0022-3565/87/2421-0354$02.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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Vol. 242, No. 1
Printed in U.S.A.

Influence of the Vitamin D Hormonal Status on the Hepatic Response to Bromobenzene[1]

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Accepted for publication April 6, 1987

ABSTRACT

Hypocalcemic vitamin D-depleted rats received either 1,25-dihydroxy-vitamin D_3 [1,25$(OH)_2D_3$] or calcium p.o. in order to study the effects of plasma calcium normalization, resulting from hormone or dietary calcium administration, on the hepatic response to bromobenzene (BB). Results showed that 1,25$(OH)_2D_3$ administration induced a rise in the circulating aspartate aminotransferase, alanine aminotransferase and sorbitol dehydrogenase after BB administration significantly greater than in unsupplemented rats. The volumic density of necrosis was not, however, increased by 1,25$(OH)_2D_3$ whereas the proportion of acidophilic cells surrounding the necrotic area and the ratio of acidophilic to necrotic cells were significantly increased suggesting the presence of regenerating parenchyma. Oral calcium yielded an increase comparable to that of 1,25$(OH)_2D_3$ in apparent BB toxicity which was accompanied by a significant rise in both the volumic density of necrosis and of acidophilic cells but the ratio of acidophilic to necrotic cells was not increased by dietary calcium. The amount of cytochrome P-450 lost after BB administration, the covalent binding of BB metabolites to cellular proteins *in vitro* and the total liver glutathione content were not changed by either 1,25$(OH)_2D_3$ or calcium supplementation. Results show that hypocalcemic vitamin D-depleted rats are protected partially against BB toxicity; this protection does not seem to be due to a decrease in the hepatic metabolism of BB but seems to be related to the hypocalcemic state; on the other hand, the active regenerating process which seemed more apparent in 1,25$(OH)_2D_3$-treated than in all other animals may have contributed to offset partly the cellular damage induced by the toxin in the hormone-treated group.

BB is a hepatotoxic agent that is known to induce selective perivenous (zone 3) damage (Brodie *et al.*, 1971). The regional necrogenic effect of BB is believed to be linked to the heterogeneous intra-acinar distribution of the oxidative (activating) and conjugative (detoxifying) enzyme systems involved in its intermediate metabolism. Hence, BB is metabolized into reactive epoxides by the microsomal cytochrome P-450-dependent MFO (Reid and Krishna, 1973) which seem to be predominantly localized near the terminal hepatic or central veins (Gumucio *et al.*, 1978). On the other hand, hepatic content of glutathione, a tripeptide involved in major detoxifying conjugation reactions, has been shown to be concentrated in more periportal areas, acinar zones 1 and 2 (Gumucio *et al.*, 1978). Thus, it is thought that toxic metabolites preferentially accumulate in perivenous regions and induce the selective zone 3 necrosis normally encountered after BB administration.

The liver MFO also metabolize numerous other xenobiotics as well as many endogenous substances such as vitamin D_3 (cholecalciferol). Vitamin D_3 is a secosteroid normally produced in the skin after UV irradiation of its precursor 7-dehydrocholesterol (Holick *et al.*, 1980). The hepatic microsomal and/or mitochondrial cytochrome P-450-dependent MFOs then catalyze the C-25 hydroxylation of endogenous or dietary vitamin D_3 (Bhattacharyya and DeLuca, 1974; Bjorkhem and Holmberg, 1978). This metabolic step is the first in a series which eventually leads to the formation of 1,25$(OH)_2D_3$, a secosteroid hormone which is known to be essential for the calcium and phosphorus homeostasis of the body (Henry and Norman, 1984). Vitamin D depletion therefore leads to many disturbances, one of the most important being hypocalcemia accompanied by secondary hyperparathyroidism. On the other hand, a growing body of evidence is also accumulating in favor of modulatory effects of 1,25$(OH)_2D_3$ on cell growth and differ-

Received for publication August 28, 1986.

[1] This work was supported by grants from the Medical Research Council of Canada.

[2] These studies have been completed in partial fulfillment of a Ph.D. thesis from the Universite de Montreal. Recipient of a Studentship award from the Medical Research Council of Canada.

[3] Recipient of a Scientific Award from the Medical Research Council of Canada.

ABBREVIATIONS: BB, bromobenzene; MFO, mixed function oxidase; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; AST, aspartate aminotransferase; ALT, alanine aminotransferase; SDH, sorbitol dehydrogenase.

entiation, most particularly in bone marrow stem cell populations (Henry and Norman, 1984). Thus, the physiologic role of the hormone is now expanding to include possible immunoregulatory and trophic functions.

It is already known that the activity of many liver enzymes can be modified by several dietary or nutritional factors (Campbell and Hayes, 1974) which, in turn, can influence the chemically induced hepatocellular damage of several toxins. Furthermore, changes in the concentration of calcium have also been shown to affect the hepatotoxic potential of various chemicals (Farber, 1982) including BB (Jewell *et al.*, 1982; Lamb and Schwertz, 1982) whereas recent studies conducted in our laboratory have pointed out that hypocalcemic vitamin D-depleted rats seemed to be less susceptible to BB intoxication than normocalcemic vitamin D-replete animals (Haddad *et al.*, 1985). The purpose of the present studies was, therefore, to compare the effects due to normalization of the plasma calcium concentrations brought about by 1,25(OH)$_2$D$_3$ or calcium supplementation p.o. in order to investigate the role of the hormone or of dietary calcium in the hepatic response to BB intoxication in vitamin D-depleted rats.

Materials and Methods

Animals and pretreatment procedures. Weanling male Sprague-Dawley rats were ordered (Charles River Canada Ltd, St. Constant, Québec, Canada) and housed in individual hanging stainless steel wire cages. Vitamin D depletion was induced by feeding *ad libitum* a vitamin D-deficient diet (Guroff *et al.*, 1963), containing 0.4 and 0.2% of elemental calcium and phosphorus, respectively, as well as demineralized water. The animals were depleted for 8 to 10 weeks before being assigned randomly to the supplementation regimens and treatment groups. Hormonal supplementation was achieved through the i.p. implantation of osmotic minipumps ALZET 2001 (ALZA Corp., Palo Alto, CA) which delivered constant infusions of 1,25(OH)$_2$D$_3$ at doses of 7 or 65 pmol·day^{-1} for a period of 8 days. Control animals were implanted with minipumps containing only the vehicle (ethanol-propylene glycol-saline, 15:65:20). In a second set of experiments, calcium supplementation p.o. was achieved by supplying a 3 to 5% solution of calcium gluconate as drinking water for a period of 14 days.

BB intoxication. On day 6 of 1,25(OH)$_2$D$_3$ infusion or day 12 of calcium supplementation p.o., the animals were divided randomly into two groups which received a single i.p. injection of either BB (1:4, v/v in corn oil), 500 mg·kg^{-1}, or, for those serving as controls, corn oil, 1.34 ml·kg^{-1}; all animals were sacrificed 48 hr later (which corresponded with peak BB toxicity in all groups) by exsanguination through the abdominal aorta under light ether anesthesia. The livers were flushed immediately with ice-cold isotonic saline and excised. Samples were taken for histological evaluation of the lesion; the livers were then homogenized at 4°C in 4 volumes of 0.25 M sucrose using a Potter-Elvehjem type glass-Teflon homogenizer. The homogenate was centrifuged immediately in a J-21 centrifuge (Beckman Instruments Inc., Palo Alto, CA) and the cell debris and nuclei sedimented at 600 × *g* for 10 min and the mitochondria at 15,700 × *g* for 15 min. The supernatant was centrifuged in a L5-75 ultracentrifuge (Beckman Instruments Inc.) and the microsomal fraction was obtained as the 105,000 × *g* pellet. The microsomal pellet was washed once by resuspension in ice-cold 100 mM buffered sodium pyrophosphate followed by recentrifugation. The microsomal pellet was then covered with 2 cm of 0.25 M sucrose and frozen immediately at −78°C.

