TERATOLOGY 50:214–228 (1994)

In Vitro Embryotoxicity of the Cysteine Proteinase Inhibitors Benzyloxycarbonyl-Phenylalanine-Alanine-Diazomethane (Z-Phe-Ala-CHN_2) and Benzyloxycarbonyl-Phenylalanine-Phenylalanine-Diazomethane (Z-Phe-Phe-CHN_2)

JEFFREY L. AMBROSO AND CRAIG HARRIS

Toxicology Program, Department of Environmental and Industrial Health, University of Michigan, Ann Arbor, Michigan 48109-2029

ABSTRACT This study makes use of whole embryo culture to investigate the potential embryotoxicity of benzyloxycarbonyl-phenylalanine-alanine-diazomethane (Z-Phe-Ala-CHN_2) and benzyloxycarbonyl-phenylalanine-phenylalanine-diazomethane (Z-Phe-Phe-CHN_2), two low molecular weight, active site-directed and irreversible inhibitors of the lysosomal cysteine proteinases. Peptidyl diazomethanes are the most specific inhibitors available for lysosomal cysteine proteinases and can be hypothesized to interrupt visceral yolk sac (VYS)-mediated nutrition during early organogenesis. When added directly to the culture medium of gestational day 10–11 rat conceptuses, both compounds inhibited lysosomal cysteine proteinase activity in the VYS in a concentration-dependent fashion that correlated with the degree of embryotoxicity observed. Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 were also found to increase the protein content of the VYS, even though all other conceptal growth parameters decreased. This effect was dependent on the serum content of the culture medium and the exposure time. Histological examination of Z-Phe-Ala-CHN_2-treated conceptuses revealed a dramatic increase in the size and number of vacuoles in the VYS endoderm epithelium, suggestive of inhibition of VYS proteolysis. At the same time, excessive cell death was observed throughout the neuroepithelium and in specific regions of the mesenchyme of the corresponding embryos. This cell death manifested morphological characteristics of apoptosis and could be detected by supravital staining with Nile Blue Sulphate. These findings provide additional evidence in support of the hypothesis that lysosomal cysteine proteinases play a critical role in VYS-mediated histiotrophic nutrition and suggest that peptidyl diazomethanes may be useful in further characterization of these enzymes. The possible direct effects of these inhibitors on embryonic cells and the relationships between interruption of VYS-mediated nutritional processes and embryonic cell death are discussed.

In vitro studies performed by Freeman and colleagues ('81; Freeman and Lloyd, '83a) have shown that during early organogenesis and prior to the establishment of the functional placenta, the rat visceral yolk sac (VYS) supplies the rapidly developing conceptus with amino acids used for de novo protein synthesis. This process, known as histiotrophic nutrition, involves the endocytosis and degradation of exogenous proteins by the VYS endoderm epithelium. More recent experiments have demonstrated the quantitative significance of this pathway: it has been estimated that 86% of the serine (Rowe and Kalaizis, '85) and 99% of the leucine (Beckman et al., '90, '91) incorporated into new conceptal proteins is derived from VYS proteolysis. The importance of histiotrophic nutrition for normal embryonic development in vivo has been suggested by the teratogenicity of several agents known to disrupt this process (Freeman and Lloyd, '83b, '86; Brent et al., '90).

Notable among the group of embryotoxicants shown to interrupt histiotrophic nutrition is the protease inhibitor leupeptin. Leupeptin has been used not only to demonstrate that inhibition of VYS proteolysis leads to embryotoxicity, but also to make inferences about the identity of the proteolytic enzymes involved. The potent inhibitory effect of leupeptin on VYS proteolysis has been taken as evidence that the lysosomal cysteine proteinases (cathepsins B, H, and L) are responsible for

Received January 12, 1994; accepted July 5, 1994.

Address reprint requests to Dr. Craig Harris, Toxicology Program, Department of Environmental and Industrial Health, University of Michigan, 1420 Washington Heights, Ann Arbor, MI 48109-2029.

VYS proteolysis (Knowles and Ballard, '81; Freeman and Lloyd, '83b; Freeman and Brown, '85). It is known, however, that in addition to the lysosomal cysteine proteinases, leupeptin can also inhibit a number of serine proteases such as plasmin, trypsin, and kallikrein (Ishii and Kasai, '81; Umezawa, '82), as well as the calcium-activated neutral proteases (calpains) (Parkes et al., '85; Rich, '86). This lack of specificity of leupeptin and related peptide aldehydes has led researchers to search for new inhibitors for the characterization of lysosomal cysteine proteinases.

Several classes of cysteine proteinase inhibitors displaying greater specificity have subsequently been developed, including the peptide epoxides (Hanada et al., '78) and the peptidyl diazomethanes (Leary et al., '77). Unlike leupeptin, both the peptide epoxides and peptidyl diazomethanes can distinguish between cysteine and serine proteinases. However, the peptide epoxides (of which E-64 is a member) have also been found to be potent inhibitors of calpains (Parkes et al., '85). The peptidyl diazomethanes are the most specific inhibitors of lysosomal cysteine proteinases currently available, being inactive against all other classes of proteinases including calpains (Green and Shaw, '81; Crawford et al., '88). The peptide portion of these inhibitors enables them to enter the active site of the enzyme where irreversible inactivation occurs via alkylation of the catalytic cysteine moiety (for reviews see Rich, '86; Shaw, '90). Mason et al. ('89) have shown that these inhibitors act as affinity labels for lysosomal cysteine proteinases. After exposing cultured cells to a radiolabeled peptide diazomethane, they found only the active forms of cathepsins B and L were labeled in cell extracts. This illustrates the specificity of these reagents since even the precursor forms of the target enzymes (procathepsins) remained unlabeled until they were processed to an active form. Another advantage of the peptidyl diazomethanes is the ability to distinguish between the enzymes cathepsin B and cathepsin L through alterations in the peptide group, since these two enzymes are indistinguishable in terms of their sensitivity to leupeptin and the peptide epoxides (all three inhibitor classes are much less effective against cathepsin H). These unique properties of the peptidyl diazomethanes make them useful tools for the investigation of proteolytic enzymes in the rat conceptus.

If the lysosomal cysteine proteinases are indeed the target for embryotoxicants such as leupeptin, it could be hypothesized that the peptidyl diazomethanes would also cause embryotoxicity in the rat and manifest evidence of perturbed histiotrophic nutrition. In order to test this hypothesis, we exposed early organogenesis-stage rat conceptuses growing in whole embryo culture to two peptidyl diazomethanes, benzyloxycarbonyl-phenylalanine-alanine-diazomethane (Z-Phe-Ala-CHN_2) and benzyloxycarbonyl-phenylalanine-phenylalanine-diazomethane (Z-Phe-Phe-CHN_2), and evaluated their response biochemically and morphologically.

MATERIALS AND METHODS

Chemicals

Benzyloxycarbonyl-phenylalanine-arginine-7-amino-4-methyl coumarin (Z-Phe-Arg-7AMC) and Z-Phe-Phe-CHN_2 were from Bachem Biochemical (Cambridge, MA); Z-Phe-Ala-CHN_2 and 7-amino-4-methyl coumarin (7AMC) were obtained from Bachem California (Torrance, CA). Nile Blue Sulphate (NBS) was from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest purity available and were obtained from common sources.

