TOXICOLOGIC PATHOLOGY, vol. 26, no. 3, pp. 395–402, 1998

Glycine Modulates the Toxicity of Benzyl Acetate in F344 Rats*

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ABSTRACT

The influence of supplemental glycine on benzyl acetate (BA; a compound metabolized via the hippurate pathway)-induced toxicity was investigated. Groups of male F344 rats were fed NIH-07 diet containing 0, 20,000, 35,000, or 50,000 ppm BA for up to 28 days. Two additional groups were fed NIH-07 diet with 50,000 ppm BA and 27,000 ppm glycine or 50,000 ppm BA and 32,000 ppm L-alanine; supplemental glycine and L-alanine were equimolar. The L-alanine group served as an amino nitrogen control. A third group was fed NIH-07 diet with 32,000 ppm L-alanine and served as an untreated isonitrogenous control. BA caused increase in mortality, body weight loss, the incidence of abnormal neurobehavioral signs such as ataxia and convulsions, along with astrocyte hypertrophy and neuronal necrosis in the cerebellum, hippocampus, and pyriform cortex of the brain. These effects were reduced significantly by supplementation with glycine but not with L-alanine. These results suggest that the neurodegeneration induced by BA is mediated by a depletion of the glycine pool and the subsequent excitotoxicity.

Keywords. Benzyl acetate; glycine; rats; brain; neurodegeneration

INTRODUCTION

Benzyl acetate, a colorless liquid, occurs naturally in flowers of jasmine, hyacinth, gardenia, and alfalfa (21). It is widely used as a flavoring agent in foods such as baked goods and candy and as a fragrance in soaps, lotions, and perfumes (12). In both humans and rodents, benzyl acetate is first hydrolyzed to benzyl alcohol, subsequently oxidized to benzoic acid, conjugated with glycine, and excreted primarily as hippuric acid in the urine; a small fraction of the dose is excreted as benzoyl glucuronide, benzyl mercapturic acid, and benzoic acid (1, 3, 4). Benzyl acetate at high doses (≥1,000 mg/kg) has been reported to produce significant body weight depression, ataxia, tremor, convulsions, and mortality in Fischer 344 (F344) rats (14, 15). Following 13 wk of exposure by either gavage or diet, the observed toxicity was accompanied by neuronal necrosis in the cerebrum and hippocampus of male and female rats. Because these toxic effects occurred at high doses only and because benzyl acetate is detoxified by glycine conjugation, it was speculated that the effects observed were the results of depletion of body reserves of glycine. Results of a more recent investigation on the effects of gavage versus dosed food administration on the toxicokinetics of benzyl acetate in rats and mice also suggest that the observed toxicity from benzyl acetate may be due to a reduction in the pool of available glycine (21). We designed the present study to investigate the influence of dietary glycine supplementation on the toxic responses and pattern of neuronal necrosis associated with the administration of high doses of benzyl acetate in F344 rats.

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MATERIALS AND METHODS

Chemical. Benzyl acetate (Aldrich Chemicals, Milwaukee, WI), glycine (EM Science), and L-alanine (Sigma Chemical Co., St. Louis, MO) purities were >99.0%. The identity was confirmed by infrared spectrophotometry. The identity of the L form of alanine was ascertained by chiral column high performance liquid chromatography. The purity of benzyl acetate was determined by gas–liquid chromatography and that of glycine and L-alanine was determined by thin layer chromatography.

Animals. Thirty-five-day-old male F344 rats (Taconic Farms, Germantown, NY) were individually housed in polycarbonate cages in an animal facility at a constant temperature (72 ± 3°F), humidity (50% ± 5%), and light–dark cycle (12 hr/12 hr). Ground NIH-07 diet (Zeigler Bros., Gardners, PA) and tap water were available *ad libitum*. All diets were stored at −20°C. One hundred seventy male rats were randomly assigned to 7 exposure groups. Groups I, II, III (n = 30 each group), and IV (n = 50) received NIH-07 diet containing 0, 20,000, 35,000, or 50,000 ppm benzyl acetate, respectively. Group V (n = 10) received NIH-07 diet containing 50,000 ppm benzyl acetate supplemented with 27,000 ppm glycine. To determine whether glycine effects were due to added amino nitrogen, group VI (n = 10) received a diet containing 50,000 ppm benzyl acetate supplemented with 32,000 ppm L-alanine. Group VII (n = 10) received a diet containing only 32,000 ppm L-alanine and served as an isonitrogenous control for groups V and VI. All diets were stored at −20°C and provided fresh daily for 29 days of *ad libitum* feeding. Body weights and observations of clinical signs of toxicity were recorded at initiation of the study and at weekly intervals including time of terminal euthanasia. Cage-side observations of coat condition, color of eyes and ears, secretions from eyes, nose, or mouth, oral and perianal excretions, leth-

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argy, body position, muscle tone, ataxia, tremor, convulsions, and labored respiration were made for individual animals at weekly intervals.

