethoxycoumarin O-deethylase and mono-oxygenase, and the intestinal aryl hydrocarbon hydroxylase (Hietanen *et al.*, 1983). Uncoupling of oxidative phosphorylation was observed in isolated rat liver mitochondria incubated with glyphosate *in vitro* (Bababunmi *et al.*, 1979). It has been postulated that uncoupling of mitochondrial oxidative phosphorylation may play a major role in glyphosate intoxication (Olorunsogo *et al.*, 1979). Support of the hypothesis was provided by studies demonstrating inhibition of the energy-linked nicotinamide nucleotide transhydrogenase reaction in intact mitochondria isolated from the livers of rats 5 hours following intraperitoneal dosing with 15 mg/kg or more glyphosate. In these studies, glyphosate probably exerts its toxic effect first by uncoupling oxidative phosphorylation, which in turn interferes with the energy-requiring transhydrogenase reaction in the cell (Olorunsogo 1982a, 1982b).

Glyphosate was nominated for study by the California Regional Water Quality Control Board-North Coast Region, State of California, because it was found in water runoff in areas of glyphosate use. The NTP selected glyphosate for toxicity evaluation because of widespread use, its potential for human exposure, and the lack of published reports concerning comprehensive toxicity or carcinogenicity evaluations. The NTP studies included genetic toxicity studies, disposition studies in F344/N rats, and 13-week dosed feed toxicity studies in F344/N rats and B6C3F₁ mice. A 14-day study with male F344/N rats also was conducted to investigate a possible adrenergic mechanism in the pathogenesis of a salivary gland change, as noted in the 13-week studies. Copies of proprietary reports of toxicity studies performed by Monsanto Corporation were made available to the NTP for use in designing its glyphosate studies.

MATERIALS AND METHODS

Procurement and Characterization of Glyphosate

The glyphosate used in all studies was obtained from Monsanto Agricultural Products (St. Louis, MO). Samples of glyphosate were analyzed at Midwest Research Institute and found to be approximately 99% pure. The infrared, ultraviolet/visible, and nuclear magnetic resonance spectra were consistent with the structure of glyphosate and available literature references. Elemental analysis results for carbon, hydrogen, nitrogen, and phosphorous agreed with theoretical values. Karl Fischer titrimetry indicated $0.18 \pm 0.04\%$ water. Titration of the acidic functional groups with tetrabutylammonium hydroxide indicated a purity of $98.6 \pm 0.4\%$. Analysis by thin-layer chromatography indicated a major spot and 2 trace impurities. Analyses indicated glyphosate, when mixed with feed and stored in at room temperature in the dark, was stable for at least 3 weeks.

The ^{14}C -glyphosate [N-(phosphono- ^{14}C -methyl)-glycine, 1.97 mCi/mM, radiochemical purity 99%] and Roundup® used in the disposition studies also were obtained from Monsanto.

Disposition Studies

Male F344/N rats (170-280 g, purchased from Harlan-Sprague-Dawley (Indianapolis, IN), were fasted overnight before dosing. Between 8 a.m. and 10 a.m., each rat received a single gavage dose of ^{14}C -glyphosate in deionized, distilled water, at levels of either 5.6 or 56 mg/kg body weight. The rats were housed individually in metabolic cages and fed Wayne Lab Blox rat chow and deionized water *ad libitum*.

Urine and feces were collected for 72 hours, at 24-hour intervals. One hundred μl of urine was mixed with 20 ml of Betaphase scintillation cocktail and analyzed for ^{14}C using a Beckman LS 2800 liquid scintillation counter (Beckman Instruments, Inc., Fullerton CA). Feces were weighed and mixed in 15 ml of 0.5 M NaOH for 24 hours before homogenization. Aliquots of fecal homogenate were oxidized in a United Technologies Packard Model 306 oxidizer (Packard Instrument Co., Downers Grove, IL), then analyzed for ^{14}C with the Beckman LS 2800.

At termination, aliquots of brain, heart, lung, liver, kidney, spleen, testes, muscle, skin, fat, small and large intestine, stomach, and blood were collected. The samples were weighed, oxidized, and analyzed for ^{14}C as described above; the contents of the small and large intestines and the stomach were analyzed separately for radioactivity. The resulting values were combined and added to the last fecal time point.

Groups of rats were given a single dose of 5.6 mg glyphosate/kg intravenously via the tail vein (dose volume 1.0 ml/kg), intraperitoneally, or orally to study the elimination of glyphosate following various routes of administration. Urine and feces were collected and analyzed for radioactivity over a 24-hour period.

Additional groups of rats were pretreated with Roundup® at 0.5 or 10 ppm in drinking water. for 16 days to determine the effect of the surfactants and inert ingredients on glyphosate absorption. The rats received a single oral dose of [¹⁴C]-glyphosate (5.6 mg/kg), either on day one, prior to treatment with Roundup®, or on day 16 of treatment.

Blood samples were obtained by cardiac puncture from rats given the oral doses of glyphosate at 5.6 or 56 mg/kg, to determine the effect of dose on the absorption of glyphosate from the gastrointestinal tract. The samples were analyzed for radioactivity according to previously described procedures.

13-Week Study Design

Groups of 10 male and 10 female F344/N rats and B6C3F₁ mice were given glyphosate in feed at dietary concentrations of 0 ppm (0%), 3125 ppm (0.3125%), 6250 ppm (0.625%), 12500 ppm (1.25%), 25000 ppm (2.5%), or 50000 ppm (5.0%). Ten additional rats/sex were included at each dietary level for evaluation of hematologic and clinical pathology parameters. Male and female F344/N rats and B6C3F₁ mice used in this study were produced under strict barrier conditions at Simonsen Laboratories (Gilroy, CA). The animals were progeny of defined microflora-associated parents that were transferred from isolators to barrier-maintained rooms. Rats and mice were shipped to the study laboratory at 31 and 38 days of age, quarantined at the study laboratory for 12 and 11 days, and placed on study at 43 days and 49 days of age, respectively. Blood samples were collected and the sera analyzed for viral titers from 5 animals per sex and species at study start and at termination in the 13-week studies. Data from 5 viral screens performed in rats and 12 viral screens performed in mice showed that there were no positive antibody titers (Boorman *et al.*, 1986; Rao *et al.*, 1989, 1989a). Additional details concerning study design and performance are listed in Table 1.

Animals surviving to the end of the studies were killed with carbon dioxide. The heart, right kidney, liver, lung, right testis, and thymus were weighed. Sperm morphology and vaginal cytology evaluations were performed at the end of the study and during the preceding 2 weeks on rats and mice from the untreated controls and 3 highest dose groups (0, 12500, 25000, and 50000 ppm). Blood smears were prepared from mice for determination of micronuclei in erythrocytes.

A necropsy was performed on all animals. Organs and tissues were examined for gross lesions (Table 1). Tissues were preserved in 10% neutral buffered formalin. Following dehydration and embedding, tissues were sectioned at approximately 5 μM, stained with hematoxylin and eosin, then examined microscopically. A complete histopathologic evaluation was conducted on all animals in the untreated control group and the highest dose group (50000 ppm). The single identified target organ, the salivary gland, was examined in all dosed groups. Tissues examined for rats and mice of both sexes are listed in Table 1.

Upon completion of the histologic evaluation of the 13-week study by the laboratory pathologist, the slides, paraffin blocks, and residual wet tissues were sent to the NTP Archives for inventory, slide/block match, and wet tissue audit. The slides, individual animal data records, and pathology tables were sent to an independent pathology laboratory where quality assessment was performed; the results were reviewed and evaluated through an NTP Pathology Review. The final diagnoses represent a consensus of contractor and review pathologists.

For clinical pathology studies, male and female rats were anesthetized with a mixture of carbon dioxide and oxygen (70%:30%), and blood samples were collected from the retroorbital sinus using heparinized microcapillary tubes. Samples for determination of hematologic and biochemical variables were collected from additional study animals on study days 5 and 21, and from the regular study animals at 13 weeks. Blood samples for hematologic analyses (approximately 0.5 ml) were collected in plastic tubes coated with potassium EDTA (Microvette CB 1000, Sarstedt, Numbrecht, Germany) and held at room temperature. Samples for biochemical analyses (approximately 0.75 ml) were collected in plastic tubes containing serum separator gel (Microtainer serum separator tube, Becton Dickinson, Rutherford, NJ). These samples were allowed to clot for 30 minutes at room temperature. At the end of this period, samples were centrifuged at 5000 g for 10 minutes and serum was removed for biochemical analyses.