Embryo culture

Time-mated primigravida Sprague-Dawley rats were obtained from the University of Michigan Reproductive Sciences Program Animal Core Facility on gestational days (GD) 6–9 (sperm positive vaginal smear = GD 0) and maintained on a 14 hour light/10 hour dark cycle with free access to food and water. Conceptuses were explanted as previously described (Fantel et al., '79) between 8–11 A.M. on GD 10, and placed in culture bottles containing 33% rat serum in Hank's balanced salt solution (HBSS; pH = 7.4) supplemented with penicillin G (100 IU/ml) and streptomycin (50 IU/ml). Embryos had elevated head folds and 7–9 somites at the beginning of the culture period. A maximum of 1 conceptus/ml was used in a total volume of 10–15 ml of medium. The medium had been warmed to 37°C and gassed with 20% O_2:5% CO_2:75% N_2 at the start of the culture period. Stock Z-Phe-Ala-CHN_2 or Z-Phe-Phe-CHN_2 in dimethylsulfoxide (DMSO) was added directly to the culture medium just prior to adding the conceptuses. Control cultures received an amount of DMSO equivalent to the highest concentration of inhibitor stock. This volume did not exceed 0.01% of the volume of culture medium. The culture bottles were sealed and placed in a roller incubator overnight and regassed with 95% O_2:5% CO_2 on the following morning (GD 11). Assessments took place at approximately 1 P.M. on GD 11.

Morphology assessment

Conceptuses were removed from culture, rinsed once with HBSS, and placed in warm HBSS for assessment of viability (heartbeat and yolk sac circulation) and malformations. Assessments were performed under a dissecting microscope and conducted blind in order to minimize bias. Yolk sac morphology was scored numerically according to the criteria of Seegmiller et al. ('91), with 0 being given to a healthy VYS and scores of 1–4 to increasingly severe VYS abnormalities (diminished pigment, vasculature, and circulation). The yolk sac was removed by dissection and measurements were taken of embryonic crown-rump length and head size

with an ocular micrometer. The embryos were further evaluated for somite number, neural tube closure, axial rotation, and morphologic abnormalities. The embryos and yolk sacs were individually placed in sodium phosphate buffer (50 mM containing 1 mM EDTA, pH = 6.0) on ice, sonically disrupted, and frozen at −70°C for subsequent determinations of protein content, DNA content, and cathepsin activity.

Protein and DNA assays

Protein content was determined by the method of Bradford ('76) and modified for 96-well microtiter plates as described in Harris et al. ('88). Bovine gamma globulin was used as a standard. DNA content was determined fluorometrically by the method of Labarca and Paigen ('80), using bovine DNA as a standard.

Lysosomal cysteine proteinase activity

The combined hydrolytic activity of the lysosomal cysteine proteinases cathepsin B and L was determined using the fluorometric substrate Z-Phe-Arg-7AMC. A modification of the method of Barrett and Kirschke ('81) for cathepsin L was employed. Briefly, aliquots of VYS homogenates were diluted in 0.1% Triton X-100, and then mixed with 8 mM dithiothreitol (DTT) in order to activate the enzyme. After a 1 minute activation period, the enzyme solution was combined with substrate and incubated for an additional 10 minutes at 37°C. The final reaction mixture contained 5 mM Z-Phe-Arg-7AMC, 100 mM sodium acetate buffer, 1 mM EDTA, and 2 mM DTT (pH = 5.5). The reaction was stopped by adding ice-cold 200 mM sodium monochloroacetate (pH = 4.3), and the fluorescence of the 7AMC product determined at 370 nm excitation and 460 nm emission using a Perkin-Elmer Model LS-5 fluorescence spectrophotometer (Perkin-Elmer, Oak Brook, IL). The instrument was standardized with authentic 7AMC. One milliunit (mU) of activity is defined as that which liberates 1 nmol of product/min.

Histology

Representative samples from control, 2.5 μM, 5.0 μM, and 10 μM Z-Phe-Ala-CHN_2-treated conceptuses from two separate experiments were processed and evaluated. At the end of the culture period (GD 11.5), conceptuses were rinsed in warm HBSS and placed in Karnovsky's fixative at room temperature. The VYSs were dissected free of the embryo and the amnion was ruptured to allow fixative to reach the embryos. After fixation, the embryos and VYSs were dehydrated and embedded in glycol methacrylate polymer, cut in 5 μm transverse sections, and stained with hematoxylin and eosin (H&E). A Leitz Aristoplan microscope with a Wild camera attachment was used for examination and photography of the sections.

NBS staining

At the end of the culture period, control and Z-Phe-Ala-CHN_2-treated conceptuses were removed from the culture medium and rinsed in warm HBSS. The embryos were dissected free of their extraembryonic membranes and placed in 50 ml roller bottles containing a 0.002% (w/v) solution of NBS in lactated Ringer's solution. The bottles were roller incubated for 30 minutes at 37°C, after which the embryos were removed and rinsed well in HBSS. Examination and photography were performed on a Leitz M8 dissecting microscope with a Wild camera attachment.

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine overall differences between treatment means. This was followed by Fisher's protected least significant differences test (PLSD) test for pairwise comparisons. The chi-square test was used for analysis of malformation incidence. $P < 0.05$ was considered significant.

RESULTS

The addition of Z-Phe-Ala-CHN_2 to culture medium containing whole conceptuses on GD 10 inhibited the overall growth of the VYS and the development of the vitelline vasculature by GD 11 (Fig. 1A). Embryos taken from these conceptuses likewise display concentration-related growth retardation and dysmorphogenesis (Fig. 1B). Table 1 summarizes the effects of Z-Phe-Ala-CHN_2 on the development of organogenesis-stage rat conceptuses grown in vitro for 26 hours. Initial inhibitor concentrations of 1–20 μM caused concentration-related decreases in crown-rump length (to 62% of control at 20 μM, $P < 0.001$), head length (to 53% of control at 20 μM, $P < 0.001$), and somite number (to 69% of control at 20 μM, $P < 0.001$). The incidence of abnormal VYSs and dysmorphogenic embryos (100% at 10 μM) increased in a concentration-related fashion, while viability (as assessed by heartbeat and VYS circulation) was not dramatically reduced (90% at 20 μM). Similar results were obtained if Z-Phe-Phe-CHN_2 was used in the same experimental protocol (Table 2), although this inhibitor appeared slightly less potent than Z-Phe-Ala-CHN_2.

The type and incidence of dysmorphogenesis produced by Z-Phe-Ala-CHN_2 exposure is shown in Table 3. Abnormal morphology in the craniofacial region was the most prominent effect noted, with anophthalmia and lack of cranial neural tube expansion occurring in 100% of the embryos exposed to 20 μM inhibitor. These changes, together with blisters and opaque necrotic areas in the craniofacial region, increased in incidence and severity as the concentration of Z-Phe-Ala-CHN_2 was increased. Z-Phe-Ala-CHN_2 treatment also produced abnormal axial rotation and shortening of the trunk in a smaller percentage of treated embryos (22% and 33%, respectively, at 20 μM), but these effects did