Functional Observational Battery. Although the cage side observations offered a general description of the state of the animal, screening by the Functional Observational Battery (FOB) was conducted as a systematic evaluation of nervous system effects. Ten animals from each exposure group were randomly selected for screening of behavioral and neurological effects at days 5, 12, 20, and 26 by the FOB (13). In addition, moribund animals in each dose group were also screened for behavioral and neurological effects. The FOB was conducted outside of the home cage and consists of tests for assessment of various aspects of neurological functioning such as autonomic nervous system functions (lacrimation, salivation, respiration, pupil response, palpebral closure), convulsive measures (tremors, convulsions), excitability measures (arousal, handling reactivity, ease of removal from a cage), neuromuscular measures (gait score, righting reflex), and sensorimotor measures (approach and touch response).

Histological Procedures. Whole brains (including cerebellum and brain stem) from all animals at euthanasia on day 29 and from all animals that died or were euthanatized in moribund condition prior to study termination were collected, weighed, and immersion fixed in 10% neutral buffered formalin. Following 48 hr of fixation, brains were cut by hand to obtain 3 coronal sections: 1 at the level of the anterior cerebral cortex and basal ganglia, the second at the level of the posterior cortex, hippocampus, and thalamus, and a third to include the medulla and cerebellum (14). Trimmed samples were dehydrated in graded ethanols, embedded in paraffin, and cut to 6 μm thickness. Brain sections were stained with hematoxylin and eosin (H&E) for light microscopic examination of neuronal necrosis.

Immunohistochemistry. For all brain samples, 6-μm sections were cut serial to the sections stained by H&E, and astrocyte reactivity was evaluated by immunohistochemistry using a polyclonal antibody specific to the structural protein of astrocytes, glial fibrillary acidic protein (GFAP) (Dako Corp., Carpinteria, CA). Rehydrated sections were treated with 3% H_2O_2 for 10 min to remove endogenous peroxidase activity, rinsed in phosphate-buffered saline (PBS) for 20 min, and incubated with nonimmune goat serum for 20 min, followed by a 60 min incubation with anti-rabbit GFAP (1:850). Sections were then incubated with a biotinylated secondary anti rabbit IgG antibody for 30 min, washed in PBS, incubated in avidin–biotin–peroxidase complex reagent for 30 min, rinsed with PBS, and stained with a diaminobenzoic acid substrate containing CO_2^+ ions. Areas of neuronal necrosis previously identified were examined by light microscopy for increased level of GFAP immunostaining and astrocyte morphology.

Statistical Methods. Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Brain and body weight data were analyzed using the parametric multiple comparison procedures of Williams (19, 20) and Dunnett (9). Lesion data were analyzed using nonparametric multiple comparison methods of Dunn (8).

TABLE I.—Survival, food intake, body weight, and brain weight of male F344 rats in the benzyl acetate (BA) study.

Group, treatment	Survival (%)[a]	Food intake (g/rat/day)	Body weight (g)	Brain weight (g)	
				Absolute	Relative
I: 0 ppm BA	100	16.0	223.0	1.63	9.9
II: 20,000 ppm BA	100	15.2	199.0[b]	1.60	10.7
III: 35,000 ppm BA	90	8.7[b]	87.4[b]	1.43[b]	17.1[b]
IV: 50,000 ppm BA	0	NA	NA	NA	NA
V: 50,000 ppm BA + 27,000 ppm glycine	90	11.9[b,c]	166.7[b,c]	1.47[b]	9.9
VI: 50,000 ppm BA + 32,000 ppm alanine	0	NA	NA	NA	NA
VII: 32,000 ppm alanine	100	16.3	216.9	1.71	7.9[b,c]

Abbreviation: NA = not available; all rats died before the end of the study.
[a] Percentage of animals that survived to end of study.
[b] Different from group I ($p < 0.05$).
[c] Different from group III ($p < 0.05$).