Automated hematologic analyses were performed with an Ortho ELT-8 hematology system (Ortho Diagnostics Systems, Inc., Westwood, NJ). The following variables were measured: erythrocyte, leukocyte, and platelet counts; mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular hemoglobin (MCH); hematocrit (HCT); and hemoglobin concentration (HGB). Leukocyte differentials were determined by microscopic evaluation of Wright-stained blood smears. Reticulocytes were stained by mixing equal volumes of blood with new methylene blue stain. Relative numbers of reticulocytes, determined by microscopic examination of approximately 1000 erythrocytes, were converted to absolute counts based on the total erythrocyte count.

Analyses of biochemical variables in serum were performed using a Roche Cobas Fara chemistry system (Roche Diagnostics Systems, Nutley, NJ). For the following variables, reagent kits and applications developed by the manufacturer were used: alanine aminotransferase (ALT), total protein, albumin, urea nitrogen (UN), creatinine, creatine kinase (CK), and alkaline phosphatase (AP). For determinations of sorbitol dehydrogenase (SDH) and total bile acids, reagent kits were obtained from Sigma Chemical Company (St. Louis, MO) and applications were developed in-house for the chemistry analyzer.

Reproductive Toxicity

In screening for potential reproductive toxicity, the caudal, epididymal, and testicular weights, sperm motility, sperm count per gram caudal tissue, and testicular spermatid head count were evaluated at necropsy. Vaginal cytology was evaluated on animals during the 2 weeks just preceding necropsy, using procedures outlined by Morrissey *et al.* (1988). For the 12 days prior to sacrifice, females were subject to vaginal lavage with saline. The aspirated cells were air-dried onto slides, stained with Toluidine Blue O, and cover slipped. The relative preponderance of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were used to identify the stages of the estrual cycle.

Sperm motility was evaluated at necropsy as follows: The left epididymis was removed and quickly weighed; the cauda epididymis was removed at the junction of the vas deferens and the corpus

TABLE 1 Experimental Design and Materials and Methods in the 13-Week Studies of Glyphosate

Study Dates May -- September, 1988	Type and Frequency of Observation Observed 2 x d for mortality/moribundity; 1 x wk for clinical signs of toxicity; weighed initially, 1 x wk, and at necropsy; food consumption was measured.
Strain and Species F344/N rats; B6C3F ₁ mice	Diet NIH-07 feed and water <i>ad libitum</i>
Animal Source Simonsen Laboratories, Gilroy, CA	Animal Room Environment Temp.: 67 - 74oF; relative humidity 40 - 89%; 10 air exchanges/hour; 12 h fluorescent light/day
Study Laboratory Southern Research Institute, Birmingham, AL	Time Held Before Study Rats -- 12 days; Mice -- 11 days
Size of Study Groups 10 males and 10 females of each species per dose group. Rats were housed 5 per cage; mice were individually caged.	Age When Placed on Study Rats - 43 days; Mice - 49 days
Doses Rats and mice -- 0, 3125, 6250, 12500, 25000, or 50000 ppm in feed	Duration of Dosing Rats - daily for 13 weeks; Mice - daily for 13 weeks
Method of Animal Distribution Animals were assigned to groups using a stratified weight method and then assigned to study groups in random order.	Age When Killed Rats -- 135-137 days; Mice -- 142-144 days
Necropsy and Histologic Examinations: Complete necropsies were performed on all animals. Complete histopathologic examination was conducted on the control and the highest treatment group (50000 ppm); the target organ, salivary gland, was examined in all lower dose groups; the following tissues were examined microscopically for all controls and 50000 ppm group animals: adrenal glands, bone (femur, including marrow and epiphysis), brain (three sections: frontal cortex and basal ganglia, parietal cortex and thalamus, cerebellum and pons), esophagus, eyes (if grossly abnormal), gall bladder (mice), gross lesions and tissue masses with regional lymph nodes, heart, intestine (duodenum, jejunum, ileum, cecum, colon, rectum), kidneys, liver, lungs and mainstem bronchi, lymph nodes (mandibular, mesenteric), mammary	gland and adjacent skin, nasal cavity and turbinates (three sections), ovaries, pancreas, parathyroid glands, pituitary gland, preputial or clitoral glands, prostate gland, salivary glands, spinal cord and sciatic nerve (if neurologic signs were present), spleen, stomach (including forestomach and glandular stomach), testes/epididymis, seminal vesicle, thigh muscle, thymus, thyroid gland, trachea, urinary bladder, uterus, vagina (from animals used in SMVCE). Organ weights (to the nearest mg) obtained from all core study animals include: liver, thymus, right kidney, right testis, heart and lungs. Hematologic and serum chemical analyses were performed; sperm motility and vaginal cytology was evaluated in rats and mice exposed to 0, 12500, 25000, and 50000 ppm.

epididymis, then weighed. Warm (37°C) Tyrodes buffer (mice) or test yolk buffer (rats) was applied to two pre-warmed slides, and a small cut was made in the distal cauda epididymis.

The sperm that extruded from the epididymis were dispersed throughout the solution, cover-slipped, and counted immediately on a warmed microscope stage. Two independent observers counted the number of moving and non-moving sperm in 5 fields of 30 sperm or less per field. After sperm sampling for motility evaluation, the cauda was placed in phosphate buffered saline (PBS), gently chopped with a razor blade, and allowed to sit for 15 minutes. The remaining clumps of tissue were removed, the solution was mixed gently, then heat-fixed at 65°C. Sperm density was subsequently determined using a hemocytometer.

To quantify spermatogenesis, the left testis was weighed, frozen and stored. After thawing, testicular spermatid head count was determined by removing the tunica albuginea and

homogenizing the testis in PBS containing 10% DMSO. Homogenization-resistant spermatid nuclei were enumerated using a hemocytometer; the data were expressed as spermatid heads per total testis, and per gram of testis.

Study of the Mechanism of Induction of Salivary Gland Lesions by Glyphosate

Because of the morphologic similarity between a salivary gland change noted in the 13-week studies of glyphosate and a salivary gland lesion previously reported to result from treatment with the adrenergic agonist, isoproterenol, a study was designed to test the hypothesis that the salivary gland effect of glyphosate was mediated through an adrenergic mechanism. For this study, male F344/N rats (200-250 g) were obtained from Charles River Laboratories (Raleigh, NC) and were randomized to 5 groups with 4 animals per group. Glyphosate was administered to the appropriate groups by dosed feed, while control groups were fed control NIH-07 diet. The adrenergic agents, isoproterenol and propranolol, were administered by continuous subcutaneous infusion by osmotic minipumps. Treatment groups are shown in Table 2.

TABLE 2 Treatment Groups in the Study to Determine the Mechanism of Induction of Salivary Gland Lesions by Glyphosate

Group	Feed	Pump
1	control	vehicle (water + 0.1% ascorbate)
2	glyphosate (50000 ppm)	vehicle
3	glyphosate (50000 ppm)	propranolol (~1.2 mg/kg/day)
4	control	isoproterenol (~1.0 mg/kg/day)
5	control	isoproterenol + propranolol

One day prior to initiating glyphosate-dosed feed, all rats were anesthetized with methoxyflurane and osmotic minipumps (Alzet model 2002, pumping rate 0.55 ± 0.03 ml/h, Alza Corporation, Palo Alto, CA) were implanted subcutaneously. Group 1 (negative control) was fed standard NIH-07 diet and implanted with pumps containing vehicle (sterile water + 0.1% ascorbic acid). Group 2 was fed NIH-07 diet containing glyphosate (50000 ppm) and implanted with vehicle pumps. Group 3 was fed 50000 ppm glyphosate-dosed feed and implanted with pumps containing the adrenergic antagonist propranolol (Sigma Chemical Co., St. Louis, MO, 25 mg/ml vehicle). As a positive control, Group 4 was administered the adrenergic agonist, isoproterenol (Sigma Chemical Co., St. Louis, MO, 20 mg/ml vehicle), by pump and fed normal diet. Group 5 animals (blocking controls) were implanted with both isoproterenol and propranolol pumps and fed normal diet. The rats were identified by tail tattoo and weighed one day prior to initiation of dosed feed and at study termination. Food consumption was measured every other day. After 14 days of treatment, the left parotid and submandibular/sublingual glands were removed and weighed separately, after which the glands were cut into small pieces, placed into a 2.5% glutaraldehyde/2.0% paraformaldehyde solution, and processed for electron microscopy. The right parotid and submandibular/sublingual glands were removed and placed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) and Alcian Blue (pH 2.5)-periodic acid Schiff (AB-PAS).