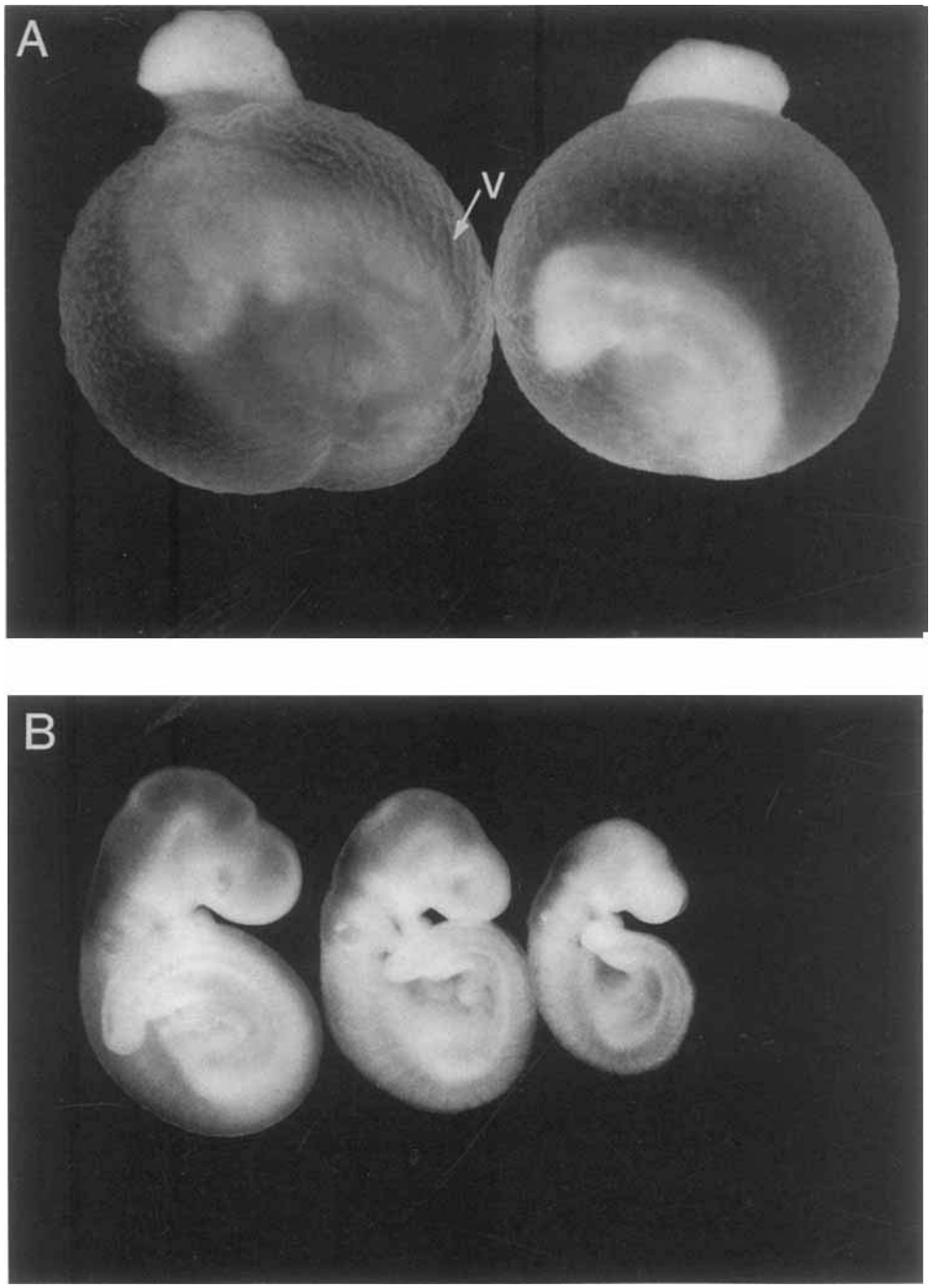

Fig. 1. Effect of Z-Phe-Ala-CHN_2 on VYS and embryo morphology. **A:** Control and 10 μM Z-Phe-Ala-CHN_2-treated whole conceptuses at the end of the culture period (GD 11.5). Control is on the left. Note the decreased VYS vasculature (V) in the treated conceptus. ×14. **B:** Examples of control, 2.5 μM, and 5.0 μM Z-Phe-Ala-CHN_2-treated embryos at the end of the culture period. The extraembryonic membranes have been removed for photography. ×13.

not appear to follow a clear concentration-response relationship. The morphologic effects of Z-Phe-Ala-CHN_2 were closely paralleled by those observed for Z-Phe-Phe-CHN_2 (data not shown).

Biochemical growth parameters are presented in Figure 2. Z-Phe-Ala-CHN_2 exposure decreased both DNA and protein content of embryos to 26% and 27% of control at 20 μM, respectively, by the end of the culture period. In the VYS, however, changes in protein and DNA content did not parallel one another. Only DNA content decreased in a typical concentration-response pattern (to 61% of control at 20 μM, $P < 0.001$).

TABLE 1. Effects of Z-Phe-Ala-CHN_2 on rat embryonic development in vitro[1]

Inhibitor concentration	% Viable[2]	% Dysmorphic[3]	Crown-rump length (mm ± SD)	Head length (mm ± SD)	Somites (±SD)	Mean yolk sac score	Yolk sac diameter (mm ± SD)
DMSO control	100 (31)	6 (2/31)	3.4 ± 0.3	1.7 ± 0.2	23 ± 1	0	3.6 ± 0.2
1.0 μM	100 (5)	0 (0/5)	3.2 ± 0.2	1.7 ± 0.1	22 ± 1	0	3.5 ± 0.2
2.5 μM	100 (21)	43 (9/21)*	3.0 ± 0.2**	1.5 ± 0.2**	21 ± 1*	1	ND
5.0 μM	96 (25)	96 (23/24)*	2.8 ± 0.2**	1.4 ± 0.2**	20 ± 2*	1	3.4 ± 0.2
10.0 μM	93 (27)	100 (25/25)*	2.4 ± 0.2**	1.2 ± 0.1**	19 ± 3**	2	3.4 ± 0.2
20.0 μM	90 (10)	100 (9/9)*	2.1 ± 0.1**	0.9 ± 0.1**	16 ± 3**	3	3.3 ± 0.2

[1]All measurements were made on GD 11.5 after a 26-hour exposure to Z-Phe-Ala-CHN_2 as described in Materials and Methods. Data represent pooled samples from five replicate experiments, except yolk sac diameter, which is from two experiments. ND = not determined.
[2]Indicates percentage of conceptuses with active heartbeat and yolk sac circulation at the end of the culture period (number cultured).
[3]Indicates percentage of dysmorphic embryos at the end of the culture period (number dysmorphic/number surviving). For type and incidence of dysmorphogenesis, see Table 3.
*Significantly different from control, $P < 0.05$.
**Significantly different from control, $P < 0.001$.

TABLE 2. Effect of Z-Phe-Phe-CHN_2 on rat embryonic development in vitro

Initial inhibitor concentration	% Viability[1]	% Dysmorphic[2]	Crown-rump length (mm ± SD)	Mean yolk sac score	Total head length (mm ± SD)
DMSO control	100 (17)	0 (0/17)	3.4 ± 0.2	0	1.7 ± 0.1
1.0 μM	100 (5)	0 (0/5)	3.3 ± 0.2	0	1.6 ± 0.1
2.5 μM	100 (10)	30 (3/10)	3.1 ± 0.2*	1	1.5 ± 0.2*
5.0 μM	100 (17)	76 (13/17)	2.8 ± 0.2**	1	1.3 ± 0.2**
10.0 μM	100 (11)	82 (9/11)	2.6 ± 0.2**	2	1.2 ± 0.1**
20.0 μM	100 (5)	100 (5/5)	2.5 ± 0.2**	1	1.2 ± 0.1**

[1]Indicates number of conceptuses possessing an active heartbeat and yolk sac circulation/total number cultured (n).
[2]Indicates number of morphologically abnormal embryos at the end of the culture period/total number surviving. (Abnormalities consisted of any of the following singly or in combination: anophthalmia, microphthalmia, craniofacial hypoplasia, craniofacial blisters, shortened tail, abnormal axial rotation, clear neural tube.)
*Significantly different from control, $P < 0.05$.
**Significantly different from control, $P < 0.001$.

TABLE 3. Type and incidence of dysmorphogenesis in embryos exposed to Z-Phe-Ala-CHN_2 in vitro[1]

Inhibitor concentration (n)	MO[2]	AO	HP	FH	BL	NC	AAR	ST	CE
DMSO control (31)	0	0	0	0	0	0	3	0	6
1 μM (5)	0	0	0	0	0	0	0	0	0
2.5 μM (21)	18	9	27	27	9	0	9	18	0
5.0 μM (24)	58	29	58	58	8	4	25	33	0
10.0 μM (25)	8	92	92	92	24	20	28	44	0
20.0 μM (9)	0	100	100	100	67	78	22	33	0

[1]Data represent percentage of surviving embryos possessing the listed abnormality at the end of the 26-hour culture period. MO = microphthalmia; AO = anophthalmia; HP = prosencephalic hypoplasia; FH = flat head; BL = prosencephalic blisters; NC = necrosis (head or trunk); AAR = abnormal axial rotation; ST = short, blunt trunk; CE = cloacal extrophy.
[2]The incidence of microphthalmia peaks in embryos treated with 5.0 μM Z-Phe-Ala-CHN_2; at higher concentrations anophthalmia was observed in nearly every case.