RESULTS

Survival and Food Consumption

All control rats, all rats receiving L-alanine alone or 20,000 ppm benzyl acetate (BA), and 90% of rats receiving 35,000 ppm BA or 50,000 ppm BA with glycine survived until the end of the study. Rats receiving 50,000 ppm BA survived a maximum of 12 days, and rats receiving 50,000 ppm BA supplemented with L-alanine did not survive beyond day 6 (Table I). BA administration caused a dose-related decrease in food consumption that, at the 50,000 ppm dose, was partially reversed by the coadministration of glycine (Table I).

Body and Brain Weights

Body weight gain of rats exposed to either 35,000 or 50,000 ppm BA was significantly decreased (Fig. 1). Absolute brain weights were significantly lower in these groups. Relative to body weight, brain weights of rats receiving 35,000 ppm BA were significantly greater than those of the controls. Compared with controls, rats exposed to L-alanine alone showed significant increases brain weights and a significant decrease in its relative weight (Table I). Supplementation with glycine resulted in body weight and brain weight similar to those of control animals (Fig. 1 and Table I).

Neurobehavioral Observations

Significant neurobehavioral signs are summarized in Table II. In the 35,000 ppm BA dose group, 37% of rats showed ataxia and 60% showed tremors. Because of generalized systemic toxicity, the distinct signs of ataxia was evident in 8% and tremors in 26% of rats in the 50,000 ppm BA dose group. No neurobehavioral signs were observed in rats given 50,000 ppm BA supplemented with glycine. Tremors were observed in 40% of rats receiving 50,000 ppm BA supplemented with 32,000 ppm L-alanine.

The functional observations conducted prior to death or at termination of the study showed that BA induced

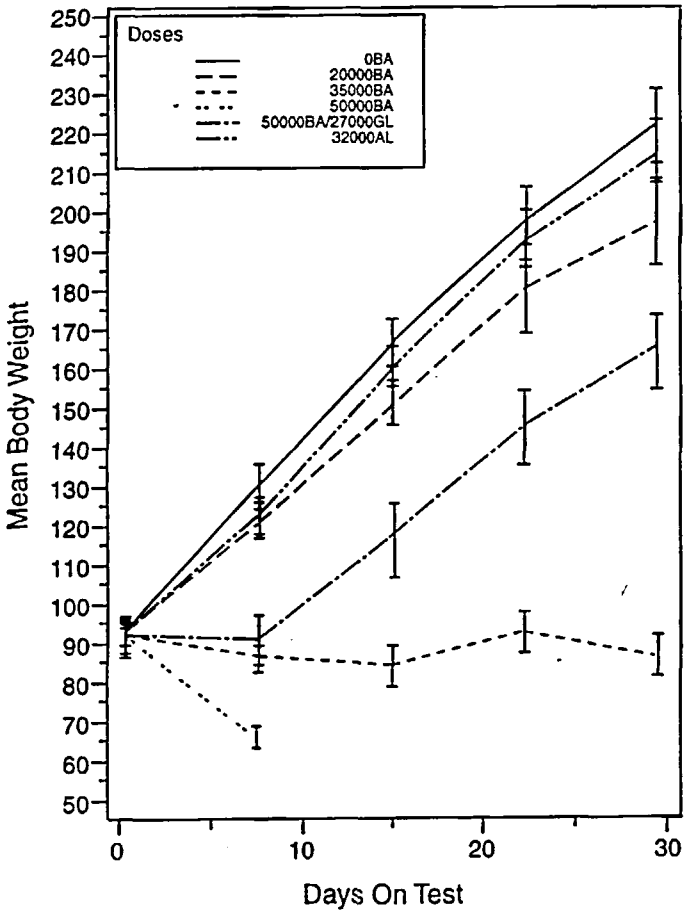

FIG. 1.—Growth curves. Numbers refer to dose in ppm of the specified chemical. BA = benzyl acetate; GL = glycine; AL = alanine. The curve for the BA + AL group is not shown because all rats in that group died before the first scheduled weigh date.

alterations in gait, increased incidence of tremors, and signs of increased respiration at dose levels of 35,000 ppm or 50,000 ppm (Table III). For each end point, supplementation with L-alanine did not moderate the BA effect, but dietary supplementation with glycine ameliorated all behavioral signs of neurotoxicity. Control rats and rats receiving 20,000 ppm BA or L-alanine also did not show any signs of neurotoxicity.