Genetic Toxicity Studies

Mutagenicity Studies

Mutagenicity studies of glyphosate in *Salmonella typhimurium* were conducted as described in Zeiger *et al.* (1988). Glyphosate was tested for genotoxicity in *S. typhimurium* strains TA100, TA1535, TA97, and TA98 using the plate-incorporation assay in both the absence or presence of Aroclor 1254-induced S9 from male Syrian hamster liver or male Sprague-Dawley rat liver. Glyphosate was dissolved in distilled water and tested at doses up to 10,000 µg/plate. A positive response is defined in this assay as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants which was not dose-related, not reproducible, or of insufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment.

Mouse Peripheral Blood Micronucleus Test

At the termination of the 13-week study, blood smears were prepared from peripheral blood samples obtained by cardiac puncture of dosed and control mice. The slides were stained with Hoechst 33258/pyronin Y (MacGregor *et al.*, 1983). Ten thousand normochromatic erythrocytes from each animal were scored for micronuclei.

Statistical Methods

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Organ and body weight data, which are approximately normally distributed, were analyzed using the parametric multiple comparisons procedures of Williams (1971, 1972, 1986) and Dunnett (1955). Clinical pathology and hema-tology data, which typically have skewed distributions, were analyzed using the nonparametric multiple comparisons methods of Shirley (1977) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of dose-response trends and to determine whether a trend-sensitive test (Williams, Shirley) was more appropriate for pairwise comparisons than a test capable of detecting departures from monotonic dose-response (Dunnett, Dunn). If the P-value from Jonckheere's test was greater than or equal to 0.10, Dunn's or Dunnett's test was used rather than Shirley's or Williams' test.

The outlier test of Dixon and Massey (1951) was employed to detect extreme values. No value selected by the outlier test was eliminated unless it was at least twice the next largest value or at most half of the next smallest value.

Analysis of Vaginal Cytology Data

Since the data are proportions (the proportion of the observation period that an animal was in a given estrous state), an arcsine transformation was used to bring the data into closer conformance with normality assumptions. Treatment effects were investigated by applying a multivariate analysis of variance (Morrison, 1976) to the transformed data to test for the simultaneous equality of measurements across dose levels.

Analysis of Micronuclei Data

Statistical analyses for micronuclei were completed using linear trend tests on polychromatic erythrocytes data and log-transformed data for normochromatic erythrocytes, and analysis of variance on ranks (ANOVA) for percentage polychromatic cells among total erythrocytes. The frequency of micronuclei in the dosed groups was compared with the frequency determined for the concurrent untreated control animals using the Student t-test.

Quality Assurance

The 13-week toxicity studies of glyphosate were performed in compliance with FDA Good Laboratory Practices regulations (21 CFR 58). The Quality Assurance Unit of Southern Research Institute performed audits and inspections of protocols, procedures, data, and reports throughout the course of the studies. The operations of the Quality Assurance Unit were monitored by the NTP.

RESULTS

Disposition Studies

More than 90% of the radioactivity from either a 5.6 or 56 mg/kg oral dose of [¹⁴C]-glyphosate was eliminated within 72 hours. Approximately 50% was eliminated in the feces in the first 24 hours; urinary elimination of radioactivity was essentially complete by 12 hours. The apparent decrease in cumulative percentage eliminated in urine after the 5.6 mg/kg oral dose probably is due to interindividual variation, and variances (from 10 to 3) in the number of animals per time point. In contrast, following an intravenous dose of [¹⁴C]-glyphosate at 5.6 mg/kg, 90% of radioactivity was eliminated in urine in the first 6 hours (Table 3).

TABLE 3 Cumulative Percentage of Oral or I.V. Dose of Glyphosate Eliminated in Urine and Feces^a

Time (Hours)	Oral 5.6 mg/kg		Oral 56 mg/kg		I.V. 5.6 mg/kg	
	Urine	Feces	Urine	Feces	Urine	Feces
6	10 ± 5	7 ± 11			90 ± 7	0.3 ± 0.2
12	31 ± 10	28 ± 10			95 ± 9	0.5 ± 0.5
24	26 ± 14	55 ± 13	28 ± 10	47 ± 12	98 ± 11	3 ± 2
48	18 ± 2	71 ± 8	33 ± 12	57 ± 15		
72	19 ± 2	74 ± 5	34 ± 12	58 ± 15		

^a N = 3-10

TABLE 4 Percentage of Dose in Tissues Following Oral Administration of Glyphosate at 5.6 mg/kg^a

Tissue	Time (h)				
	3 ^b	6 ^b	12 ^b	24 ^c	96 ^c
Small Intestine	7.72 ± 1.74	10.20 ± 5.49	4.12 ± 2.25	0.48 ± 0.51	0.03 ± 0.01
Large Intestine	1.21 ± 1.07	0.51 ± 0.01	0.46 ± 0.28	0.17 ± 0.17	0.01 ± 0.00
Liver	0.10 ± 0.00	0.07 ± 0.04	0.11 ± 0.01	0.14 ± 0.08	0.05 ± 0.05
Kidney	0.36 ± 0.19	0.48 ± 0.42	0.31 ± 0.06	0.10 ± 0.07	ND
Skin	0.70 ± 0.45	0.18 ± 0.25	0.21 ± 0.12	ND ^d	ND
Blood	0.28 ± 0.01	0.18 ± 0.06	0.31 ± 0.10	0.03 ± 0.06	ND
Tissue Total	12.00 ± 0.33	11.67 ± 6.29	5.54 ± 2.35	0.89 ± 0.84	0.10 ± 0.06

^a Data represented as percent of dose administered ± standard deviation.

^b N = 2 rats.

^c N = 3 rats.

^d ND notes that the values were not determined as the amount of radioactivity in the samples was below the level of accurate analytical measurement (<100 dpm).

The tissue distribution of radioactivity from a single oral 5.6 mg/kg dose of [¹⁴C]-glyphosate is presented in Table 4. At time points up to 24 hours, most of the radioactivity was found in the gastrointestinal tract; only 1% remained in the tissues at 24 hours.

In animals given a 56 mg/kg oral dose, the peak blood level of radioactivity occurred later than in those given a 5.6 mg/kg oral dose (1 hour vs. 2 hours); the peak blood concentration was more

than 30 times higher following the 56 mg/kg oral dose (Figure 1). Radioactivity rapidly declined in blood following a 5.6 mg/kg i.v. dose (Figure 2). The blood radioactivity vs. time plot fits a 2-compartment model with an alpha (distribution) phase of about 0.5 hour and a beta (elimination) phase of 13 hours.

Rats were exposed to Roundup® (the isopropylamine salt of glyphosate and added surfactants) in drinking water at concentrations of 0.5 to 100,000 ppm for 9 to 16 days. No differences were observed in the elimination of an oral dose of 5.6 mg/kg [¹⁴C]-glyphosate following any of these exposures, as compared with the elimination of a similar dose 1 day prior to beginning administration of Roundup® (data not shown).

13-Week Studies in F344/N Rats

All animals survived until the end of the study. Diarrhea was observed in the 50000 ppm groups of both sexes for the first 50 days, though not thereafter. In males, reduced weight gains were observed in the 25000 and 50000 ppm groups. The final mean body weight of the 50000 ppm group was approximately 18% less than that of controls (Table 5 and Figure 3). In females, there was only a marginal effect on body weight gain, with the high dose group 5% lighter than controls at the end of the study (Figure 3). In male rats, small increases in relative organ weights were observed for liver, kidney, and testicle; a decrease in relative weight was observed in the thymus (Appendix A, Table A1). In females, changes in organ weights were minor and could not be related definitely to treatment. There were no treatment-related effects on food consumption throughout the study. The mean, time-weighted chemical consumption for each group, based on food intake, is given in Table 5.

TABLE 5 Survival, Weight Gain, and Feed Consumption of F344/N Rats in the 13-Week Dosed Feed Study of Glyphosate

Dose (ppm) In Feed	Survival ^a	Mean Body Weight (grams)			Final Weight Relative to Controls (%) ^c	Average Feed Consumption ^d	Glyphosate Consumed ^e
		Initial	Final	Change ^b			
MALE							
0	10/10	115	353	238		17	0
3125	10/10	111	352	241	100	17	205
6250	10/10	111	338	227	96	17	410
12500	10/10	113	345	232	98	17	811
25000	10/10	108	332	224	94	17	1678
50000	10/10	112	290	178	82	15	3393
FEMALE							
0	10/10	95	191	96		11	0
3125	10/10	92	190	98	100	11	213
6250	10/10	94	194	100	102	11	421
12500	10/10	96	193	97	101	11	844
25000	10/10	92	186	94	97	11	1690
50000	10/10	95	181	86	95	10	3393

^a Number of animals surviving at 13 weeks/number/dose group.