VYS protein content showed a biphasic response, being increased in the 2.5 μM group (to 113% of control, $P < 0.05$), no different than control in the 5 μM group (100% of control), and decreased in VYSs exposed to higher concentrations of Z-Phe-Ala-CHN_2 (to 72% of control at 20 μM, $P < 0.001$). All the treatment groups showed an increase in VYS protein content when normalized to DNA content, however. Figure 2 also indicates that conceptuses exposed to Z-Phe-Phe-CHN_2 throughout the culture period likewise showed a biphasic response in VYS protein content that did not correlate with other growth parameters. VYS protein content appeared to be increased in all Z-Phe-Phe-CHN_2-treated groups except the 20 μM group, al-

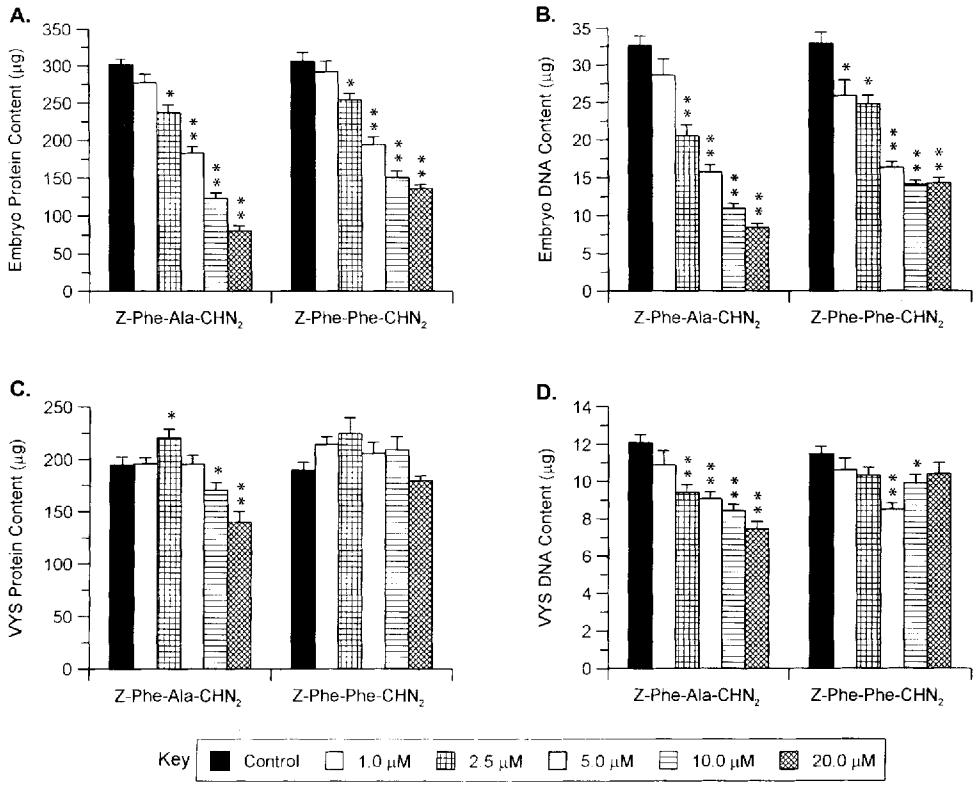

Fig. 2. Effect of peptidyl diazomethanes on biochemical growth parameters. Changes in embryonic protein content (**A**), embryonic DNA content (**B**), VYS protein content (**C**), and VYS DNA content (**D**) following 26-hour conceptal exposure to Z-Phe-Ala-CHN_2 or Z-Phe-Phe-CHN_2. Columns represent means ± SEM from "n" samples listed in Tables 1 and 2. *Significantly different from control, $P < 0.05$. **Significantly different from control, $P < 0.001$.

though none of these increases were found to be statistically significant.

The ability of the peptide diazomethanes to differentially affect embryonic and VYS protein content became more apparent if the percentage of serum in the culture medium was increased or if the length of exposure time to the inhibitor was decreased. As illustrated in Figure 3, doubling the serum content of the culture medium led to much larger increases in VYS protein content in conceptuses exposed to 2.5 µM of Z-Phe-Ala-CHN_2 than those observed originally (increased to 134% of control in 66% serum vs. 113% of control in 33% serum), while embryonic protein content in these conceptuses was decreased to an extent similar to that observed originally (to 73% of control in 66% serum vs. 79% of control in 33% serum). Moreover, whereas conceptuses exposed to 5 and 10 µM concentrations of Z-Phe-Ala-CHN_2 for 26 hours showed no increase in VYS protein content in our original experiments (100% of control at 5 µM; 88% of control at 10 µM), exposure to these same inhibitor concentrations and media composition (33% serum) for only the final 6 hours of culture showed a significant increase in protein content (increased to 136% of control at 5 µM, $P < 0.01$; 130% of control at 10 µM, $P < 0.01$).

The effect of peptidyl diazomethanes on lysosomal cysteine proteinase activity in GD 11.5 conceptal extracts is shown in Figure 4. As expected, Z-Phe-Ala-CHN_2 treatment decreased cathepsin B and L activity in a concentration-dependent fashion in the VYS (to 13% of control at 20 µM, $P < 0.001$). With the exception of the 1 µM group, Z-Phe-Phe-CHN_2 treatment also inhibited VYS cathepsin B and L activity in a concentration-dependent fashion (to 13% of control at 20 µM, $P < 0.001$). As noted previously (Ambroso and Harris, '94), VYS lysosomal cysteine proteinase activity exceeded that measured in the embryo by nearly 100-fold (approximately 350 mU/mg DNA in control VYSs vs. 3 mU/mg DNA in control embryos). Embryonic activity was not inhibited by Z-Phe-Ala-CHN_2 treatment, and was significantly increased at the highest inhibitor concentration tested (to 170% of control at 20 µM, $P < 0.05$). Z-Phe-Phe-CHN_2 treatment produced a biphasic response in embryonic cathepsin B and L activity, increasing activity to 213% of control in the 2.5 µM group ($P < 0.05$), but inhibiting this activity in the 10 µM group (29% of control, $P < 0.05$).

Conceptuses exposed to Z-Phe-Ala-CHN_2 throughout the 26 hour embryo culture period showed dramatic histological changes in both the embryo and VYS. In

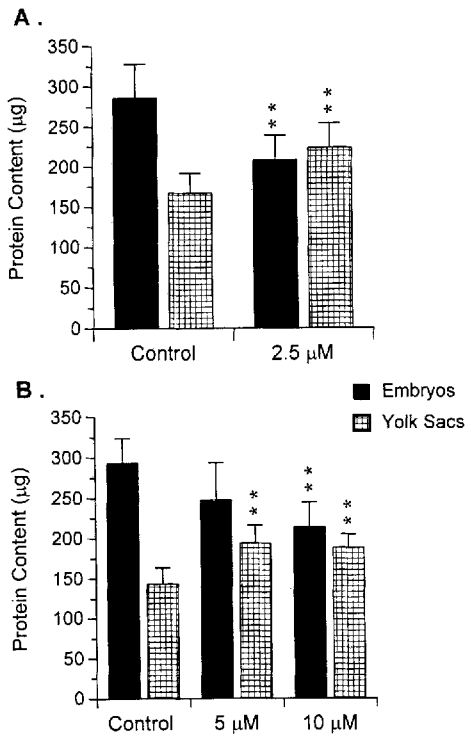

Fig. 3. Effect of medium composition and duration of exposure to Z-Phe-Ala-CHN_2 on VYS protein accumulation in organogenesis-stage conceptuses. Conceptuses grown for 26 hours in 66% serum exposed to Z-Phe-Ala-CHN_2 continuously (**A**) and conceptuses grown for 26 hours in 33% serum and exposed to Z-Phe-Ala-CHN_2 for only the final 6 hours of culture (**B**). Columns represent means ± SD of n = 11 (A) or n = 5 (B) samples. **Significantly different from control, $P < 0.01$.