Circulating parameters of vitamin D depletion and of supplementation regimens. Plasma vitamin D metabolites were measured on 2-ml samples after extraction and chromatography as described earlier (Gascon-Barré and Huet, 1982). 25-Hydroxyvitamin D was analyzed by a competitive protein binding assay using sheep serum in a dilution of 1:40,000 as binding protein; the lower limit of sensitivity of the method was 10 pg·ml^{-1}. 1,25(OH)$_2$D$_3$ was measured by a competitive protein binding assay using a cytosolic receptor from bovine thymus (Reinhardt *et al.*, 1982); the lower limit of sensitivity of the method was 1 pg·ml^{-1}. Plasma calcium was measured using the colorimetric kit No. 204382 from Boehringer Mannheim (Dorval, Québec, Canada).

Circulating parameters of liver toxicity. Plasma L-AST (EC 2.6.1.1) and L-ALT (EC 2.6.1.2) activities were evaluated by the methods of Bergmeyer (1974) and Siegel and Montgomery (1959) using the UV system A from Boehringer Mannheim Corp. Plasma SDH [E.C. 1.1.1.14 (L-iditol dehydrogenase)] activity was evaluated according to the method of Gerlach and Hiby (1974).

Determination of glucose-6-phosphatase activity, BB epoxidation and glutathione content. Glucose-6-phosphatase activity was determined on the 20% liver homogenate diluted 10 times with 0.1 M tris-maleate buffer, pH 6.2, according to Traiger and Plaa (1971). The quantity of inorganic phosphate formed was measured using the Sigma Kit No. 670 (Sigma Chemical Co., St. Louis, MO).

Covalent binding arising from BB epoxidation by the liver MFO was measured by a modified version of the method of Lau and Zannoni (1981). Microsomal pellets were resuspended in 0.05 M potassium-phosphate buffer containing 1 mM EDTA (pH 7.4) to achieve a final protein concentration of 1.2 mg·ml^{-1}. One milliter of microsomal suspension was preincubated for 5 min at 37°C with 10 µl of acetonitrile containing 2.4 µmol of [^{14}C]BB ([U-^{14}C]BB 25 mCi·mmol^{-1}, ICN Radiochemicals, Montreal, Québec, Canada). The reaction was started by the addition of 200 µl of a buffered NADPH-generating system (glucose-6-phosphate, 12 µmol; NADP, 2.4 µmol; glucose-6-phosphate dehydrogenase, 6 U; and $MgCl_2$, 12 µmol). The tubes were shaken gently, oxygenated for 20 sec and incubated for 5 min at 37°C in a Dubnoff incubator at 100 oscillations/min. Blank incubations were carried out under identical conditions in the absence of the NADPH-generating system. The reaction was stopped by deproteinization with 2 ml of a 10% trichloroacetic acid solution. The samples were then centrifuged at 2500 rpm for 10 min in a Sorvall RC-3 automatic refrigerated centrifuge (Maynard Scientific, Montreal, Québec, Canada) and the supernatant discarded. The pellets were resuspended in 5 ml of methanol-ether (3:1, v/v), heated at 60°C for 10 min, centrifuged again and the supernatant discarded. This washing procedure was repeated at least eight times or until less than 400 dpm were observed in a 0.5-ml aliquot of the supernatant added to 5 ml of Biofluor (New England Nuclear, Boston, MA) and counted in a Beckman model LS3801 liquid scintillation spectrometer (Beckman Instruments Ltd.). The pellet was then dissolved in 1 ml of 1 N NaOH and allowed to stand overnight at room temperature. Sufficient 1 N HCl was then added to the samples to obtain a final concentration of 0.1 N NaOH. A 0.5-ml aliquot was added to 10 ml of Biofluor and the amount of covalently bound radioactivity counted. Protein content was measured on another 0.5-ml aliquot by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Hepatic content in total glutathione was determined in liver homogenates according to the method of Mitchell *et al.* (1973).

Determination of cytochrome P-450 content and of microsomal enzyme activities. Cytochrome P-450 content was determined on the 105,000 × *g* pellet resuspended in 0.1 M potassium-phosphate buffer, pH 7.4, using an isosbestic point of 490 nm (ξ = 91 mM·cm^{-1}) (29); all cytochrome P-450 determinations were made on an Aminco DW-2 dual beam spectrophotometer (American Instrument Co., Silver Spring, NY).

Aniline hydroxylase and aminopyrine-N-demethylase activities were determined on the 105,000 × *g* pellet resuspended at a concentration of 1 nmol of cytochrome P-450 per assay tube; incubations were carried out at 37°C for 20 and 7 min, respectively, in a Dubnoff incubator at 100 oscillations/min. Aniline hydroxylation was determined by measuring the quantity of *p*-aminophenol formed (Chhabra *et al.*, 1972) and aminopyrine-N-demethylation was determined as described by Holtzman *et al.* (1968). All reactions were linear over the period of time studied. Protein determinations were made (Lowry *et al.*, 1951) using bovine serum albumin as standard.

Morphologic evaluation of the lesion. At the time the animals were sacrificed, liver samples were obtained, sliced, fixed in 10% buffered formalin and embedded in paraffin blocks; sections 3 to 5μm in thickness were stained with hematoxylin-fuschine-safran for light microscopic evaluation.

A morphometric evaluation of the hepatic samples was carried out in all groups after either corn oil or BB administration. The morphometric analysis was based on a point-counting approach chosen for its ease, accuracy and reproducibility in the measure of the volumic densities of determined liver parenchymal regions which were described as intact, necrotic or acidophilic areas. The test grid characteristics as well as the sampling requirements were predetermined using methods described by Weibel (1979). Single histological sections of each animal were photographed using a Zeiss Photomicroscope II at a magnification of 25×; a total of 30 randomly chosen fields were considered and provided a sampled parenchymal surface of 39 mm^2 for each animal. Each field was projected on a point-counting grid (21 × 31 cm) containing 150 points (d = 2 cm) to obtain a final magnification of 225×. The interassay variation of the method was less than 10%. All evaluations were made in a double-blind fashion.

Statistical analysis. All data are presented as the means ± S.E.M. All experiments were analyzed by a factorial analysis of variance: 2 × 2 for calcium supplementation p.o. and 2 × 3 for hormonal infusion. This statistical analysis made it possible to determine the influence of 1) the hepatotoxin, 2) the supplementation regimens and 3) the interaction between BB and the supplementation regimens on the various parameters studied. Orthogonal contrasts between subgroups were evaluated when the overall analysis of variance reached statistical significance. Dose-related linearity of data was calculated using the Fisher's *F* test.