Histopathology

Gross lesions that could be attributed to compound administration or correlated to microscopic effects in target organs were not evident at necropsy. Neuronal necrosis at various brain sites was present in rats receiving 35,000 or 50,000 BA (Table IV). Necrosis occurred at specific sites in the hippocampus and cerebral cortex (Fig. 2) and more diffusely in the cerebellum. Necrosis was present in all early death animals and in those surviving to scheduled euthanasia. In the hippocampus, necrosis primarily affected the large neurons of the dentate gyrus (Fig. 3). In mild lesions, necrotic cells were confined to the dentate gyrus and were intermingled with viable neurons. In more severe lesions, all cells of the dentate gyrus were affected, and there was also viable involvement of CA1–CA4 pyramidal cells of the hippocampus. In the majority of brains displaying hippocampal necrosis, neuronal death of similar severity was typically present in the pir-

TABLE II.—Cage-side observations[a] of male F344 rats in the benzyl acetate (BA) study.

Group, treatment	Ataxia	Tremors
I: 0 ppm BA	0/30	0/30
II: 20,000 ppm BA	0/30	0/30
III: 35,000 ppm BA	11/30 (37)	18/30 (60)
IV: 50,000 ppm BA	4/50 (8)	13/50 (26)
V: 50,000 ppm BA + 27,000 ppm glycine	0/10	0/10
VI: 50,000 ppm BA + 32,000 ppm alanine	0/10	4/10 (40)
VII: 0 ppm BA + 32,000 ppm alanine	0/10	0/10

[a] Number of rats with observation/total number of rats observed (% affected). Signs of neurotoxicity were recorded on days 5, 12, and 26 of the study.

TABLE III.—Summary of functional observations[a] in the benzyl acetate (BA) study in male F344 rats at termination of the study or death.

Group, treatment	Abnormal gait[b]	Labored and/or increased respiration	Convulsions	Tremors
I: 0 ppm BA	0/10	0/10	0/10	0/10
II: 20,000 ppm BA	0/10	0/10	0/10	0/10
III: 35,000 ppm BA	9/9 (100)	4/9 (44)	0/9	5/9 (56)
IV: 50,000 ppm BA	7/11 (64)	5/11 (45)	1/11 (9)	3/11 (27)
V: 50,000 ppm BA + 27,000 ppm glycine	0/8	0/8	0/8	0/8
VI: 50,000 ppm BA + 32,000 ppm alanine	7/10 (70)	4/10 (40)	0/10	2/10 (20)
VII: 0 ppm BA + 32,000 ppm alanine	0/10	0/10	0/10	0/10

[a] Number of rats with effect/total number of rats observed (% affected).
[b] Abnormal gait includes ataxia, hind limb impairment, forelimb or body dragging, and lameness.

TABLE IV.—Incidence and severity of neuronal necrosis in male F344 rats in the 29-day benzyl acetate (BA) study.

Group, treatment	No. rats examined	Neuronal necrosis[a] HC	PC	CB
I: 0 ppm BA (terminal euthanasia)	10	0	0	0
II: 20,000 ppm BA (terminal euthanasia)	10	0	0	0
III: 35,000 ppm BA				
Early deaths	3	3 (4.0)	3 (3.3)	3 (2.7)
Terminal euthanasia	9	9 (4.0)[b]	9 (4.0)[b]	9 (4.0)[b]
IV: 50,000 ppm BA (early death)	50	50 (3.9)	50 (2.8)	50 (1.8)
V: 50,000 ppm BA + glycine				
Early death	1	1 (4.0)	1 (1.0)	0
Terminal euthanasia	9	8 (2.8)[b,c]	7 (1.6)[b,c]	6 (1.0)[b,c]
VI: 50,000 ppm BA + L-alanine (early death)	10	10 (3.8)	9 (1.6)	10 (1.4)
VII: L-alanine (terminal euthanasia)	10	0	0	0

Abbreviations: HC = hippocampus; PC = pyriform cortex; CB = cerebellum.
[a] Number of rats affected (severity of lesion: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked).
[b] Incidence and severity greater than that in group I (control) ($p < 0.05$).
[c] Severity less than that in group III ($p < 0.05$).