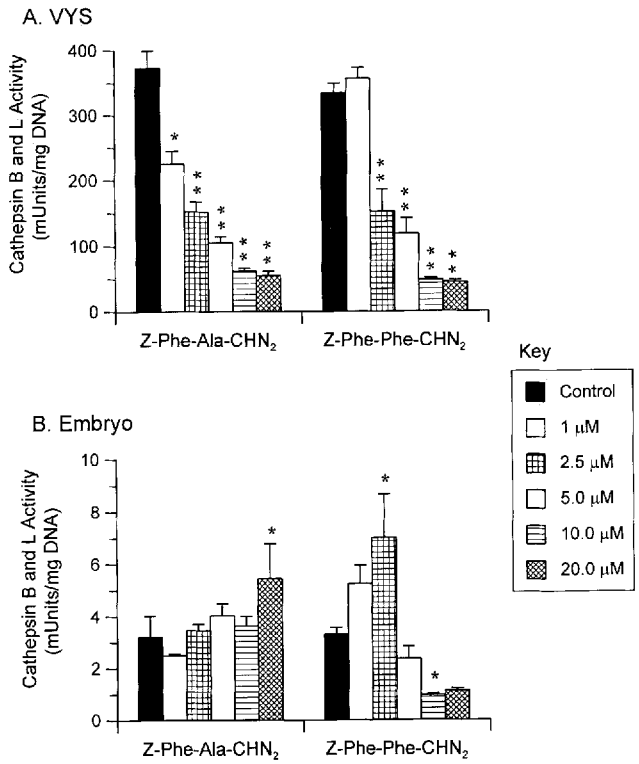

Fig. 4. Effect of 26-hour conceptal exposure to Z-Phe-Ala-CHN_2 or Z-Phe-Phe-CHN_2 on lysosomal cysteine proteinase activity. VYS (**A**) and embryo (**B**) tissue extracts. Columns represent means ± SEM from "n" samples listed in Tables 1 and 2. *Significantly different from control, $P < 0.05$. **Significantly different from control, $P < 0.001$.

the VYS endoderm epithelium, a concentration-dependent increase in both the size and number of cytoplasmic vacuoles was observed (Fig. 5). These vacuoles contained material which stained pink (eosinophilic) with H&E, and is presumably undegraded protein accumulating in lysosomes. This staining became increasingly more abundant and intense as the concentration of Z-Phe-Ala-CHN_2 was increased. The cytoplasm of the VYS epithelium was also distended and the nuclei marginalized, apparently as a result of the vacuolar expansion occurring in the apical region of these cells.

Histologic examination of transverse sections taken from the corresponding embryos revealed extensive cell death in the neuroepithelium, craniofacial mesenchyme, otic vesicles, and somites (Fig. 6) which also increased in a clear, concentration-dependent manner. Under the light microscope, morphologic changes suggestive of apoptosis were evident, including nuclear condensation, cytoplasmic blebbing, and the presence of presumptive apoptotic bodies in the cytoplasm of mesenchyme and neuroepithelial cells. When the number of cells showing evidence of apoptosis was counted per high power field, all inhibitor-treated groups were found to be increased over controls (data not shown). A pronounced reduction in the thickness of the neuroepithelium of Z-Phe-Ala-CHN_2-treated embryos was noted. Cell death appeared to be occurring throughout the neural tube, but was localized in specific regions of the mesenchyme such as the branchial arches, the primordia of the cranial nerves, and areas near the optic vesicles and the nasal placodes. In some embryos treated with higher concentrations of inhibitor, a complete absence of mesenchymal cells was observed in the rostral forebrain region, associated with blistering of the overlying surface ectoderm. These embryos also lacked any indication of optic vesicle formation.

The selectivity of Z-Phe-Ala-CHN_2-induced cell death could be appreciated by its absence in many areas of the embryonic mesenchyme and in certain other tissues, such as the heart, primitive gut, and VYS. Even at concentrations of Z-Phe-Ala-CHN_2 which severely damaged much of the neuroepithelium, somites, and cranial neural crest cells, little or no evidence of cell death was observed in these other tissues. Close examination of the regions of cell death revealed apparently phagocytic cells containing two types of cytoplasmic vacuoles-dense basophilic granules with the