Results

Effects of 1,25(OH)$_2$D$_3$ Supplementation

General systemic effects. The circulating concentrations of 1,25(OH)$_2$D$_3$ obtained after 8 days of constant i.p. infusion with vehicle or doses of 7 or 65 pmol of 1,25(OH)$_2$D$_3\cdot$day^{-1} in corn oil controls were 25.0 ± 6.4, 206.6 ± 45.0 and 407.9 ± 69.8 pg$\cdot$ml^{-1}, respectively ($P < .001$ for linear progression), whereas, in animals intoxicated with BB 48 hr before sacrifice, they were 36.0 ± 9.0, 211.9 ± 46.7 and 346.1 ± 42.9 pg$\cdot$ml^{-1}, respectively ($P < .001$ for linear progression; overall values were not significantly different between corn oil controls and BB-intoxicated rats). Plasma 25-hydroxyvitamin D concentrations averaged 0.3 ± 0.07 ng$\cdot$ml^{-1} and no differences between treatment groups were present. The plasma calcium concentrations observed at the end of the study are presented in figure 1A. From initially equivalent hypocalcemic states in all groups, 1,25(OH)$_2$D$_3$ infusion at doses of 7 or 65 pmol$\cdot$day^{-1} for 8 days was associated, in corn oil controls, with normocalcemic and significantly hypercalcemic states, respectively, whereas vehicle-infused animals remained hypocalcemic. BB intoxication did not affect the dose-related calcemic response to hormone infusion but the plasma calcium concentrations in BB-intoxicated rats remained at values some 1 mg$\cdot$dl^{-1} lower than their respective corn oil controls. Body weights (fig. 1B), which were initially similar in all groups, increased slightly more in 1,25(OH)$_2$D$_3$-supplemented than in vehicle-infused animals; however, when compared to corn oil controls BB intoxication led to significantly lower body weights in vehicle-infused animals whereas hormone infusion seemed to prevent the BB-induced weight loss. 1,25(OH)$_2$D$_3$ infusion also was associated with a gradual increase in liver weight (fig. 1C) which became statistically

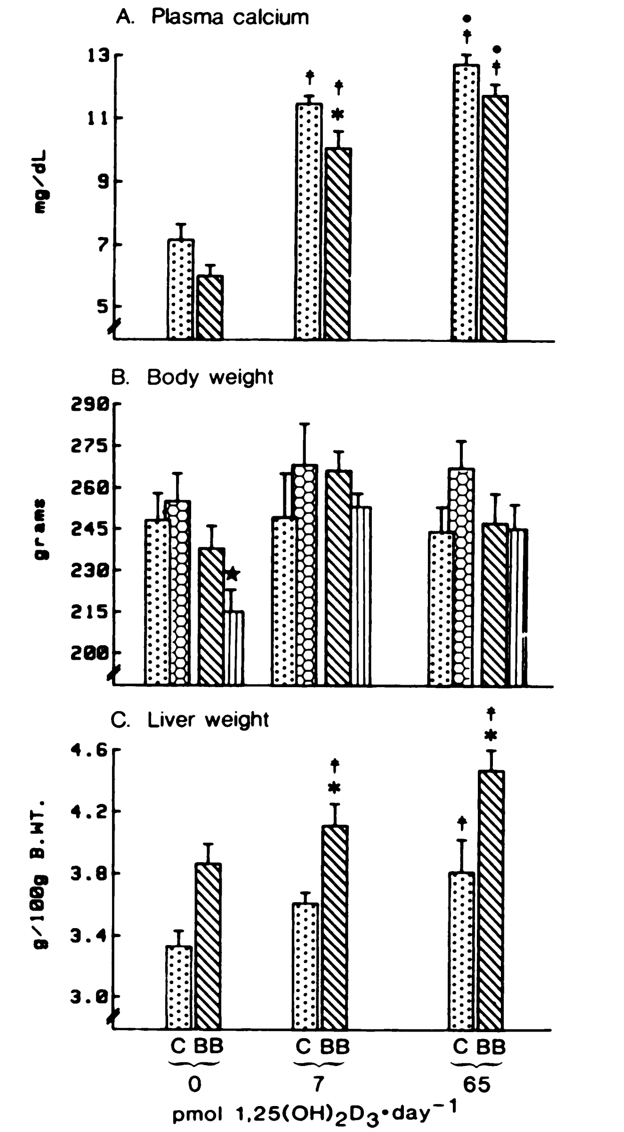

Fig. 1. Effect of constant i.p. infusion with vehicle (0) or doses of 7 or 65 pmol of 1,25(OH)$_2$D$_3\cdot$day^{-1} on general systemic parameters in corn oil (□)- and BB-treated (▨) vitamin D-depleted rats. Treatments were given i.p., 48 hr before sacrifice (corresponding to day 6 of the supplementation regimen): control (C), received corn oil, 1.34 ml$\cdot$kg^{-1}; BB was administered at a dose of 500 mg$\cdot$kg^{-1}. Results are expressed as means ± S.E.M.; N = 48 or 6 to 10 rats per subgroup. In B, initial body weights (at minipump implantation) (□) were compared with those on day 6 of the supplementation regimen (■) for corn-oil controls whereas in BB-treated groups initial body weights (▨) were compared with those at sacrifice (▥). Corn oil administration did not modify body weights significantly. Statistically significant differences between group means were analyzed by a 2 × 3 factorial analysis and were: different from C, *$P < .05$; different from vehicle-infused group, ‡$P < .001$; different from 7 pmol of 1,25(OH)$_2$D$_3\cdot$day^{-1} group, ●$P < .05$; and different from initial body weight, ★$P < .05$.

significant at the dose of 65 pmol$\cdot$day^{-1} as illustrated by the corn oil control group. BB intoxication, on the other hand, led to significantly greater liver weights on 1,25(OH)$_2$D$_3$-infused rats than in their vehicle-infused as well as corn oil-treated controls.

Effects on liver toxicity. The circulating concentrations of three liver enzymes obtained on the day the animals were sacrificed are presented in figure 2. 1,25(OH)$_2$D$_3$ infusion alone (corn oil control groups, ●) did not have any significant effect