^b Mean weight change of the animals in each dose group.

^c (Dosed group mean/Control group mean) x 100.

^d Average food consumption in gm/animal/day.

^e Estimated, mean, time-weighted chemical consumption in mg/kg/day.

Figure 1 **Blood Levels of ¹⁴C-Glyphosate Following Oral Administration of ¹⁴C-Glyphosate at 5.6 or 56 mg/kg (% dose ± standard deviation)**

Figure 2 **Levels of Radioactivity in Blood after a Single I.V. Dose of 5.6 mg/kg Glyphosate (2 rats per time point, results averaged).**

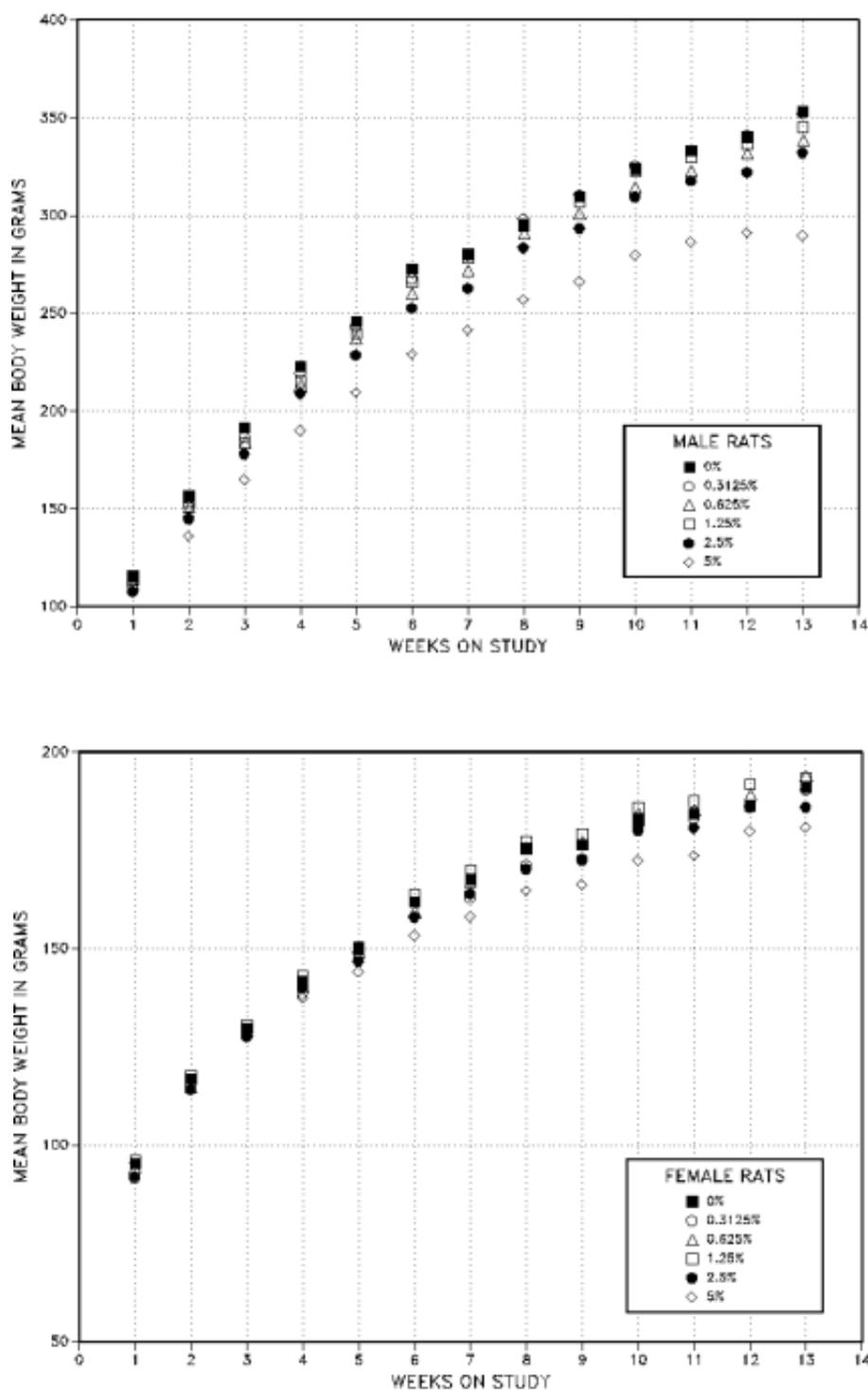


Figure 3 Body Weights of F344/N Rats Exposed to Glyphosate by Dosed Feeding for 13 Weeks

Chemically-related changes in hematological parameters observed in male rats at 13 weeks included mild increases in hematocrit and RBC at 12500, 25000, and 50000 ppm, hemoglobin at 25000 and 50000 ppm, and platelets at 50000 ppm. In female rats, minimal but significant increases occurred in lymphocyte and platelet counts, WBC, MCH, and MCV. Treatment-related alterations in clinical chemistry parameters included increases in alkaline phosphatase in males and in females at all time points, alanine aminotransferase activity in males and females at all time points except 90 days, total bile acids at days 23 and 90 in males and at day 23 in females, total protein in females at all time points, and sporadic increases in urea nitrogen and albumin (Appendix B).

In reproductive studies, male rats experienced a significant decrease (20%) in sperm counts in the 25000 and 50000 ppm groups. Left caudal, epididymal and testicular weights, epididymal sperm motility, total spermatid heads/testes, and total spermatid heads/g caudal tissue were not different from those of controls (Appendix C, Table C1). Female rats had a longer estrous cycle length (5.4 days vs. 4.9 days) in the 50000 ppm group compared to controls (Appendix C, Table C1).

At necropsy, no gross lesions were observed that were considered possibly related to glyphosate administration. Morphologic changes attributed to glyphosate were observed microscopically in the parotid and submandibular salivary glands of male and female rats. Salivary gland lesions were diagnosed as "cytoplasmic alteration" and consisted of basophilic change and hypertrophy of acinar cells. These changes were more evident in the parotid gland in which the normal granular, eosinophilic staining cytoplasm of the acinar epithelial cells was replaced by basophilic and finely vacuolated cytoplasm (Plate 1). This effect varied in distribution from multifocal in less severe cases, imparting a mottled tinctorial staining appearance to the gland, to diffuse involvement in higher dose animals. In addition, acinar cells appeared swollen, resulting in enlargement of secretory acini and a relative reduction in the number of secretory ducts seen. Nuclei of affected acinar cells were hyperchromatic. In the submandibular salivary gland, similar cytoplasmic tinctorial changes and hypertrophic effects were observed (Plate 2). The sublingual gland was not detectably altered.

A no-effect level for cytoplasmic alteration of the parotid and submandibular salivary glands in this study was not reached. One control female rat had a small basophilic focus in the parotid gland which was typical of the spontaneous lesion occasionally seen in rats. Table 6 presents incidence and severity data of glyphosate-induced cytoplasmic alteration of the salivary glands from the 13-week dosed feed study in rats. No other lesions in rats appeared related to glyphosate administration.

TABLE 6 Incidence and Severity of Cytoplasmic Alteration of the Parotid and Submandibular Salivary Glands (combined) in F344/N Rats in the 13-Week Dosed Feed Study of Glyphosate

Dose (ppm)	0	3125	6250	12500	25000	50000
MALES	0/10	6/10 (1.0)*	10/10 (1.0)	10/10 (1.8)	10/10 (2.7)	10/10 (2.9)
FEMALES	0/10	8/10 (1.0)	10/10 (1.0)	10/10 (2.1)	10/10 (2.4)	10/10 (3.0)

* Average severity score based on a scale of 1=minimal, 2=mild, 3=moderate, 4=marked.

13-Week Studies in B6C3F₁ Mice

Body weight gains were depressed in the 2 highest dose groups of both sexes (Table 7 and Figure 4). There were 2 early deaths in the study: An untreated female was accidentally killed, and a high dose female died from undetermined causes (Table 7). Increases in relative organ weights were observed in the heart, kidney, liver, lung, thymus, and testis of male mice (Appendix A, Table A2). There were no differences in food consumption between the dosed and control groups.