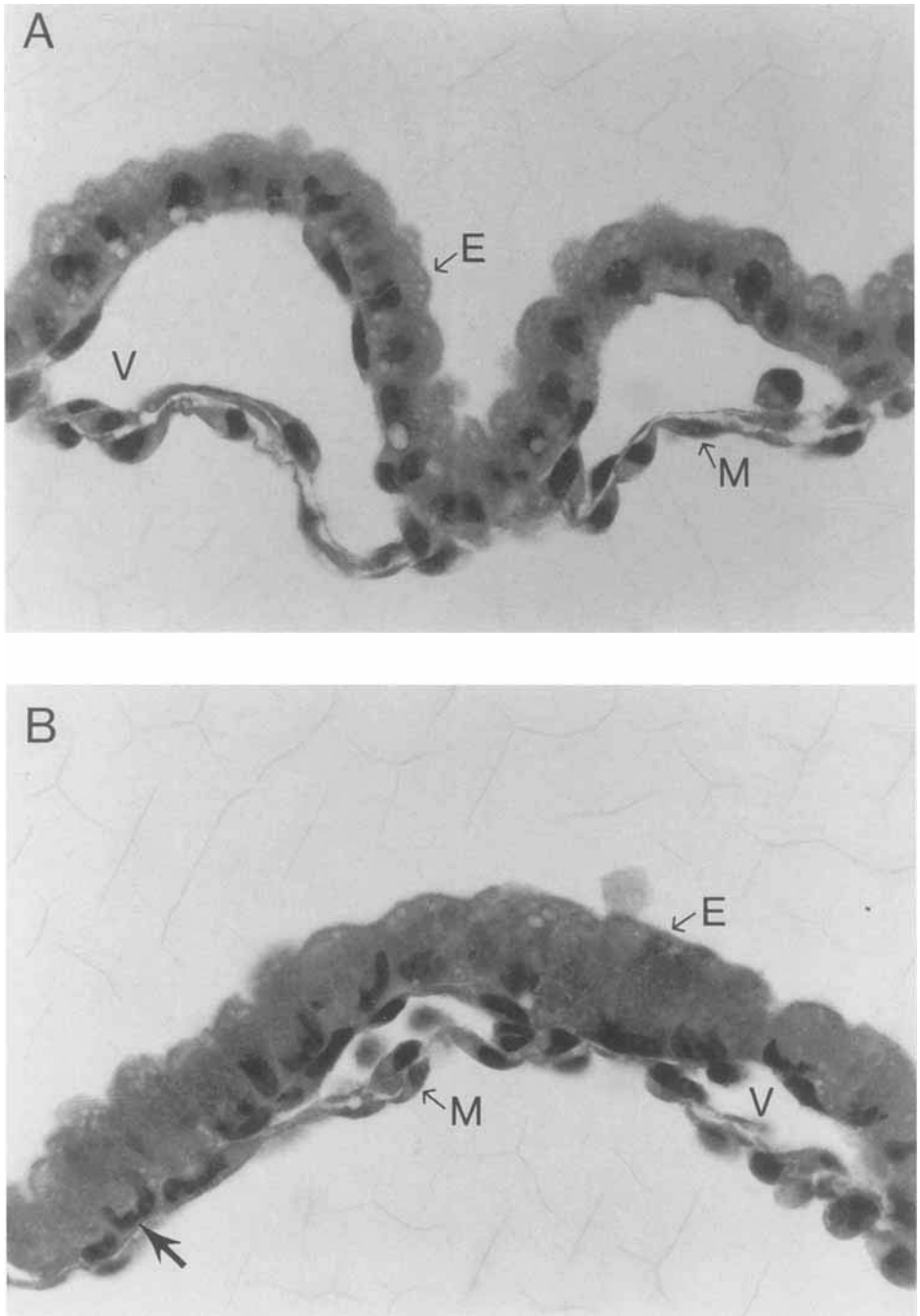

Fig. 5. Z-Phe-Ala-CHN_2 alters VYS histology. **A:** Transverse section of a control VYS. The cytoplasm of the endoderm epithelium (E) contains many small absorptive vacuoles that appear clear or stain faint pink with H&E. M = mesothelial layer; V = vitelline vasculature. ×600. **B:** Similar section of a 5 μM Z-Phe-Ala-CHN_2-treated VYS. Note reduced size of the vitelline vasculature, and distension of the epithelial cytoplasm. The epithelial nuclei (arrow) have been marginalized by large cytoplasmic vacuoles containing material that stains bright pink (eosinophilic) with H&E. ×600.

characteristic appearance of apoptotic bodies and eosinophilic vacuoles reminiscent of those observed in the VYS epithelium (Fig. 7). Figure 7 also shows massive cell death occurring in the trigeminal ganglion primordia of an inhibitor-treated embryo which has the morphologic appearance of apoptosis.

The induction of apoptosis in embryos treated with Z-Phe-Ala-CHN_2 was further evaluated by supravital

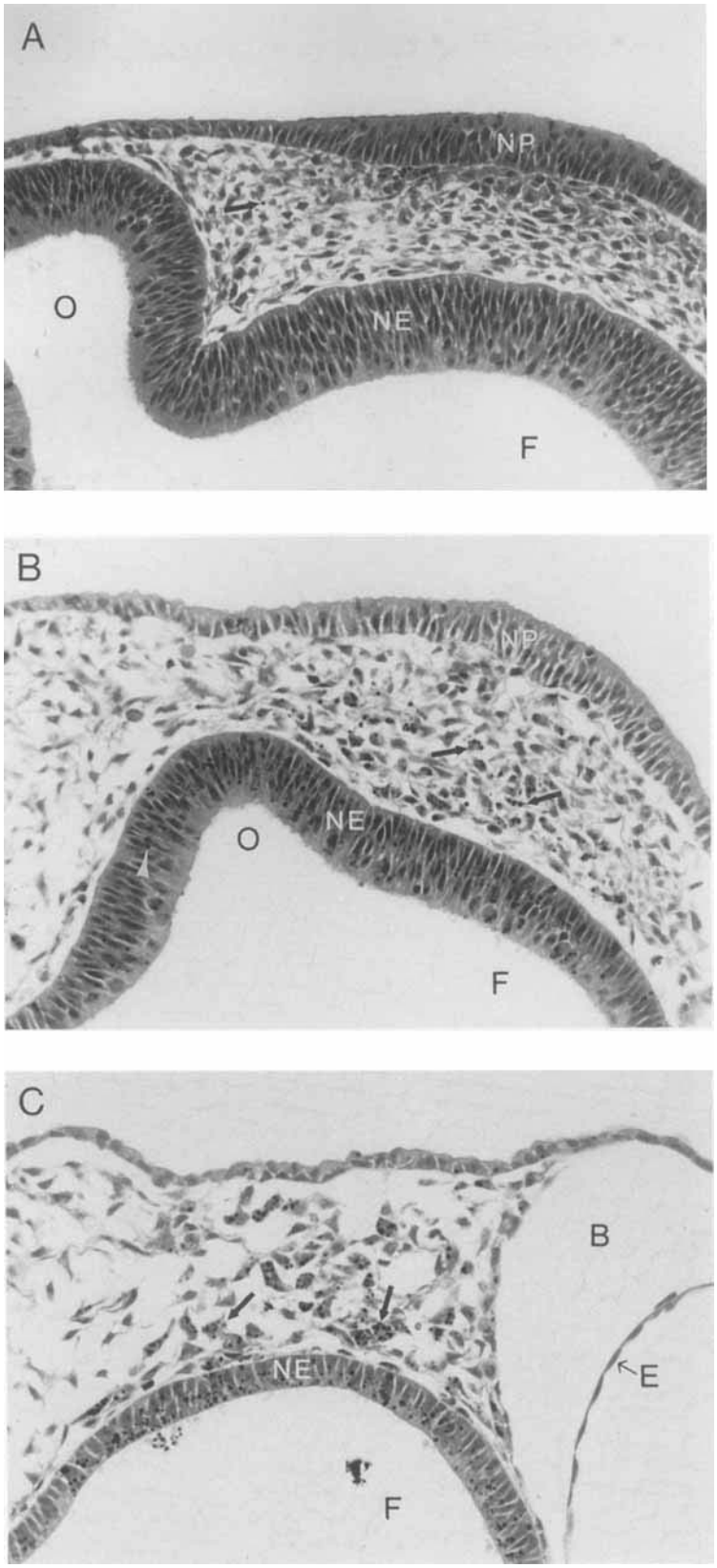

Fig. 6. Effects of Z-Phe-Ala-CHN_2 exposure on embryo histology. **A:** Transverse section of a control embryo forebrain (F) and optic vesicle (O) from the level of Rathke's pouch. Occasional cells undergoing cell death (arrow) can be observed. NE = neuroepithelium; NP = nasal placode. H&E. ×240. **B:** Similar section as A from an embryo exposed to 2.5 μM Z-Phe-Ala-CHN_2. The optic vesicle is underdeveloped, and the thickness of the neuroepithelium is reduced. Increased cell death is observed in the neuroepithelium (white arrowhead) and in the mesenchyme (black arrows) between the optic vesicle and the nasal placode. ×240. **C:** Similar section as A from an embryo exposed to 10 μM Z-Phe-Ala-CHN_2. Note the complete absence of optic vesicle formation and massive cell death in the neuroepithelium and mesenchyme. The most rostral aspect of the prosencephalon is devoid of underlying mesenchyme, and a blister (B) is apparent in the surface ectoderm (E). Arrows indicate mesenchymal cells presumed to contain phagocytized cell debris. ×240.