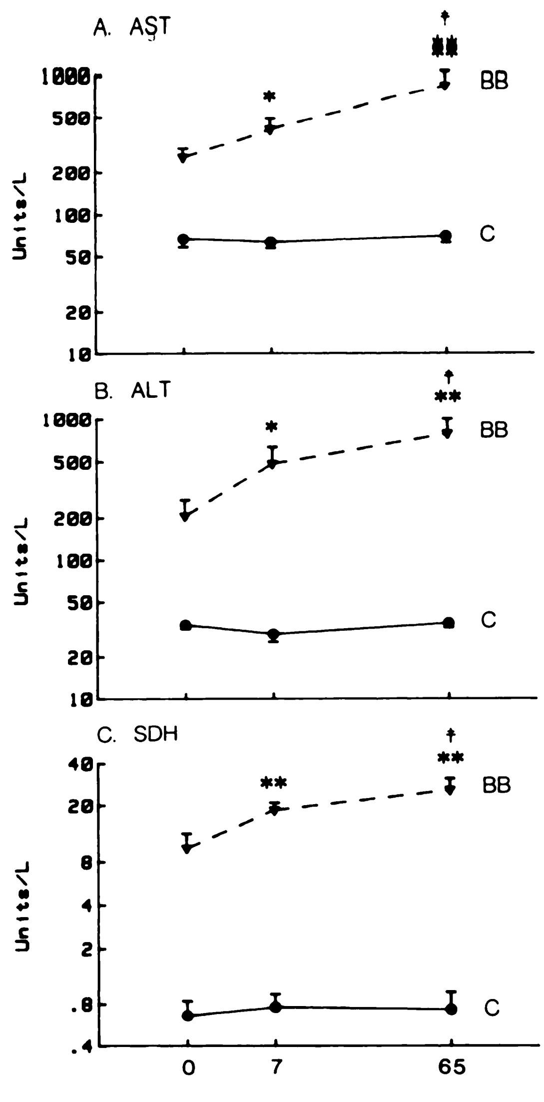

Fig. 2. Effect of constant i.p. infusion with vehicle (0) or doses of 7 or 65 pmol of $1,25(OH)_2D_3 \cdot day^{-1}$ on the plasma activity of hepatic enzymes in corn oil (●) and BB-treated (▼) vitamin D-depleted rats. Treatments were given i.p., 48 hr before sacrifice (corresponding to day 6 of the supplementation regimen): control (C); corn oil, 1.34 $ml \cdot kg^{-1}$; BB, 500 $mg \cdot kg^{-1}$. Results are expressed as means ± S.E.M.; N = 48 or 6 to 10 rats per subgroup. Statistically significant differences between group means were analyzed by a 2 × 3 factorial analysis of variance and were: different from C, *$P < .05$, **$P < .001$; and different from vehicle-infused group, ‡$P < .01$.

on the plasma concentrations of AST, ALT and SDH. However, BB intoxication led to increases in the plasma concentrations of these hepatic enzymes in all groups but the magnitude of the effect increased as the dose of $1,25(OH)_2D_3$ infused increased, as shown by the significantly greater circulating levels of AST, ALT and SDH obtained in rats supplemented with 65 pmol of $1,25(OH)_2D_3 \cdot day^{-1}$ than in those receiving the vehicle only. The hepatic activities of glucose-6-phosphatase, BB epoxidation and total liver glutathione content are presented in table 1. Neither $1,25(OH)_2D_3$ infusion nor BB intoxication exerted any significant effect on the hepatic glucose-6-phosphatase activity. As assessed in the corn oil control groups, $1,25(OH)_2D_3$ infusion also was without effect on the rate of BB epoxidation (which was determined by the amount of reactive metabolites covalently bound to microsomal proteins) as well as on the total glutathione content on the liver.

Effects on the metabolic activities of the MFO. Hepatic microsomal enzyme activities are presented in figure 3. Hormonal infusion alone did not significantly affect the microsomal cytochrome P-450 content whereas BB intoxication led to a significant, $1,25(OH)_2D_3$-independent decrease in the microsomal cytochrome P-450 content (fig. 3A). The aniline hydroxylase molar activity (fig. 3B) was not affected significantly by $1,25(OH)_2D_3$ infusion alone as seen in the corn oil control groups. On the other hand, BB intoxication led to significantly higher rates of aniline hydroxylation in vehicle-infused rats but this increase was abolished gradually by $1,25(OH)_2D_3$ infusion, with activity levels returning to corn oil control values in the BB-intoxicated 65 pmol of $1,25(OH)_2D_3 \cdot day^{-1}$ group. The aminopyrine-N-demethylase activity (fig. 3C) remained unaffected by either hormonal supplementation or BB administration.

Effects on liver morphology. The morphological appearance of the hepatic lesions observed after BB intoxication in vehicle-infused or hormone-supplemented rats is presented in figure 4. Groups of corn oil control rats did not show any sign of morphological abnormality, whether vehicle- or $1,25(OH)_2D_3$-infused, as shown by the representative photomicrograph in figure 4A. In BB-intoxicated animals, the affected zone contained cells damaged to various degrees, from cytosolic ballooning to nuclear pyknosis and cell membrane disruption. A slight to moderate inflammatory response, generally proportional to the extent of necrosis and mainly reflected by monocyte infiltration, could be observed in the affected zones. The necrotic process was always limited to the perivenous portion of the liver acinus and central-central bridging was observed occasionally. The necrotic areas were typically surrounded by a crown of acidophilic cells which featured a very dense, strongly fuschine-positive and homogeneous cytoplasm, a slightly hypertrophied round- or oval-shaped nucleus as well as one to three prominent nucleoli. These cells did not contain any lipidic or hydropic inclusions. The remainder of the liver parenchyma (that is, acinar zones 1 and 2) was morphologically intact except for a progressive lipid accumulation seemingly related to the dose of $1,25(OH)_2D_3$ infused as assessed by a semiquantitative analysis. This moderate periportal steatosis was most evident in the 65 pmol of $1,25(OH)_2D_3 \cdot day^{-1}$ group (fig. 4D).

The morphometric analysis of BB hepatotoxicity in vehicle- or $1,25(OH)_2D_3$-infused animals is presented in table 2. The volumic density of hepatocellular necrosis obtained after BB intoxication was not increased significantly by $1,25(OH)_2D_3$ administration. On the other hand, hormonal supplementation was associated with a significant dose-related increase in the proportion of hepatic parenchyma occupied by acidophilic cells. Moreover, the ratio of acidophilic to necrotic volume densities increased linearly with the dose of $1,25(OH)_2D_3$ infused to reach values significantly greater in the 65 pmol of $1,25(OH)_2D_3 \cdot day^{-1}$ group than in the vehicle-infused animals.

Effects of Calcium Supplementation

General systemic effects. From initially equivalent hypocalcemic states in all groups, calcium gluconate supplementation p.o. for 2 weeks was successful in normalizing plasma calcium concentrations whereas animals receiving demineralized water remained hypocalcemic (fig. 5A). Intoxication with BB 48 hr before sacrifice did not significantly affect overall

TABLE 1
Hepatic metabolic parameters in hypocalcemic Vitamin D-depleted or 1,25(OH)$_2$D$_3$-supplemented rats
Results are presented as means ± S.E.M.

Parameters	Treatment	N	1,25(OH)$_2$D$_3$ Infusion (pmol·day^{-1}) 0	7	65	Analysis of Variance
Glucose-6-phosphatase, mg protein·g liver^{-1}·20 min^{-1}	Control	6	7.64 ± 0.90	7.80 ± 0.99	7.06 ± 0.72	N.S.
	BB	6	6.73 ± 1.10	7.70 ± 0.86	6.46 ± 0.75	N.S.
BB epoxidation, nmol [^{14}C]BB bound·mg protein^{-1}·5 min^{-1}	Control	4	9.48 ± 0.54	9.38 ± 0.65	8.85 ± 0.70	N.S.
Total glutathione, μmol·g liver^{-1}	Control	4	7.81 ± 0.64	6.52 ± 1.20	7.85 ± 1.38	N.S.