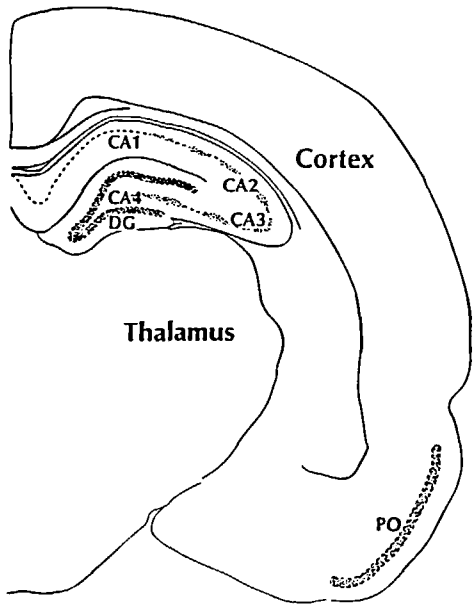

FIG. 2.—Coronal hemisection of brain. Shaded areas indicate predilection sites of benzyl acetate-induced neuronal necrosis. DG = dentate gyrus; CA1–4 = pyramidal cell regions of the hippocampus; PO = primary olfactory cortex.

iform cortex of the ventrolateral cerebral cortex. Necrosis followed the contours of the neuronal layers in this area, resulting in a laminar pattern.

Neuronal necrosis in the cerebellum was confined for the most part to the small neurons of the granular layer (Fig. 4). In minimal to mild cases, pyknotic nuclei were scattered among viable cells. In more severe lesions, there was diffuse necrosis of the granular layer, and occasional necrotic Purkinje cells were present.

In rats dying early or euthanatized at day 8, neuronal necrosis was typically more severe in the hippocampus and piriform cortex than in the cerebellum. One rat that died early and that had received benzyl acetate supplemented with glycine had marked hippocampal necrosis but sparse necrosis at other identified brain sites. At day 29, the severity of neuronal necrosis in rats supplemented with glycine was attenuated (Table IV and Fig. 3 and 4).

In areas of neuronal necrosis, there was concomitant astrocyte hypertrophy indicated by an increase in immunoreactivity for GFAP (Figs. 3 and 4). Cellular processes of hypertrophied cells were thickened and intensely immunostained. In general, regional GFAP staining intensity was proportional to the degree of necrosis and to the duration of the treatment. Hypertrophy and increased reactivity were most apparent in rats treated with 35,000 ppm BA and surviving to day 29. Dietary supplementation with glycine resulted in decrease in the degree of astrocyte hypertrophy and the severity of necrosis.

DISCUSSION

We examined the effect of glycine supplementation on specific toxic end points (weight loss, depressed food consumption, neurobehavioral effects, convulsions, survival, neuronal necrosis, astrocyte reactivity) following dietary administration of high doses of benzyl acetate to F344 rats. Administration of benzyl acetate to rats caused a decrease in body weight and in the absolute and relative weights of brain, kidney, and liver. The results of the Functional Observational Battery showed that neurological symptoms of abnormal gait including ataxia, tremors and/or convulsion and increased respiration occurred in rats at the 2 highest dose levels of benzyl acetate. The increased respiration was probably due to stress resulting from ataxia and convulsions.

The results of the present study clearly indicate that glycine supplementation of the diet protected rats from the toxic effects of benzyl acetate. The F344 rats fed glycine-supplemented diets had an increased rate of survival, food intake, and body weight and showed no abnormal neurobehavioral signs (abnormal gait including ataxia or convulsions) compared with rats that received benzyl acetate. Furthermore glycine supplementation minimized benzyl acetate induced neuronal necrosis in the hippocampus, cerebellum, and primary olfactory cortex.

L-alanine supplementation of the benzyl acetate diet resulted in increased mortality as compared with dosing with benzyl acetate alone. Because supplementation with glycine but not L-alanine reduced benzyl acetate toxicity, toxicity appears to be the result of a shortage in the available glycine and not merely a shortage of amino nitrogen. The results of earlier studies with sodium benzoate (sodium salt of benzoic acid, the main hydrolysis product of benzyl acetate) support this conclusion. Glycine supplementation protected rats against growth inhibition, ataxia, convulsion, and death caused by the administration of sodium benzoate (11, 18). The relationship between glycine supplementation and brain lesions induced by benzyl acetate or structurally related chemicals has not been previously addressed. Each of these compounds are excreted primarily as benzoylglycine (hippuric acid), further supporting the need for glycine in the metabolism and detoxification of such chemicals (1, 2, 10, 22). Thus, high doses of either compound, or of other structurally related chemicals, will result in excessive demand for glycine for