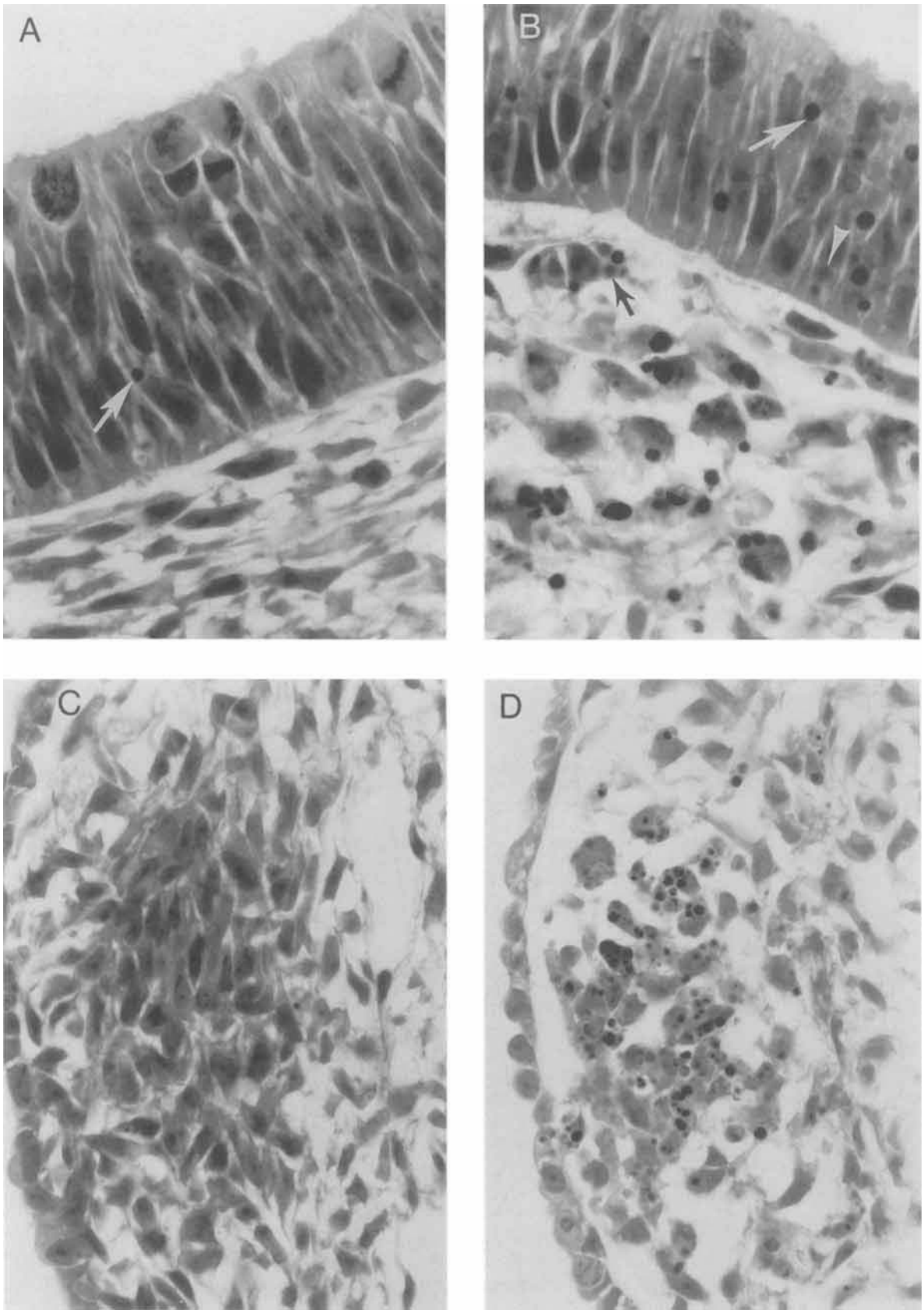

Fig. 7. Z-Phe-Ala-CHN_2 elicits selective cell death in the craniofacial mesenchyme. **A:** Photomicrograph of a control neuroepithelium and mesenchyme on GD 11.5. The area shown is just ventral to the optic vesicle. Occasional evidence of cell death can be observed (arrow). H&E. ×980. **B:** Similar section as A from a 2.5 μM Z-Phe-Ala-CHN_2-treated embryo. Note excessive cell death in both the neuroepithelium and underlying mesenchyme. Many cells contain inclusions which fit a morphologic description of apoptotic bodies (arrows), and stain with hematoxylin. Additional cytoplasmic vacuoles, containing eosinophilic material, can also be found in the same cells (arrowhead). ×980. **C:** Histology of control dorsal trigeminal neural crest. Again, occasional cell death can be observed (arrow). ×600. **D:** Similar section as C from an embryo exposed to 5.0 mM Z-Phe-Ala-CHN_2. Massive cell death is observed, with apparent phagocytosis. These alterations were not observed in adjacent populations of mesenchymal cells. ×600.

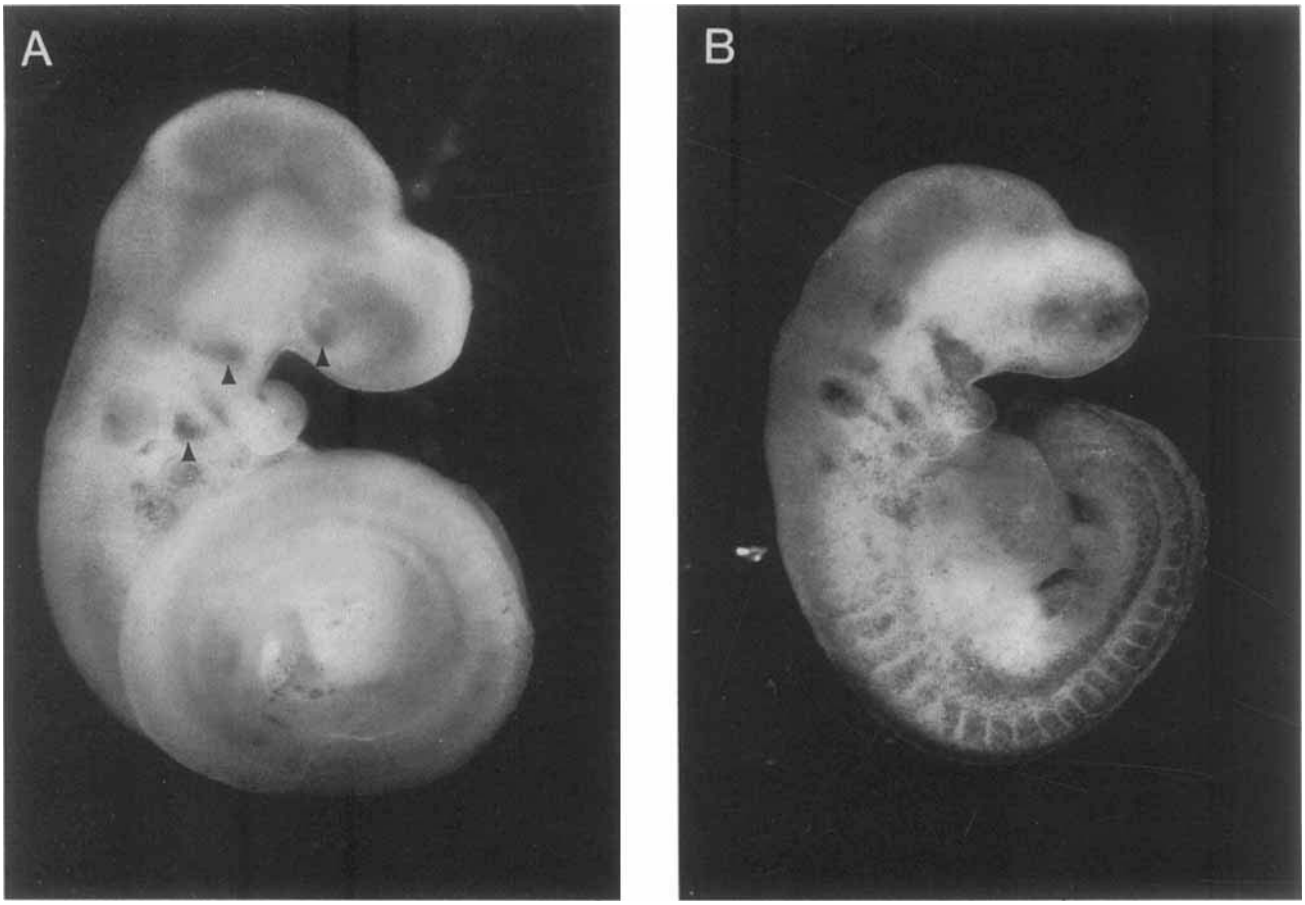

Fig. 8. NBS staining of GD 11.5 embryos, showing sites of cell death. **A:** Darkfield micrograph of a control embryo which shows staining in characteristic areas, such as the choroid fissure of the optic vesicle and cranial nerve primordia (arrowheads). The nasal placode is out of the plane of focus but also shows some staining. ×30. **B:** Embryo exposed to 2.5 μM Z-Phe-Ala-CHN_2, also stained with NBS. The cranial nerves, nasal placode, otic vesicle, and somites are particularly sensitive and are clearly delineated in this embryo, while the heart is relatively free of staining. ×30.

staining with NBS, a classic technique used to evaluate embryonic cell death. NBS has been found to stain regions of normal programmed cell death (PCD) and also chemical-induced cell death in embryos from a variety of species (reviewed by Saunders, '66; Menkes et al., '70; Sulik et al., '88). In controls exposed to DMSO vehicle alone, NBS staining was observed in areas of the embryo where normal PCD has been described, such as the nasal placode, cranial nerves, and the choroid fissure of the optic vesicle. In contrast, embryos treated with Z-Phe-Ala-CHN_2 exhibited a concentration-related increase in the area and intensity of NBS staining compared to that observed in controls (Fig. 8). Leupeptin treatment was also observed to increase the distribution and intensity of NBS staining in embryos after similar exposure regimens (data not shown).