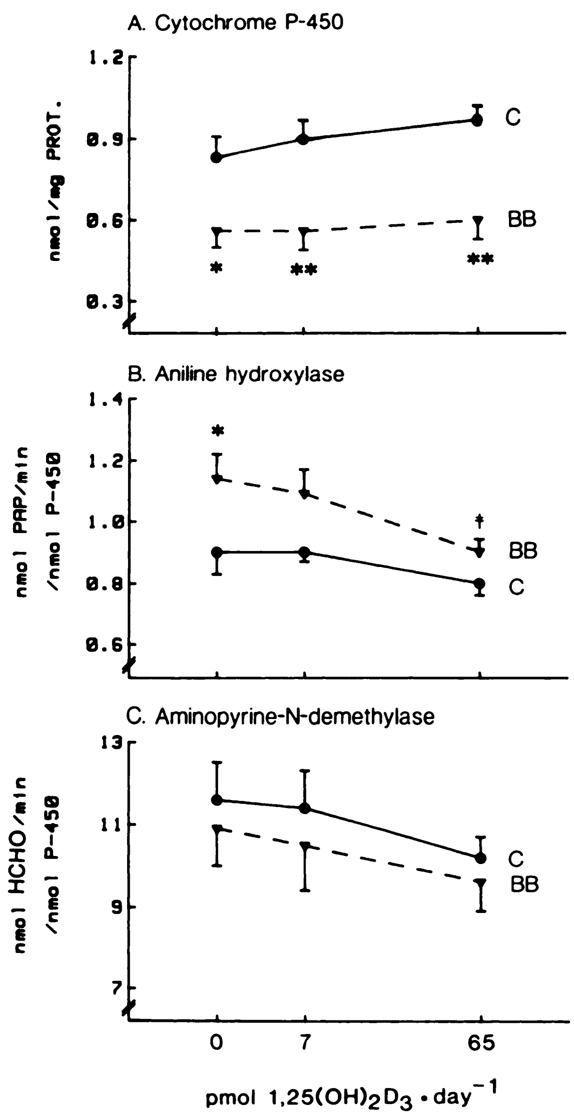

Fig. 3. Effect of constant i.p. infusion with vehicle (0) or doses of 7 or 65 pmol of 1,25(OH)$_2$D$_3$·day^{-1} on microsomal MFO activities in corn oil (●)- and BB-treated (▼) vitamin D-depleted rats. Treatments were given i.p., 48 hr before sacrifice (corresponding to day 6 of the supplementation regimen): control (C); corn oil, 1.34 ml·kg^{-1}; BB, 500 mg·kg^{-1}. Results are expressed as means ± S.E.M.; N = 48 or 6 to 10 rats per subgroup in A and B whereas N = 36 or 6 rats per subgroup in C. Statistically significant differences between group means were analyzed by a 2 × 3 factorial analysis of variance and were: different from C, *P < .02, **P < .002; and different from vehicle-infused group, ‡P < .05. PROT, protein; PAP, p-aminophenol; HCHO, formaldehyde.

plasma calcium concentrations. Body weights (fig. 5B), which were initially similar in all groups, decreased significantly after BB intoxication in both hypocalcemic and calcium-supplemented animals while remaining unchanged in corn oil controls. Liver weights were not affected significantly by calcium supplementation p.o. whereas after BB intoxication both calcium-supplemented and hypocalcemic animals displayed significantly greater liver weights than their respective corn oil controls (fig. 5C).

Effects on liver toxicity. The plasma activities of the hepatic enzymes ALT and SDH are presented in figure 6. Oral calcium supplementation alone did not modify significantly the circulating concentrations of ALT and SDH as illustrated by the data obtained in corn oil controls. In contrast, BB intoxication led to significant increases in the plasma levels of both hepatic enzymes which were, however, significantly greater in the calcium-supplemented than in the hypocalcemic group (P < .05). An evaluation of microsomal cytochrome P-450-dependent BB epoxidation in corn oil controls yielded values of 9.48 ± 0.54 and 10.53 ± 0.44 nmol of [^{14}C]BB bound·mg of protein·5 min^{-1} in hypocalcemic and calcium-supplemented animals, respectively (N.S.), whereas the hepatic total glutathione content was evaluated at 7.81 ± 0.64 and 7.80 ± 0.30 μmol/g of liver in hypocalcemic and calcium-supplemented rats, respectively (N.S.).

Effects on the metabolic activities of the MFO. The liver MFO activities are presented in figure 7. The specific content in microsomal cytochrome P-450 was diminished significantly by BB intoxication whereas calcium supplementation had no effect whether in corn oil controls or BB-intoxicated animals. The microsomal aniline hydroxylase activity was not influenced significantly by either calcium supplementation or BB intoxication.

Effects on liver morphology. The morphological appearance of the BB-induced hepatic lesion observed in calcium-supplemented rats is presented in figure 4E. Liver samples from hypocalcemic controls yielded results morphologically equivalent to vehicle-infused animals in the preceding experiment (fig. 4B). The morphometric analysis of BB hepatotoxicity revealed that the volumic density of hepatocellular necrosis was significantly greater in calcium-supplemented than in hypocalcemic rats (0.227 ± 0.023 *vs.* 0.134 ± 0.023, P < .05). Similarly, the proportion of hepatic parenchyma occupied by acidophilic cells was increased significantly by calcium supplementation (0.146 ± 0.019 *vs.* 0.077 ± 0.009 for hypocalcemic animals, P < .005). However, the ratio of acidophilic to necrotic volume densities was similar in both groups (0.716 ± 0.096 and 0.701 ± 0.103 for hypocalcemic and calcium-supplemented rats, respectively, N.S.).