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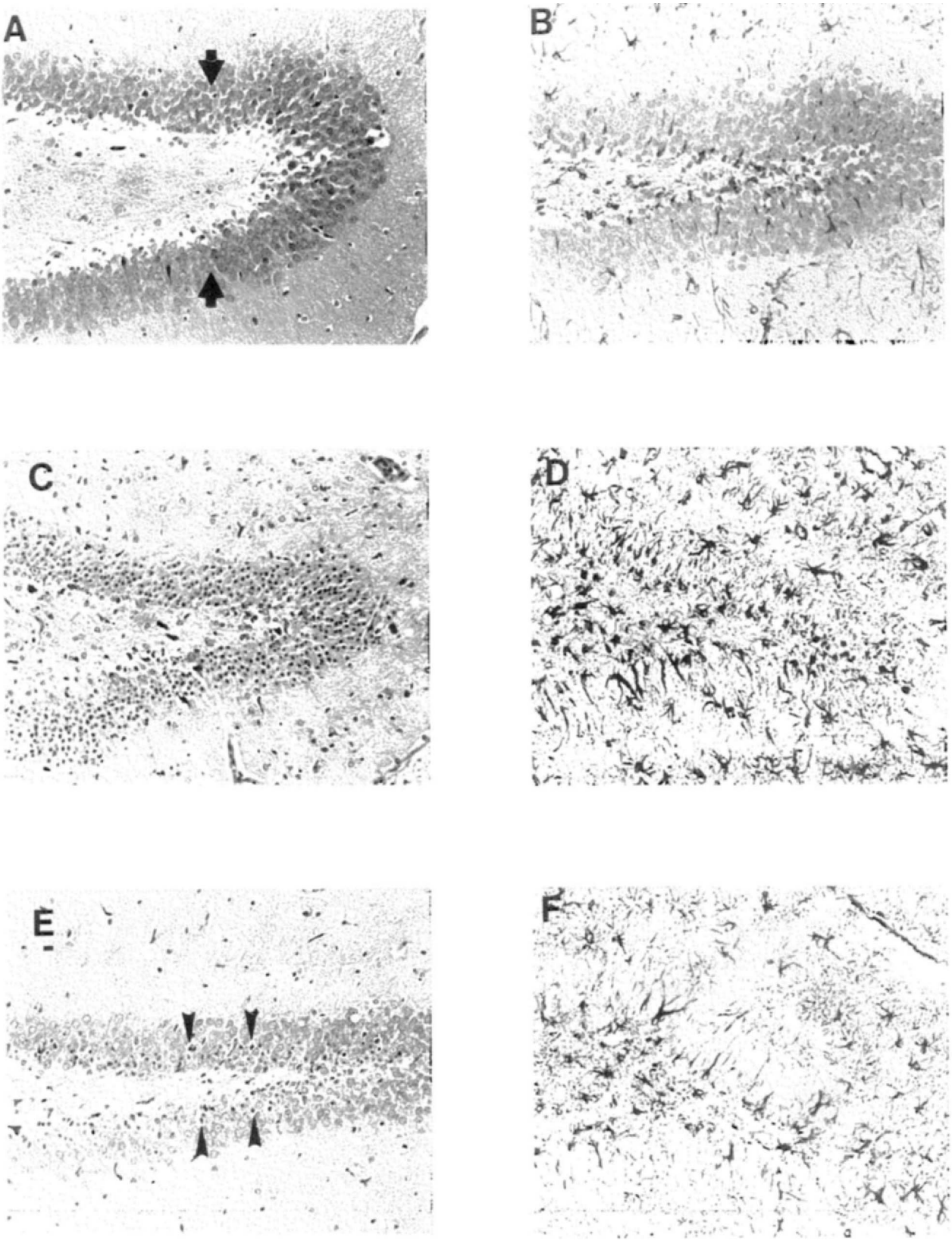
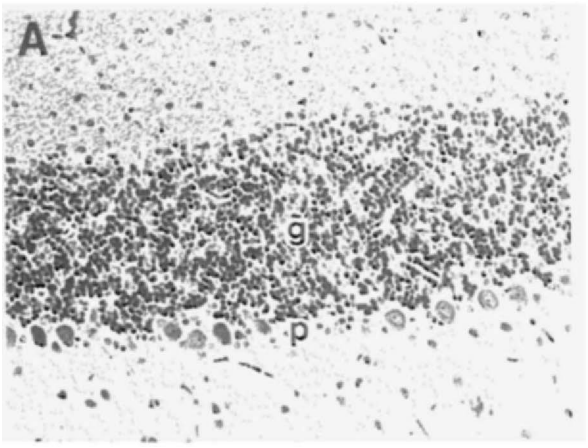
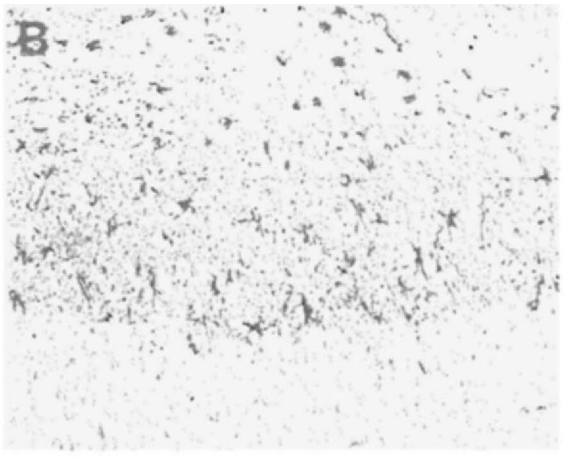
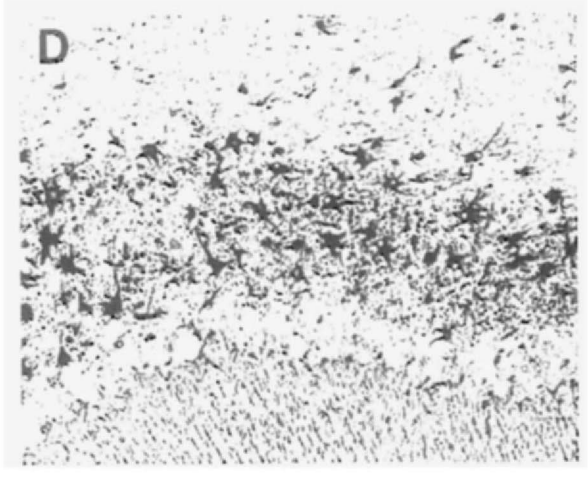
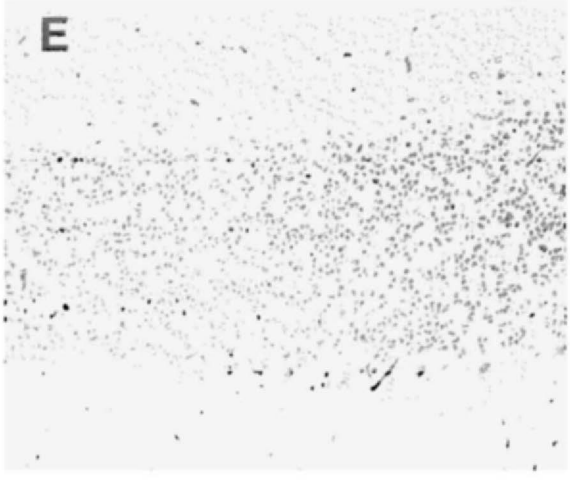
FIG. 3.—Dentate gyrus (DG) of rat. BA = benzyl acetate. A) Normal appearance of neuron cell bodies (arrows) in a control rat. H&E. ×150. B) Marked neuronal necrosis in rat dosed with 35,000 ppm BA for 29 days. Note the dark-staining pyknotic nuclei in necrotic neurons. H&E. ×150. C) Rat treated with 50,000 ppm BA and supplemented with glycine. Mild necrosis is evidenced by some dark-staining necrotic cells (arrows) scattered among viable neurons. H&E. ×150. D) Control rat. GFAP and astrocytes display slender cell processes. ×150. E) Rat treated with 35,000 ppm BA for 29 days. There is markedly increased GFAP immunoreactivity associated with astrocyte hypertrophy. ×150. F) Rat treated with 50,000 ppm BA and supplemented with glycine. GFAP staining is increased above control but the level of astrogliosis appears to be less than that of the nonsupplemented rat. ×150.