TABLE 7 Survival, Weight Gain, and Feed Consumption of B6C3F₁ Mice in the 13-Week Dosed Feed Study of Glyphosate

Dose (ppm) in Feed	Survival ^a	Mean Body Weight (grams)			Final Weight Relative to Controls(%) ^c	Average Feed Consumption ^d	Glyphosate Consumed ^e
		Initial	Final	Change ^b			
MALE							
0	10/10	23.5	32.1	8.6		4.6	0
3125	10/10	23.2	31.1	7.9	97	4.5	507
6250	10/10	23.4	31.5	8.1	98	4.7	1065
12500	10/10	23.2	30.3	7.1	94	4.9	2273
25000	10/10	23.0	28.6	5.6	89	5.1	4776
50000	10/10	23.5	26.7	3.2	83	5.3	10780
FEMALE							
0	9/10	18.9	27.9	9.0		5.4	0
3125	10/10	18.4	28.6	10.2	103	5.8	753
6250	10/10	18.2	26.2	8.0	94	5.3	1411
12500	10/10	18.8	26.9	8.1	96	5.2	2707
25000	10/10	18.5	26.2	7.7	94	5.3	5846
50000	9/10	18.5	25.1	6.6	90	5.2	11977

- a Number of animals surviving at 13 weeks/number in dose group.
 b Mean weight change of the animals in each dose group.
 c (Dosed group mean/Control group mean) x 100.
 d Average food consumption in gm/animal/day.
 e Estimated, mean, time-weighted chemical consumption in mg/kg/day.

A "dark" salivary gland in a high-dose male was the only significant gross finding at necropsy. No effects were observed on sperm motility or estrual cycle length. Treatment-related microscopic changes were limited to the parotid salivary gland; the changes consisted of a diffuse increase in basophilia of the acinar cells, diagnosed as "cytoplasmic alteration." In more severely affected glands, the cells and acini also appeared enlarged with an associated relative reduction in the number of ducts. Submandibular and sublingual glands were not detectably altered. The incidence and severity of cytoplasmic alteration of the parotid salivary gland was dose-related (Table 8).

TABLE 8 Incidence and Severity of Cytoplasmic Alteration of the Parotid Salivary Gland in B6C3F₁ Mice in the 13-Week Glyphosate-Dosed Feed Study

Dose (ppm)	0	3125	6250	12,500	25,000	50,000
MALES	0/10	0/10	5/10 (1.0)*	9/10 (1.6)	10/10 (2.8)	10/10 (4.0)
FEMALES	0/10	0/10	2/10 (1.0)	9/10 (1.3)	10/10 (2.1)	10/10 (3.1)

* Average severity score based on a scale of 1=minimal, 2=mild, 3=moderate, 4=marked.

Plate 1 Parotid salivary gland of control rat (1a) and 50000 ppm dose group rat (1b) from the 13-week dosed feed study of glyphosate. Note swelling and basophilia of acini (A) and decreased relative number of ducts (D) in the glyphosate-treated animal compared to control. 150X

Plate 2 Submandibular salivary gland of control rat (2a) and 50000 ppm dose group rat (2b) from the 13-week dosed feed study of glyphosate. Note slight enlargement and mottled staining of acini (A) and decreased relative number of ducts (D) in the glyphosate-treated animal compared to control. 150X

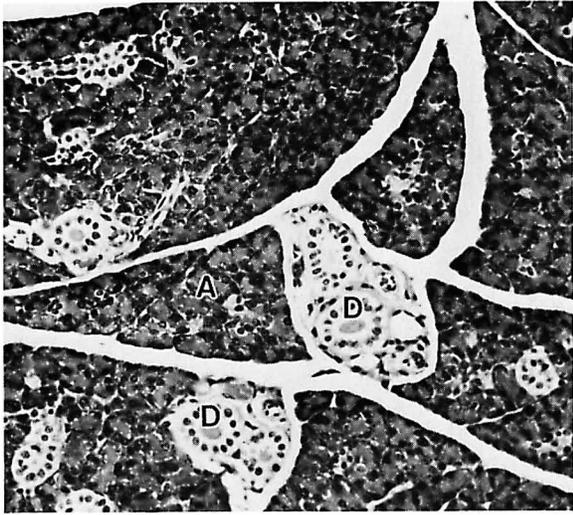


Plate 1 (a)

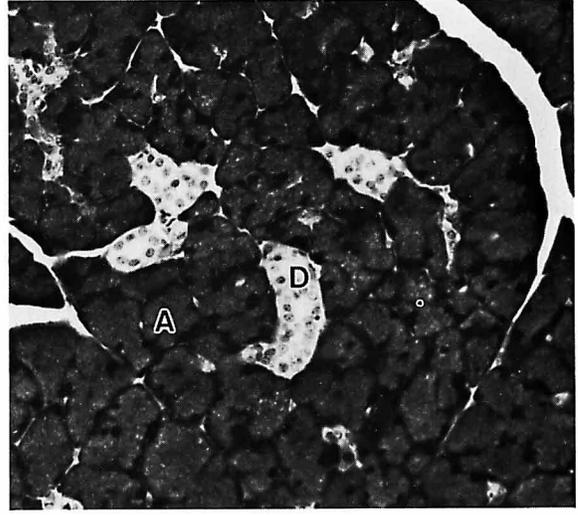


Plate 1 (b)

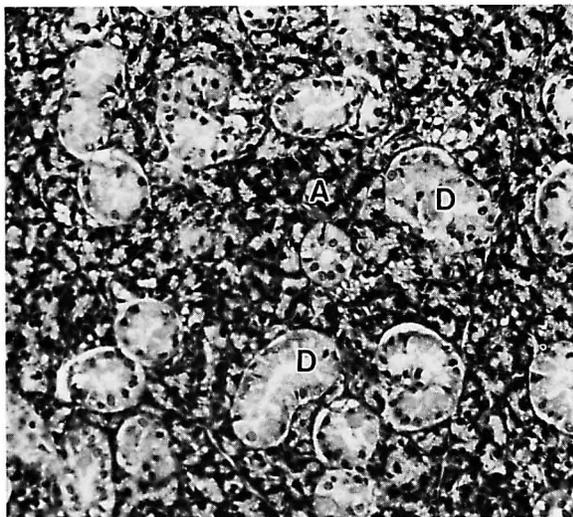


Plate 2 (a)

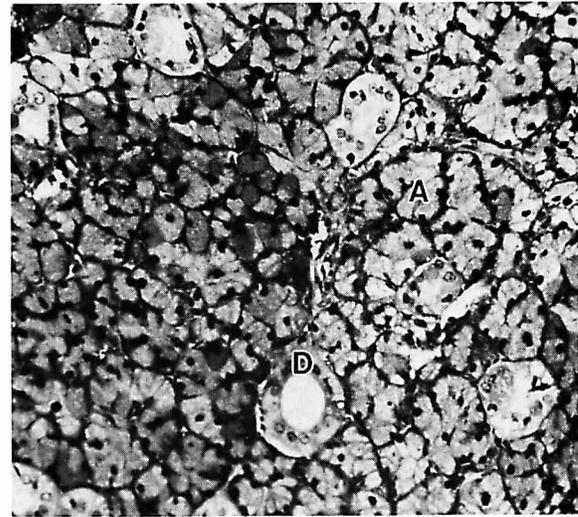


Plate 2 (b)