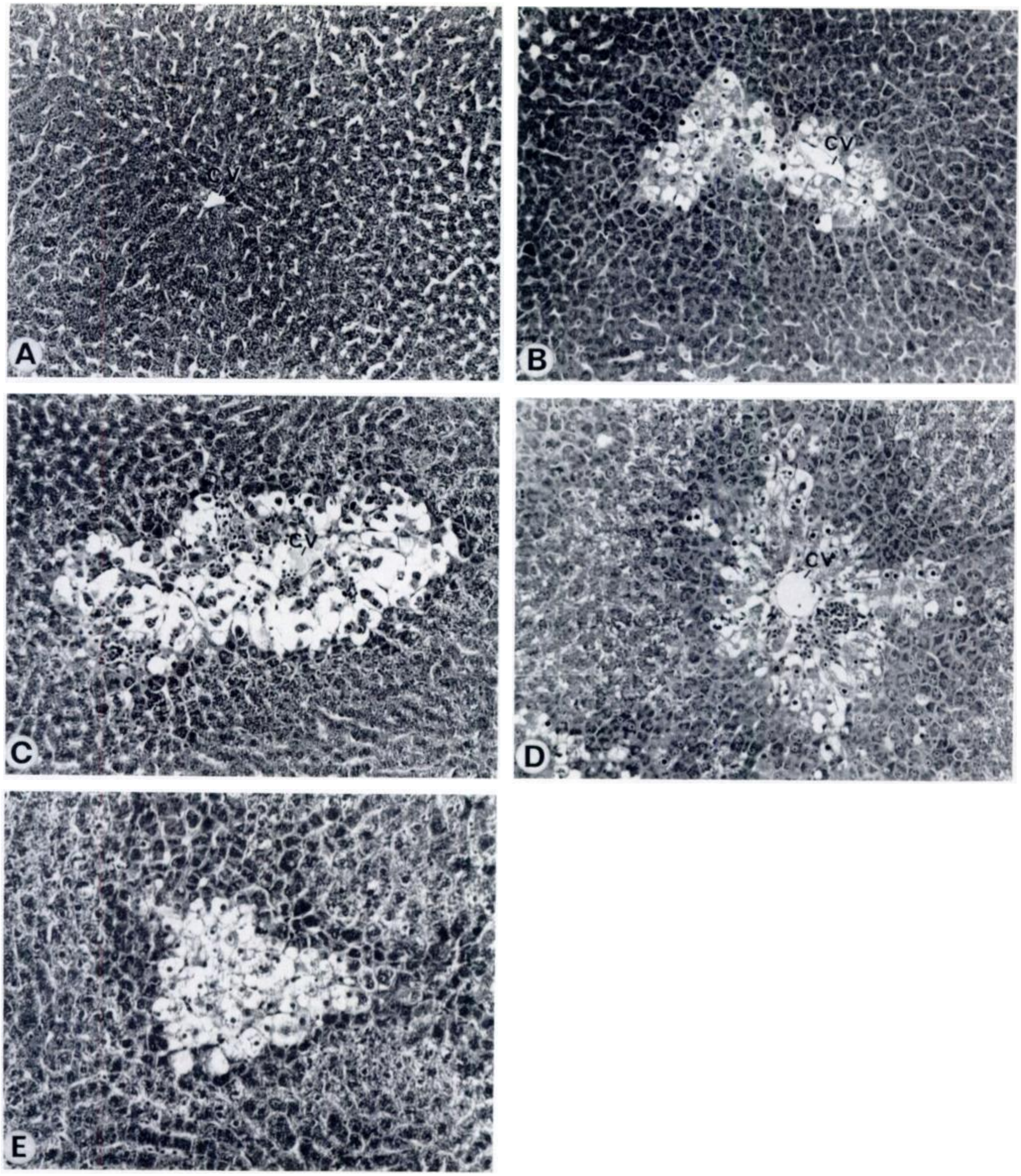

Fig. 4. Representative photomicrographs of rat liver after BB intoxication in $1,25(OH)_2D_3$- or calcium-supplemented vitamin D-depleted rats. A, nonintoxicated control; B, unsupplemented hypocalcemic animal; C, 7 pmol of $1,25(OH)_2D_3 \cdot day^{-1}$; D, 65 pmol of $1,25(OH)_2D_3 \cdot day^{-1}$; E, calcium supplemented animal. In B, C, D and E, BB was given i.p. 48 hr before sacrifice (corresponding to day 6 of hormone infusion or to day 12 of calcium supplementation): control, corn oil, 1.34 $ml \cdot kg^{-1}$; BB, 500 $mg \cdot kg^{-1}$. CV, central vein. Note the pericentral necrosis surrounded by a crown of acidophilic cells after BB treatment (B, C, D and E) whereas controls (A) had normal liver morphology.

Discussion

The severity of hepatocellular necrosis induced by halogenated hydrocarbons such as BB has been shown to be correlated to the extent of covalent binding of electrophilic metabolites to liver macromolecules (Brodie *et al.*, 1971), although a causal relationship between the two phenomena has not been unequivocally established (Farber, 1982; Popper, 1982). The chemically reactive compounds involved in the necrotic process are known to arise from the oxidative metabolism of the parent

TABLE 2

Effects of $1,25(OH)_2D_3$ infusion on the morphometric parameters of BB hepatotoxicity in Vitamin D-depleted rats

All animals received single i.p. injections of BB, 500 mg·k^{-1}, 48 hr before the end of the $1,25(OH)_2D_3$ supplementation period. Results represent volumetric proportions of liver parenchyma occupied by necrotic cells, acidophilic cells or individual ratios of these parameters; they are expressed as the means ± S.E.M. The corn oil-treated groups showed an entirely normal liver morphology and their morphometric parameters of toxicity were equal to zero in all cases. ANOVA, analysis of variance.

Dose of $1,25(OH)_2D_3$ Infused	n	Vv Necrotic Cells	Vv Acidophilic Cells	Vv Acidophilic / Vv Necrotic
pmol·day^{-1}				
0	10	0.111 ± 0.014	0.078 ± 0.014	0.668 ± 0.078
7	9	0.136 ± 0.022	0.117 ± 0.021	0.781 ± 0.116
65	10	0.150 ± 0.023	0.140 ± 0.017	1.224 ± 0.239
ANOVA		N.S.	P < .05*,†	P < .05*,†

* Statistically significant differences attributed to the 65 pmol of $1,25(OH)_2D_3$·day^{-1} group, P < .05; † results demonstrated a linear progression related to the dose of $1,25(OH)_2D_3$ infused (P < .05).

compound by the hepatic microsomal cytochrome P-450-dependent MFO system (Reid and Krishna, 1973). Aside from the rate of microsomal activation of the original toxins to various electrophilic metabolites, the extent of chemically induced hepatocellular damage will also depend on the physicochemical properties of the reactive species formed (*e.g.* stability of the molecules, amino or fatty acid residues targeted) as well as the physiological significance of the macromolecules attacked (*e.g.* leading to enzyme inactivation, lipid peroxidation or hapten formation). Furthermore, the detoxifying capacity of the liver (mainly phase II conjugation reactions such as to glutathione) and the efficacy of the processes eventually leading to cellular degeneration will greatly influence the hepatotoxic response to a given chemical agent. For instance, halogenated hydrocarbons are known to affect the calcium-sequestering function of the mitochondria (Jewell *et al.*, 1982) and of the endoplasmic reticulum (Lowrey *et al.*, 1981). In that context, it is noteworthy that disturbances in intracellular calcium homeostasis are thought to mediate the crucial irreversible events involved in cell membrane injury which eventually lead to hepatocellular degeneration and death (Popper, 1982); in fact, the two common events which are generally seen preceding cell death from hepatotoxic insult are intracellular calcium accumulation and disruption of the plasma membrane integrity (Farber, 1982).

The present studies were carried out in order to investigate the effect of the hormonal metabolite of the vitamin D endocrine system or of simple dietary calcium supplementation on the response of the liver to the toxic injury induced by BB in animals which remained depleted in vitamin D throughout the study. The data obtained indicate that constant i.p. infusion of $1,25(OH)_2D_3$ for 1 week increased the apparent hepatic toxicity of BB in these animals. Indeed, hormonal supplementation induced a dose-dependent increase in the plasma $1,25(OH)_2D_3$ and calcium concentrations which were accompanied by significant increases in the quantity of the hepatic enzymes AST, ALT and SDH leaking into the blood stream after BB administration. The effect of $1,25(OH)_2D_3$ was also accompanied by a modest increase in the volumic density of zone 3 necrosis as well as by an enhanced accumulation of lipids in more periportal acinar regions, both of which are typical of BB intoxication (Miller *et al.*, 1978). However, the $1,25(OH)_2D_3$-mediated increased hepatotoxicity of BB could not be related to the amount