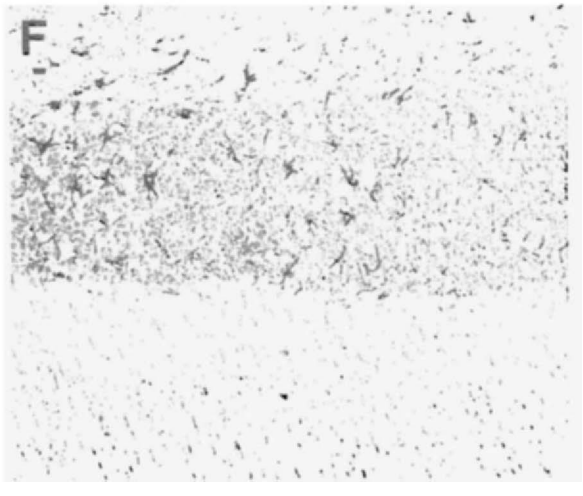












excretory purposes and thus may deplete the available glycine pool despite the ability of the animal to endogenously synthesize glycine. The glycine depleting actions of sodium benzoate have been demonstrated by the reduction in plasma glycine concentrations in exposed humans (5, 7), and liver glycine concentration in guinea pigs (6) and rats (2). Additionally, Gregus et al (10) reported that sodium benzoate administration to rats resulted in decreased hepatic concentrations of the cofactors carnitine and coenzyme A, essential for the metabolism and derivation of energy from fatty acids. Carnitine is essential for the transport of activated fatty acids into the mitochondrial matrix, and co-enzyme A is essential for the activation of fatty acids prior to oxidization by the mitochondria (17).

The pattern of neuronal degeneration in the hippocampus and cortex seen in the rats exposed to benzyl acetate is similar to that seen in models of hippocampal excitotoxicity. In these cases, stimulation of specific excitatory receptors such as the glutamate receptor, the kainic acid receptor, and the *N*-methyl-D-aspartate (NMDA) receptor can result in severe and prolonged hyperpolarization of the neuronal membrane, resulting in excitotoxicity and neuronal degeneration. Subsequent to hyperpolarization, the neuronal membrane allows the influx of Cl^- ions, raising the firing threshold and producing an inhibitory action. In this inhibitory process, glycine serves as a neurotransmitter to inhibit neuronal firing by gating Cl^- channels (13). Blocking of this process results in increased excitability of some populations of neurons and the induction of seizures. Depletion of glycine by benzyl acetate may depress the normal inhibitory pathway of glycine, resulting in an inability of the system to downregulate excitatiory hyperpolarization and producing a pattern of excitotoxicity. A similar process of nervous system injury may occur in the spinal cord, where glycine is the primary neurotransmitter of the interneurons. Perturbations at these neurons could account for the effects seen on neuromuscular function and tremor. They could also contribute to the appearance of neuronal necrosis in the cerebellum. In the absence of available morphological data, the involvement of the spinal cord interneuron in benzyl acetate neurotoxicity is only speculative but warrants further examination.

Although the activity of glycine at the glycine receptor is usually considered an inhibitory process, glycine also plays a role in the modulation of NMDA receptor activation. The binding of glycine is required for NMDA receptor activation, thus glutamate and glycine serve as coagonists for the NMDA receptor (16). This glycine site on the NMDA receptor is pharmacologically distinct from the inhibitory glycine receptor and can be activated by several small analogs of glycine, such as serine and alanine. The increased sensitivity demonstrated in animals exposed to benzyl acetate and supplemented with L-alanine may be due to modulation at the NMDA receptor site, resulting in an activation of the receptor and an exerbation of the excitotoxicity.

Although glycine is abundantly present in most tissues and participates in many metabolic reactions, a relationship between glycine depletion and neurodegeneration has yet to be addressed. The ability of a specific chemical such as benzyl acetate to severely deplete the pool of available glycine makes it possible to examine the potential role for glycine in neurodegeneration. Future studies would be needed to address alterations in receptor mediated events associated with the process of excitotoxicity, focusing on the loss of an inhibitory mechanism as a modulating factor in hippocampal neurodegeneration and spinal cord histopathology.

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FIG. 4.—Cerebellum of rat. BA = benzyl acetate. A) Normal appearance of the granular cell layer (g) and Purkinje cell layer (p) in a control rat. H&E. ×150. B) Marked neuronal necrosis in rat treated with 35,000 ppm BA for 29 days. Note the pyknotic nuclei and decreased thickness and cellularity of the granular layer (g). Purkinje cells (p) are spared. H&E. ×150. C) Rat treated with 50,000 ppm BA and supplemented with glycine. The granular cell layer (g) is similar to that of the control rat. H&E. ×150. D) Control rat. Astrocytes with slender processes are scattered in the granular layer. GFAP. ×150. E) Rat treated with 35,000 ppm BA for 29 days. Note the marked astrocyte hypertophy. GFAP. ×150. F) Rat treated with 50,000 ppm BA and supplemented with glycine. GFAP staining is increased above control but is slightly decreased relative to the non-supplemented rat. GFAP. ×150.

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