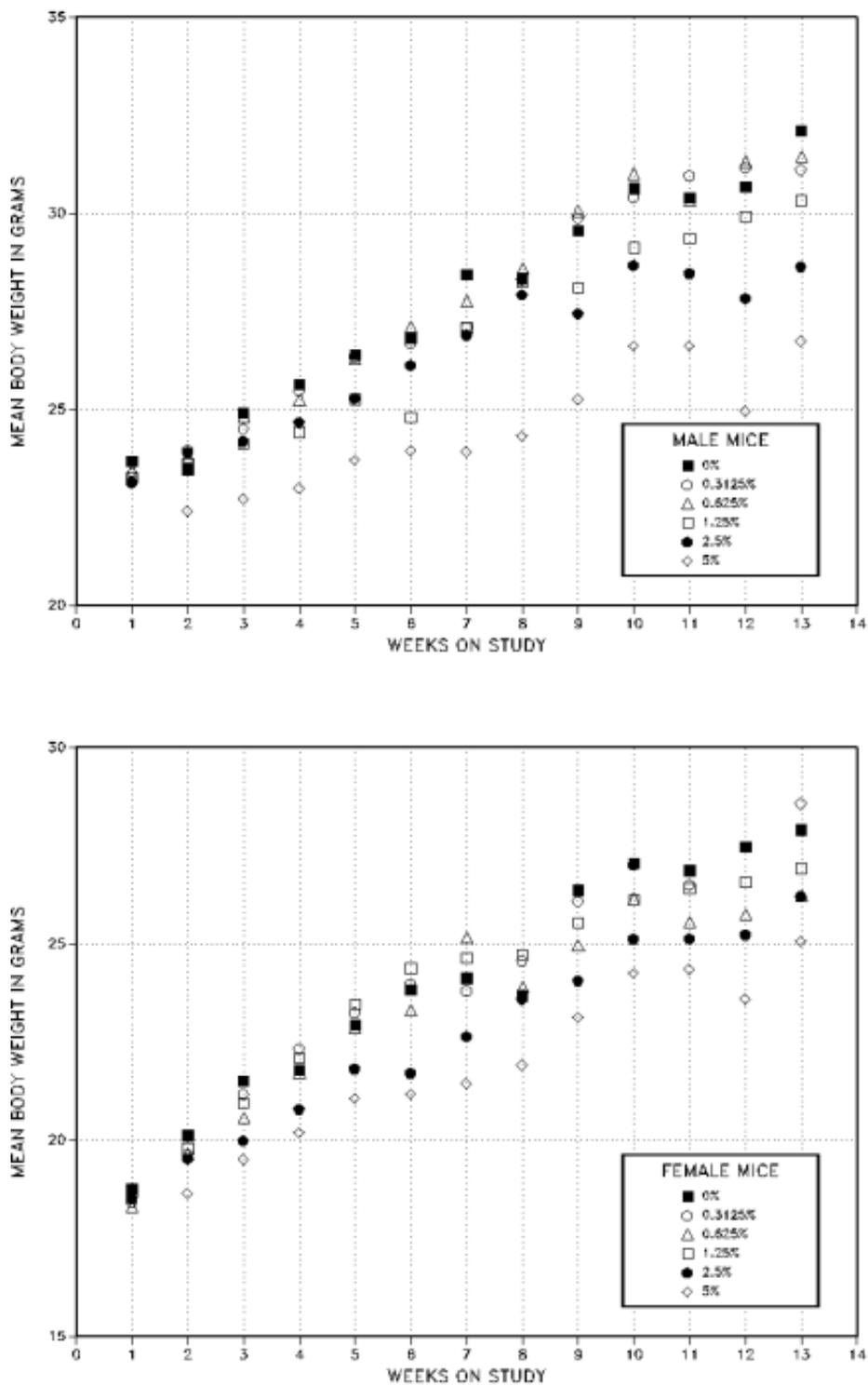


Figure 4 Body Weights of B6C3F₁ Mice Exposed to Glyphosate by Dosed Feeding for 13 Weeks

Mechanism of Induction of Salivary Gland Lesion

Cytoplasmic alteration of salivary gland acinar cells induced by glyphosate in the 13-week studies was similar morphologically to the reported effect of chronic treatment with the adrenergic mediator isoproterenol. To test the hypothesis that the salivary gland effect of glyphosate is mediated through an adrenergic mechanism, a special study was designed in which rats were concurrently administered glyphosate by dosed feed and/or adrenergic agents by subcutaneous minipump infusion.

All rats survived to the end of the 14-day study; the implanted minipumps were well-tolerated. Rats receiving isoproterenol were hypoactive and had increased respiratory rates on day 1 following pump implantation, but were normal by the following day. Feces of rats receiving glyphosate-dosed feed were observed to be slightly softer in consistency and wetter than normal in appearance by study day 7; perianal fecal staining was also evident in several of these animals. Average food consumption and body weight gains are presented by group in Table 9. It is apparent that there was no food avoidance in those groups receiving the glyphosate-dosed feed; there was a significant decrease in body weight gains in those groups, however.

TABLE 9 Feed Consumption and Weight Gain of F344/N Rats in the 14-Day Mechanism Study of Glyphosate

Treatment Group (diet/pump)	Food Consumption (gm/rat/day)	Weight Gain (gm)
1 (control diet/vehicle)	14.4	16.0 ± 2.9
2 (glyphosate/vehicle)*	17.6	6.3 ± 2.0
3 (glyphosate/propranolol)*	20.4	6.0 ± 2.4
4 (control diet / isoproterenol)	14.9	16.7 ± 1.6
5 (control diet/isoproterenol + propranolol)	15.0	17.5 ± 8.0

* Glyphosate was given in the diet at a concentration of 50000 ppm.

Parotid and submandibular/sublingual salivary gland weight data are shown in Table 10. Both isoproterenol, the adrenergic agonist given by subcutaneous infusion, and glyphosate, in dosed feed, induced significant enlargement of these glands, glyphosate having much greater effect than isoproterenol. The parotid was the much more affected of the two glands. The adrenergic antagonist, propranolol, inhibited the effect of both isoproterenol and glyphosate on salivary gland weights. In the parotid, there was approximately a 50% increase in gland weight following isoproterenol administration, an effect blocked completely by concurrent administration of propranolol. Glyphosate induced an almost 3-fold increase in parotid weight, an effect significantly inhibited, though not completely, by propranolol. These trends were paralleled by smaller changes in submandibular/sublingual gland weights.

Microscopically, both isoproterenol and glyphosate given in the 14-day study induced lesions in the parotid gland similar to those seen in the 13-week study. These lesions consisted of cytoplasmic basophilic change, fine vacuolation, and swelling of acinar cells, diagnosed collectively as cytoplasmic alteration. A distinct gradation in the severity of these lesions was possible based on the extent of involvement and degree of tinctorial alteration and cell enlargement present.

TABLE 10 Salivary Gland Weights of F344/N Rats in the 14-day Mechanism Study of Glyphosate

Group (diet/pump)	Parotid		Submandibular/Sublingual	
	Absolute (mg)	Relative*	Absolute (mg)	Relative*
1 (control diet/vehicle)	126.2 ± 16.4	0.50 ± 0.08	209.7 ± 14.8	0.83 ± 0.04
2 (glyphosate/vehicle)	354.0 ± 37.5	1.47 ± 0.12	375.0 ± 26.3	1.56 ± 0.07
3 (glyphosate/propranolol)	245.0 ± 10.4	1.06 ± 0.06	261.0 ± 6.4	1.13 ± 0.04
4 (control diet / isoproterenol)	194.2 ± 15.6	0.76 ± 0.06	259.7 ± 10.6	1.03 ± 0.03
5 (control diet/isoproterenol + propranolol)	137.2 ± 19.1	0.55 ± 0.07	225.5 ± 7.8	0.91 ± 0.05

* mg/g body weight

Glyphosate-treated animals were most severely affected; glands from all these animals were characterized by diffuse, intense basophilic change of acinar cells with clearly evident acinar enlargement, resulting in a relative reduction in the number of ducts present. Concurrently, the cytoplasm of affected cells was finely vacuolated, and nuclei were hyperchromatic and displaced more basally by increased cytoplasmic volume. In serial sections stained with Alcian Blue/periodic acid Schiff (AB/PAS), areas of cytoplasmic alteration were seen to be associated with loss of PAS positive staining of secretory granules. Animals receiving the adrenergic antagonist, propranolol, subcutaneously and concurrently with glyphosate-dosed feed were clearly protected from the more severe lesions. All animals dosed with isoproterenol were likewise affected with cytoplasmic alteration of salivary acinar cells; basophilic tinctorial change in these animals was multifocal to diffuse, and hypertrophy was less prominent than in the glyphosate group. Propranolol resulted in only modest protection from isoproterenol effects based on histomorphology. The incidence and average severity of cytoplasmic alteration of the parotid gland is shown in Table 11.

Cytoplasmic alteration of the submandibular gland was detectable by light microscopy only in the glyphosate-dosed animals (Table 11). The lesion consisted primarily of cellular and acinar swelling with a relative reduction in the number of duct profiles per field. Tinctorial change was less of a component of the submandibular lesion than in the parotid, with most acinar cells being slightly more pale staining than controls, with scattered individual cells or acini being more basophilic, imparting a mottled staining pattern to the tissue. AB-PAS reactivity was essentially unchanged in affected glands.

TABLE 11 Incidence and Severity of Cytoplasmic Alteration of the Salivary Glands of F344/N Rats in the 14-Day Mechanism Study of Glyphosate

Group (Feed/Pump)	Parotid	Submandibular	Sublingual
1 (control diet/vehicle)	1/4 (1.0) *	0/4	0/3
2 (glyphosate/vehicle)	4/4 (4.0)	4/4	0/4
3 (glyphosate/propranolol)	3/4 (1.5)	4/4	0/2
4 (control diet / isoproterenol)	4/4 (2.7)	0/4	0/1
5 (control diet/isoproterenol + propranolol)	4/4 (2.0)	0/4	0/4

* Average severity grades for parotid gland lesions in affected animals, based on the following scale:
1=Focal change; 2=Multifocal, confluent change; 3=Diffuse change;
4=Diffuse change with intense basophilia and marked acinar swelling.