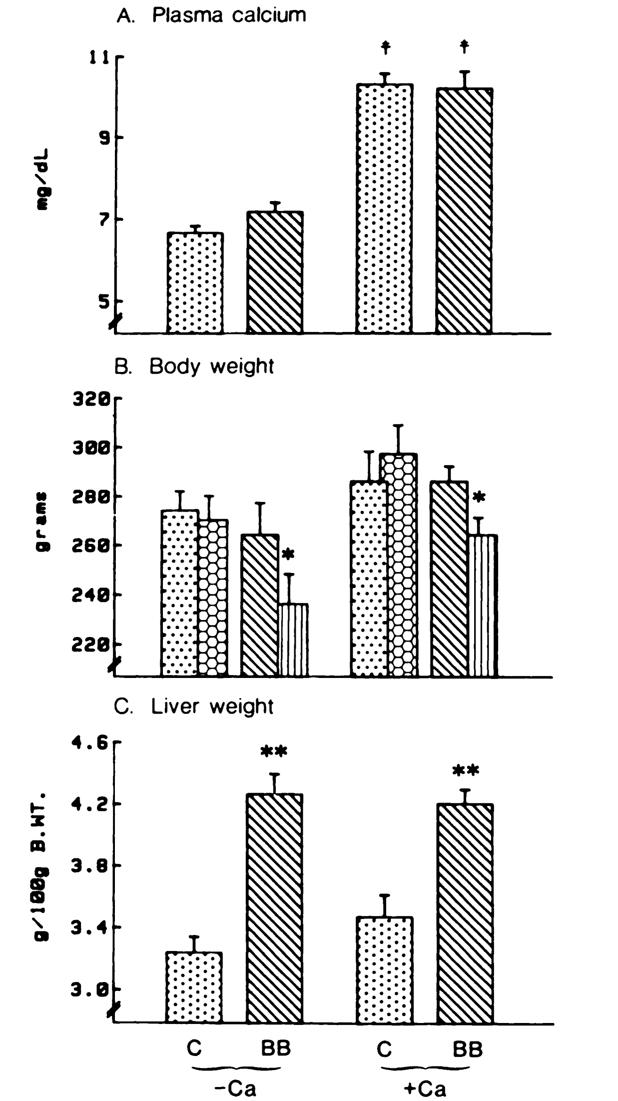

Fig. 5. Effect of calcium supplementation p.o. (+Ca) on general systemic parameters in corn oil (□)- and BB-treated (▨) vitamin D-depleted rats. Treatments were given i.p., 48 hr before sacrifice (corresponding to day 12 of the supplementation regimen): control (C) received corn oil, 1.34 ml·kg^{-1}; BB, 500 mg·kg^{-1}. Results are expressed as means ± S.E.M.; N = 26, or 6 to 7 rats per subgroup. In B, body weights on the day treatment was started (C, □; BB, ▨) were compared with those obtained on the day the animals were sacrificed (C, ■; BB, ▥). Statistically significant differences between group means were analyzed by a 2 × 2 factorial analysis of variance and were: different from C, *P < .05, **P < .001; and different from hypocalcemic (−Ca) group, ‡P < .001.

of microsomal cytochrome P-450 remaining in the liver at the time the animals were sacrificed to the extent of *in vitro* activation of BB into reactive epoxides by the liver microsomes, or to the detoxyfying capacity of the liver as judged by the total glutathione content of the organ. Similarly, hepatic glucose-6-phosphatase activity, which is known to be more sensitive to lipid peroxidation than to covalent binding (Popper, 1982), was not modified by $1,25(OH)_2D_3$ administration in either BB-treated or corn oil control rats. Furthermore, calcium supplementation p.o., which normalized the plasma calcium concentrations of the vitamin D-depleted animals in the absence of $1,25(OH)_2D_3$ replacement, also led to significant increases in the circulating concentrations of ALT and SDH as well as in the volumic density of necrosis observed after BB intoxication

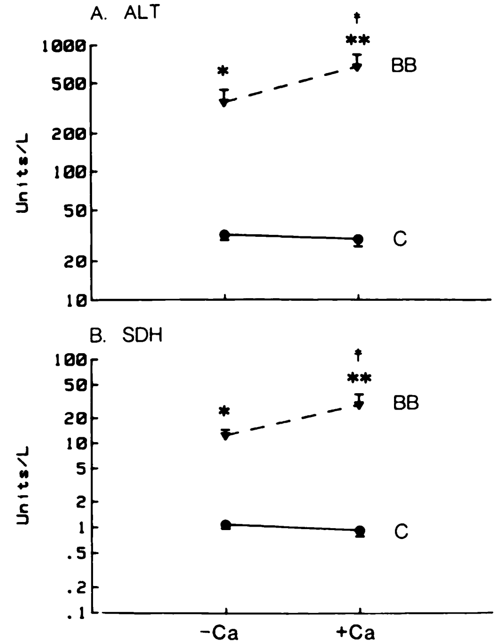

Fig. 6. Effects of calcium supplementation p.o. (+Ca) on the plasma activity of hepatic enzymes in corn oil (●)- and BB-treated (▼) vitamin D-depleted rats. All treatments were given i.p., 48 hr before sacrifice (corresponding to day 12 of the supplementation regimen): control (C), corn oil, 1.34 ml·kg^{-1}; BB, 500 mg·kg^{-1}. Results are expressed as means ± S.E.M.; *N* = 26 or 6 to 7 rats per subgroup. Statistically significant differences between group means analyzed by a 2 × 2 factorial analysis of variance and were: different from C, *$P < .05$, **$P < .001$; and different from hypocalcemic group (−Ca), ‡$P < .02$.

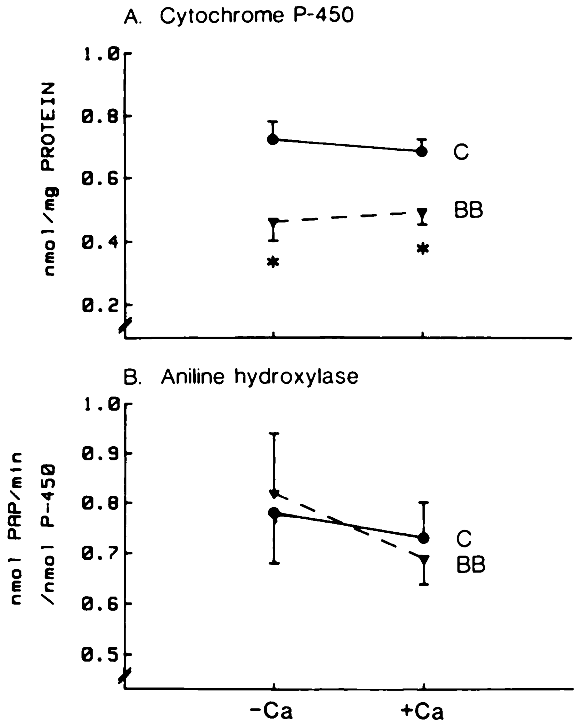

Fig. 7. Effects of calcium supplementation p.o. (+Ca) on microsomal MFO activities in corn oil (●)- and BB-treated (▼) vitamin D-depleted rats. All treatments were given i.p., 48 hr before sacrifice (corresponding to day 12 of the supplementation regimen): control (C), corn oil, 1.34 ml·kg^{-1}; BB, 500 mg·kg^{-1}. Results are expressed as means ± S.E.M.; *N* = 26 or 6 to 7 rats per subgroup. Statistically significant differences between group means were analyzed by a 2 × 2 factorial analysis of variance and were: different from C, *$P < .02$. PAP, *p*-aminophenol.

without significantly modifying the residual amount of cytochrome P-450, the extent of *in vitro* BB epoxidation or the total glutathione content of the liver. Collectively these results suggest that the greater BB hepatotoxicity observed in $1,25(OH)_2D_3$- or calcium-supplemented than in hypocalcemic rats was probably not the result of an increased intrahepatic accumulation of toxic metabolites. It has been reported previously that BB-induced plasma membrane bleb formation (Jewell *et al.*, 1982) and phospholipase C stimulation (Lamb and Schwertz, 1982) in liver cells are calcium-dependent events. The observed increase in BB toxicity in the $1,25(OH)_2D_3$- and calcium-supplemented animals could thus be related to a potentiation of the deleterious effects of an equivalent amount of activated reactive BB metabolites on the cell membrane integrity which could have been induced by changes in the extracellular (and possibly also in the intracellular) calcium concentrations brought about by the pretreatment regimens. These observations confirm the previously reported partial protection afforded by vitamin D depletion against BB-mediated hepatotoxicity (Haddad *et al.*, 1985) and further strengthen the fact that the hypocalcemic state rather than the $1,25(OH)_2D_3$ status may be responsible for the protection of the hepatic parenchyma against BB.