Plate 3 Electron micrograph of parotid acinar cells from a control male rat. Note electron dense secretory granules (S) and parallel arrays of rough endoplasmic reticulum (R). 5520X

Plate 4 Electron micrograph of parotid acinar cell from a male rat treated with isoproterenol, 1 mg/kg/day subcutaneously for 14 days. There is an increase in cell size and in electron lucency of secretory granules (S). Rough endoplasmic reticulum (R) is abundant. 5520X

Plate 5 Electron micrograph of parotid acinar cell from male rat treated with glyphosate, 50000 ppm dosed feed for 14 days. Cellular changes are similar to those of the isoproterenol-treated animal but with an even greater increase in cell size and electron lucency of secretory granules (S). Granules are also increased in size. 5520X

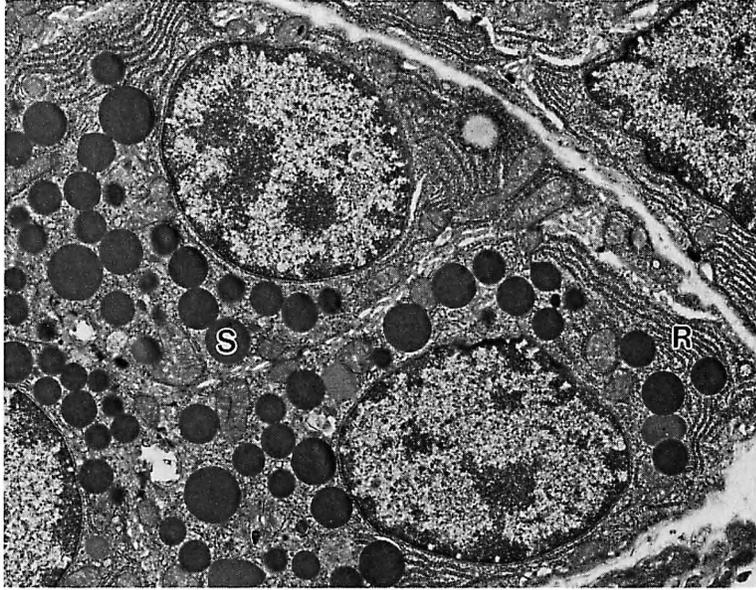


Plate 3

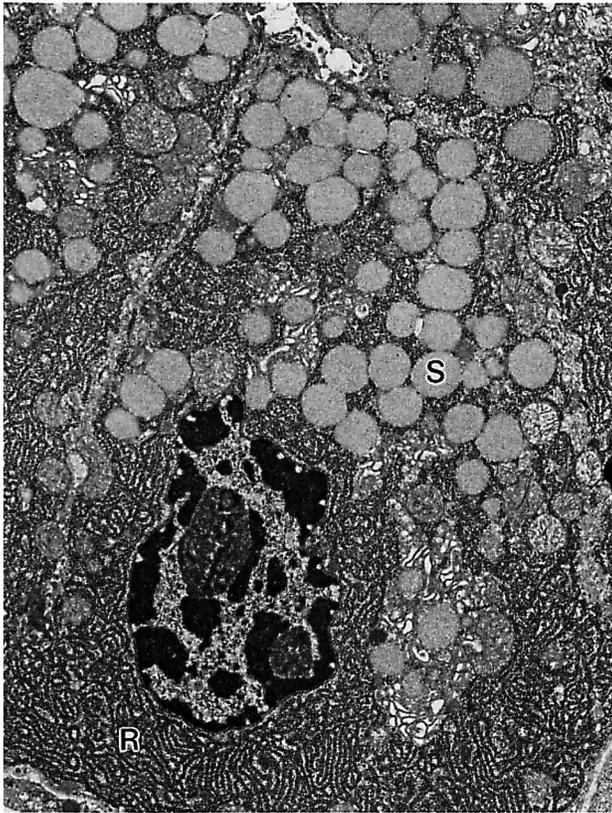


Plate 4



Plate 5

The lesions of the submandibular gland were more subtle than those in the parotid; differences in the severity of the cytoplasmic alteration in this gland were not appreciable by light microscopy. There was no definite, inhibitory effect of propranolol on the incidence of the glyphosate-induced change detected histologically in the submandibular gland. No microscopic change was observed in this gland in rats treated with isoproterenol. No changes in morphology or Alcian blue-periodic acid Schiff reactivity were seen in the sublingual glands examined from any groups.

Parotid and submandibular acinar cells from control, isoproterenol-treated, and glyphosate-treated animals were examined ultrastructurally. Parotid acinar cells of the control animals were of typical appearance, with basally oriented nuclei surrounded by rough endoplasmic reticulum (Plate 3). Electron dense secretory granules were concentrated in the apical cytoplasm. In contrast, secretory granules from the isoproterenol-treated animals were electron lucent in affected cells (Plate 4). Also, these cells obviously were enlarged, as evident from the increased cell area when compared to control cells at equivalent magnification; the number of secretory granules and volume of rough endoplasmic reticulum seemed to be increased concurrently. Similar changes, though of greater magnitude, were seen in parotid acinar cells from the glyphosate-dosed rats (Plate 5). There was a further progression in the lucency of the secretory granules, and the granules were noticeably enlarged and coalescent. Abundant rough endoplasmic reticulum surrounded the granules and nuclei, and the overall cell area was increased.

Ultrastructurally, control submandibular acini contained both mucous- and serous-type cells. Mucous cells were more prominent due to their larger size, central location within the acinus, and the large number of confluent, electron-lucent mucous granules. Serous cells were small and peripherally located in the acinus, and the electron-dense granules were few in number and relatively inconspicuous. Both cell types were dark-staining and contained abundant rough endoplasmic reticulum. In submandibular acini from the isoproterenol-treated animals, cells appeared swollen due to an increase in the number of granules; granules were heterogeneously stained, most with finely granular contents and others with granular stippling surrounding a more electron-dense core. Submandibular cells and acini from the glyphosate-exposed animals were markedly enlarged due to cytoplasmic engorgement with secretory granules, mostly of the lucent type, with some more heterogenous as seen in the isoproterenol animals. In these cells, granules were not limited to apical areas as in the controls but diffusely present throughout the cytoplasm. It could not be determined if the serous or mucous glandular acini were selectively affected by glyphosate.

Genetic Toxicology

Glyphosate (0-10000 µg/plate) did not induce gene mutations in *Salmonella typhimurium* strains TA100, TA1535, TA97, or TA98 when tested in a preincubation protocol in the presence and the absence of Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver S9 (Appendix D, Table D1). Peripheral blood normochromatic erythrocytes from male and female mice were analyzed at the termination of the 13-week feed study for frequency of micronuclei; no increase in micronuclei was observed in either males or females at any dietary concentration of glyphosate (Appendix D, Table D2).

DISCUSSION

Disposition studies showed that after a dose of glyphosate at either 5.6 or 56 mg/kg, over 70% of the administered dose was eliminated within 24 hours. Tissue distribution data indicate most of the radioactivity was in the gastrointestinal tract following oral administration, indicating the compound may not be completely absorbed. Comparison of the pattern of elimination following i.v. and oral administration of [¹⁴C]-glyphosate also supports the conclusion that the compound is incompletely absorbed. Radioactivity is eliminated primarily in feces after oral administration and primarily in urine following i.v. administration. If the usual assumption is made that i.v. administration represents the fate of a completely absorbed dose, then about 30% of the 5.6 mg/kg oral dose of glyphosate was absorbed; there is some evidence that a relatively higher percentage of the 56 mg/kg dose was absorbed. The 10-fold increase in dose resulted in a 30-fold increase in peak blood concentration. There also was a trend toward a higher percentage of the 56 mg/kg dose being eliminated in urine, but the differences were not statistically significant. Perhaps there is some interaction between glyphosate and the stomach/intestinal contents that binds a relatively larger percentage of the low dose, making it less available for absorption.

In the 13-week studies, glyphosate did not affect survival of F344/N rats or B6C3F₁ mice. Body weight gains were depressed in rats and mice at the 2 highest dose levels; weight gain depression was more severe in males than in females. Kubena *et al.* (1981) reported that body weight gains were reduced (about 50%) in male and female chicks fed a diet containing 6080 ppm of the isopropylamine salt of glyphosate for 21 days, beginning at 1 day of age; the calcium and magnesium content of the tibiotarsus bone was increased compared to controls. There were no differences in body weights in chicks fed a dose of 608 ppm or lower. In the Kubena study (which did not mention feed palatability) and in our 13-week study, the possibility of reduced food intake in the high dose groups cannot be ruled out; more food tends to be spilled when it is not palatable, and our food consumption measurements did not account for scattered feed. Poor palatability of feed containing high concentrations of glyphosate is suggested by the finding that rats drank less water containing Roundup[®] at 10000 ppm or higher. Another possibility is that the higher concentrations of glyphosate in feed result in poor absorption of dietary components from the GI tract. However, if uncoupling of oxidative phosphorylation, as proposed by Olorunsogo *et al.* (1979) and Bababunmi *et al.* (1979), is occurring as a result of glyphosate ingestion, then a reduction in weight gain for a given amount of food consumed would be expected.

Hematologic effects in rats dosed with glyphosate were unremarkable and generally consistent with mild dehydration (increases in RBC counts, hematocrit, and hemoglobin concentrations). This conclusion also is supported by the mild increases that occurred at various time points in serum concentrations of urea nitrogen, total protein and albumin. Mild but significant increases in concentrations of TBA and in activities of serum alanine aminotransferase and alkaline phosphatase at multiple time points in male and female rats are consistent with an hepatobiliary effect. These findings likely reflect hepatocellular leakage or perhaps single cell necrosis (ALT) and cholestasis (TBA and ALP). Increases in absolute and relative liver weights in female rats also were suggestive of an effect of glyphosate on the liver, and support the

clinical pathology findings. However, the lack of histopathologic evidence for a treatment-related effect on the liver indicates the mild nature of the hepatotoxicity. Vainio *et al.* (1983) reported an absence of peroxisome proliferation or hypolipidemia in male Wistar rats given Roundup[®] daily by gavage at 300 mg/kg, 5 times a week for 2 weeks; these daily doses were more than 10-fold lower than those achieved in the highest dose groups in the current study.

Measures of sperm density, or the number of sperm/g caudal epididymal tissue, were reduced somewhat in male rats in the 2 highest dose groups; other spermatozoal measurements were not different from controls in rats or mice. There was a slight lengthening of the estrous cycle in high dose female rats, but the biologic significance of these findings, if any, is not known.

It is noteworthy that the U.S. Environmental Protection Agency, after reviewing an unpublished 2-year carcinogenicity study of glyphosate in CD-1 mice, announced that there was "an equivocal carcinogenic response, possibly causing a slight increase in the incidence of renal tubular adenomas in male mice at the highest dose tested (30000 ppm)." A carcinogenicity study in rats has yet to be reviewed (Anonymous, 1991). In the present study, however, the salivary gland was identified as the sole target organ for glyphosate toxicity in both rats and mice. The lesion was diagnosed as cytoplasmic alteration of the acinar epithelial cells, consisting of increased basophilic staining and vacuolation of cytoplasm, and enlargement of cells and acini. This lesion was limited to the parotid gland in mice but affected both parotid and submandibular glands in rats; the sublingual gland was not affected. Salivary gland lesions are relatively uncommon in toxicity studies; however, both spontaneous and chemically-induced changes of a similar nature to those seen in the glyphosate study have been described. So-called "basophilic hypertrophic foci" occasionally may be seen as a spontaneous lesion in the parotid gland of rats and mice (Chiu and Chen, 1986); however, these are infrequent and focal in nature. More extensive and diffuse basophilic and hypertrophic change has been described in subchronic studies with some chemicals, such as doxylamine (Jackson and Blackwell, 1988) and methapyrilene (Jackson and Sheldon, 1984). By far, the most extensive and detailed studies of these changes in salivary glands have been done with sympathomimetic agents -- for example, the adrenergic agonist, isoproterenol, which induces striking morphologic changes in salivary glands (Schneyer, 1962; Fukuda, 1968). As with glyphosate's effects on the salivary glands, isoproterenol affects the parotid and submandibular glands but not the sublingual. This is due to the fact that, in the rat, the acini of the parotid and submandibular are richly supplied with adrenergic fibers, while the sublingual gland is devoid of adrenergic innervation (Nordenfelt, 1967). Because glyphosate and isoproterenol are similar in both the morphologic effects induced in the salivary glands and the gland specificity of those effects, it was hypothesized that glyphosate-related lesions were mediated through an adrenergic mechanism. A study was designed to test this hypothesis.

Two weeks' exposure to glyphosate by dosed feed resulted in marked increases in parotid and submandibular salivary gland weights. This effect on salivary gland weights is similar to that of isoproterenol, both as described in the literature (Schneyer, 1962) and as seen in the positive control group of this study. Increased salivary gland weights were associated histologically with cytoplasmic alteration of acinar cells. This effect was more marked in the parotid than in the submandibular gland. In the parotid, the cytoplasmic change induced by both glyphosate and isoproterenol was associated with a loss of the normal PAS-positive reactivity of the secretory

granules, indicating either a loss of the granules or a change in their chemical composition. The sublingual gland was not affected histologically by either glyphosate or isoproterenol, demonstrating target specificity of glyphosate- and isoproterenol-associated lesions to those salivary glands which are innervated by adrenergic fibers (Nordenfelt, 1967).

The effect of adrenoreceptor stimulation on parotid acinar cells has been described by ultrastructural and morphometric criteria to be increases in cell size, primarily due to increases in the number and size of secretory granules, as well as changes in the staining of these granules from electron dense to lucent, interpreted to represent a mucoid transformation of the cell (Schneyer, 1962; Henriksson, 1982; Carlsoo *et al.*, 1984). These findings are identical to those found upon electron microscopic examination of parotid cells from animals treated with both glyphosate and isoproterenol in this study, the effects varying only in degree between the chemicals. Ultrastructural effects in the submandibular gland were similar between these compounds, though of a less well-defined nature. These effects consisted of cell enlargement due to accumulation of lucent or heterogenous staining mucoid type granules, although it was not clear whether the serous or mucous cells of the acinus were being affected. This study led to the conclusion that the salivary gland effect is mediated through an adrenergic mechanism, as evidenced by (1) inhibition of the glyphosate-induced effect by the adrenergic antagonist, propranolol; (2) the similarity between the effects of glyphosate and the adrenergic agonist, isoproterenol; and (3) the specificity of those effects for salivary glands with adrenergic innervation. The biologic significance of this finding is unknown. In addition to basophilic and hypertrophic morphologic changes of acinar cells, treatment with isoproterenol has been associated with increased cell proliferation in the parotid gland (Schneyer *et al.*, 1967). This suggests that if glyphosate is acting through an adrenergic pathway, it may likewise induce hyperplasia in this gland, possibly predisposing it to neoplastic change; however, this is not considered likely, since spontaneous basophilic, hypertrophic foci of the parotid, as well as of the pancreas (an anatomically similar tissue) are not considered to be preneoplastic lesions. Moreover, there was no increased incidence in rats of salivary gland tumors in a 2-year study of methapyriline (personal communication, Dr. I. Hirono, Fujita Gakuen Health University, Japan, May 17, 1991), a chemical which induced similar salivary gland lesions as glyphosate in subchronic studies.

The results of the *Salmonella typhimurium* assays and micronuclei tests showed no evidence that glyphosate is genotoxic. A similar conclusion was drawn by Li and Long (1988) after evaluating glyphosate in a battery of genotoxicity assays including *Salmonella typhimurium* reversion, *E. coli* WP-2 reversion, CHO/HGPRT gene mutation, hepatocyte/DNA repair, and *in vivo* rat bone marrow cytogenetics. Moriya *et al.* (1983) also reported negative findings in *Salmonella* (TA100, TA98, TA1535, TA1537, and TA1538) and *E. coli* (WP2 hcr) assays.

In summary, these studies demonstrated that glyphosate was incompletely absorbed from the gastrointestinal tract and excreted in the urine after oral administration. The unabsorbed portion of the dose was excreted in feces. There was no evidence of genetic or reproductive toxicity of glyphosate. At doses of 25000 and 50000 ppm in the feed, glyphosate reduced body weight gain, caused cytoplasmic alteration and hypertrophy of salivary gland acinar cells, and elevated serum bile acids, alkaline phosphatase, and alanine aminotransferase activities, although there was no histopathologic evidence of liver injury. The effects on salivary glands appeared to be adrenergically mediated and could be counteracted by the adrenergic antagonist propranolol. The no-observed-adverse effect level (NOAEL) for the salivary gland lesion was 3125 ppm in the feed for mice, but the lesion was observed at all dose levels studied in rats.

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