The role played by the increased circulating levels of parathyroid hormone which were most likely present in the vitamin D-depleted hypocalcemic rats has not, however, been investigated. In fact, it is known that the liver is not only involved in parathyroid hormone inactivation (D'Amour and Huet, 1984) but is also a target organ for the hormone (Canterbury *et al.*, 1974; Moxley *et al.*, 1984; Hruska *et al.*, 1979; Bergeran *et al.*, 1981; Bachelet *et al.*, 1985); its role as an added parameter to the low circulating calcium concentrations in the protective effect against BB toxicity should, therefore, not be neglected.

The data obtained during the present studies also uncovered a dose-related stimulatory influence of $1,25(OH)_2D_3$ infusion on the liver weight (as judged by the response of the corn oil control animals) whereas calcium supplementation p.o. was without effect. The trophic property of the hormone is not without precedent (Henry and Norman, 1984; Haddad *et al.*, 1986) and was further suggested, in the present studies, by a dose-related increase in the volumic density of acidophilic cells surrounding the perivenous necrotic area after BB intoxication. Several lines of evidence suggest that the observed crown of acidophilic cells probably represented regenerating liver parenchyma. Indeed, these cells were hypertrophied, featured a higher than usual adherence to the coloring agents hematoxylin (specific for basophilic structures such as nucleic acids) and fuschine (specific for acidophilic structures such as basic amino acid residues) and they were characterized by the presence of many prominent nucleoli; all of these parameters are morphologically indicative of active DNA and protein synthesis which are normally associated with different phases of cellular proliferation. It is also known that the hepatic parenchymal zone taking part in tissue repair and regeneration after cryo- or thermonecrosis is limited to a very narrow area adjacent to the lesions (Helpap and Grouls, 1979). Similarly, the contribution of different acinar zones to the regenerative process after allyl alcohol or BB intoxication has been shown to be dependent upon the intra-acinar distribution of the liver injury (Nostrant *et al.*, 1978). Interestingly, it has been reported previously that $1,25(OH)_2D_3$ is essential for the proper hepatic proliferative response of thyroparathyroidectomized rats after partial hepatectomy whereas the simple restoration of normal circulating calcium concentrations remained without effect (Youdale *et al.*, 1985) indicating that $1,25(OH)_2D_3$ may be involved in the liver regeneration processes after surgical trauma. Indeed, the acidophilic cells surrounding the BB-induced zone 3 necrosis which were observed during the present studies may have been, as mentioned above, actively taking part in the regenerative processes and their proportion was found to be significantly increased by $1,25(OH)_2D_3$ administration as well as calcium supplementation. However, the ratio of acidophilic to necrotic volume densities, which relates the putative proliferative response to the extent of the damage inducing it, was seen to increase significantly as a function of the dose of $1,25(OH)_2D_3$ infused whereas calcium supplementation was without effect on this parameter. This phenomenon may then have contributed to partly offset the cellular damage induced by BB administration in $1,25(OH)_2D_3$-treated animals, and could lead, in the long term, to a better recovery of hepatic function in hormone-supplemented than in unsupplemented animals. In support of this concept, the plasma concentrations of the hepatic enzymes AST, ALT and SDH after BB administration have, in fact, been found to return to normal levels faster in vitamin D replete than in hypocalcemic vitamin D-depleted rats (Haddad, 1986).

The data obtained on hepatic microsomal enzyme activities during the present studies also are indicative of the presence of an effect of $1,25(OH)_2D_3$ on certain cytochrome P-450-dependent systems. Indeed, in BB-intoxicated rats (reflecting "periportal-enriched" activity due to the BB-induced perivenous destruction), aniline hydroxylation decreased in a dose-dependent fashion after $1,25(OH)_2D_3$ administration. This corroborates our previous finding that aniline hydroxylase activity after BB intoxication was greater in hypocalcemic vitamin D-depleted than in normocalcemic vitamin D-replete rats (Haddad *et al.*, 1985). However, correction of the hypocalcemic state alone, as was done after calcium supplementation p.o. in the present studies, did not significantly affect aniline hydroxylase activity. Furthermore, the effect of $1,25(OH)_2D_3$ on certain MFO enzyme activities seemed to be relatively specific inasmuch as neither aminopyrine-N-demethylation nor BB epoxidation were affected by hormonal infusion although the latter activities have been reported to be located mainly in the pericentral (Gumucio *et al.*, 1978; James *et al.*, 1981) rather than in the periportal region of the liver acinus as is the case for aniline hydroxylation (James *et al.*, 1981). $1,25(OH)_2D_3$ could thus be associated with a modulation of the intra-acinar localization of certain metabolic activities which may involve the synthesis of either specific isoenzymes of cytochrome P-450 or of other essential components of the intracellular organelles involved in their activities; alternatively, the $1,25(OH)_2D_3$-induced modulation of certain enzyme activities may simply reflect changes in the microenvironment of the enzymes which are brought about by the hormone.

The mode of action of the hormone $1,25(OH)_2D_3$ in the modulation of certain intra-acinar enzyme system(s) in the apparent hepatocellular injury and also possibly in tissue repair is not known. However, it is important to mention that as of now, no receptors to $1,25(OH)_2D_3$ have been reported in the mammalian liver (Norman *et al.*, 1982) making it difficult to postulate that the effect of the hormone could be due to DNA-directed specific changes in hepatocellular events. It is, therefore, not excluded that many of the apparent intrahepatic changes induced by $1,25(OH)_2D_3$ could be linked, at least in part, to hormone-mediated modifications in the extracellular and/or intracellular concentrations of calcium which the hormone is known to increase (Baran and Milne, 1986). Finally, the implication of some intracellular receptor-independent action such as on the composition of certain membranes, with which the hormone has been associated in other organs (Nemere and Norman, 1982), remains a possibility to be considered.

Acknowledgments

The authors are grateful to Jocelyne Boudrias-Belgiorno for her assistance in the histomorphological techniques, Robert Boileau in the computer analysis, Elizabeth Pérès-Nault for the preparation of the graphs, Jean Marchi and Jacques Bernier for the photography as well as Hélène Camirand and Rachel Viau for their excellent secretarial assistance. Crystalline reference 25-hydroxyvitamin D_3 was a gift from Dr. John Babcock (The Uphjohn Company, Kalamazoo, MI) whereas crystalline reference $1,25(OH)_2D_3$ was generously provided by Dr. Milan Uskokovic (Hoffmann La Roche Company Inc., Nutley, NJ).

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