DISCUSSION

We have provided biochemical and histological evidence indicating that Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 inhibit VYS proteolysis in organogenesis-stage conceptuses in vitro. VYS cathepsin B and L activity in conceptuses treated with the inhibitors decreased in a concentration-dependent fashion and appeared to correlate with other indicators of embryotoxicity such as decreased embryonic protein content, DNA content, and crown-rump length. Although Daston et al. ('91) and Grubb et al. ('91) have measured constitutive lysosomal cysteine proteinase activity in GD 12.5 conceptuses, we believe that the present study is the first to demonstrate inhibition of this activity in conceptuses exposed to proteinase inhibitors. It should be noted, however, that these measurements of enzyme activity were made at the end of the culture period, and may not reflect the degree of inhibition achieved at earlier time points or the total amount of enzyme present (but rather only active enzyme). Because of the ability of rat conceptuses to upregulate lysosomal cysteine proteinase activity following exposure to reversible inhibitors (Ambroso and Harris, '94), we would predict that in

conceptuses treated with peptidyl diazomethanes, VYS cathepsin B and L activity might be inhibited by an even greater extent at earlier time points, and thus toxicity may correlate better with the duration rather than extent of inhibition.

A characteristic finding in conceptuses exposed to protease inhibitors such as leupeptin is an increase in VYS protein content, due to an accumulation of undegraded protein in the vacuoles of the VYS epithelium (Freeman and Lloyd, '83b, '86; Daston et al., '91). This change is observed even though the overall growth of the VYS (yolk sac diameter) is retarded by leupeptin treatment. In the present study, we observed only a small increase in VYS protein content in conceptuses exposed to 2.5 μM Z-Phe-Ala-CHN_2 after a 26 hour exposure period, although the pattern of changes observed in VYS DNA and protein content in response to peptidyl diazomethane treatment was suggestive of inhibition of VYS proteolysis. If the serum content of the medium was increased, however, more obvious increases in VYS protein content were observed. This was also true if Z-Phe-Ala-CHN_2 exposure was limited to only the final 6 hours of culture (as in Freeman and Lloyd, '83b), rather than the full 26 hour period. Thus, the ability of Z-Phe-Ala-CHN_2 (and most likely Z-Phe-Phe-CHN_2) to increase VYS protein content in vitro is dependent upon both the protein content of the medium and the exposure time. The lack of a dramatic increase in VYS protein content in our initial studies is at least partly due to the lower serum content of our culture medium (33%) compared with previous studies (which typically used 50–100% serum), and it appears that shorter exposure periods may also contribute to the more dramatic increases in protein content observed in some cases (Freeman and Lloyd, '83b, '86). Ibbotson and Williams ('79) have shown that fluid-phase pinocytosis in cultured GD 17.5 VYSs is increased in serum-free medium, but it is unknown whether this phenomenon is a factor in our experiments.

Although VYS protein content in Z-Phe-Ala-CHN_2-treated conceptuses showed a biphasic response, a concentration-dependent accumulation of eosinophilic material (presumably undegraded protein) in vacuoles of the VYS epithelium was clearly apparent in histological sections prepared from these conceptuses. Such changes in VYS histology are consistent with changes observed in conceptuses exposed to other agents known to inhibit VYS proteolysis (Beck and Lowy, '82; Daston et al., '91; Hunter et al., '91). Protein/DNA ratios in the VYS were also found to be increased in all treatment groups. These data suggest that protein accumulation does occur in the vacuolar system of Z-Phe-Ala-CHN_2-treated VYSs and that the decrease of VYS protein content seen at higher concentrations is most likely due to decreased overall growth of the VYS.

The data presented above argue in favor of inhibition of VYS proteolysis and therefore disruption of histiotrophic nutrition as the likely cause of Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 embryotoxicity in vitro. This conclusion is further supported by recent experiments in which conceptuses exposed to Z-Phe-Ala-CHN_2 accumulated exogenously administered fluorescein-labeled proteins in their VYSs (Ambroso and Harris, unpublished observations). Thus the present study appears to support the contention that lysosomal cysteine proteinases are responsible for VYS proteolysis and are the target for leupeptin- and peptidyl diazomethane-induced embryotoxicity. Although no previous studies have distinguished between cathepsins B, H, and L during the critical period of early organogenesis, available evidence suggests that cathepsin H is not of primary importance for VYS proteolysis. First, cathepsin H is known to be relatively resistant to inhibition by leupeptin compared to cathepsins B and L (Barrett and Kirschke, '81); second, both Daston et al. ('91) and Grubb et al. ('91) found very low levels of cathepsin H activity in GD 12.5 VYSs. Our results further discount the role of cathepsin H as a primary factor in VYS proteolysis because the peptidyl diazomethanes have also been found to be poor inhibitors of this enzyme compared to cathepsins B and L (Kirschke et al., '80).

Distinctions have been made between cathepsin B and cathepsin L on the basis of the relative sensitivities of these enzymes to Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 (Kirschke et al., '80; Kirshke and Shaw, '81; Kirschke and Barrett, '87). The latter studies found cathepsin L to be inactivated much more rapidly than cathepsin B by both of these inhibitors. Unfortunately, the exposure times used in the present study are too long to allow us to make such distinctions for the rat VYS. Further studies with these inhibitors are underway which aim to clarify the roles of cathepsin B and cathepsin L in VYS proteolysis.

In addition to providing information about the identity of proteolytic enzymes involved in histiotrophic nutrition, the embryotoxic potential of peptidyl diazomethanes and related cysteine proteinase inhibitors is also of interest because of their possible use as therapeutic agents in animal and human disease. Remarkably, lysosomal cysteine proteinases have been identified in a number of human parasites and are believed to be important in their pathogenesis: *Plasmodium falciparum* (Rosenthal et al., '88), *Trypanosoma cruzi* (Cazzulo et al., '90), *Leishmania amazonensis* (Alfieri et al., '91), and *Clonorchis sinensis* (Song and Rege, '91), among others. Altered cellular production and activity of lysosomal cysteine proteinases have also been associated with tumor progression and metastatic potential (Sloane et al., '92; Lah et al., '92; van der Stappen et al., '91), as well as with arthritic lesions in both rodents and man (Van Noorden et al., '88; Huet et al., '92). In at least two of the examples listed above, cysteine proteinase inhibitors resembling peptidyl diazomethanes have been successful as therapeutic agents

in animal models of human disease (Van Noorden et al., '88; Rosenthal et al., '93).

A striking and unexpected histological observation was the extensive cell death occurring in embryonic tissues of conceptuses exposed to Z-Phe-Ala-CHN_2. Embryos recovered after the 26 hour culture period showed evidence of massive apoptosis in the neuroepithelium and in selective regions of the craniofacial mesenchyme. Follow-up experiments demonstrated that embryos exposed to Z-Phe-Ala-CHN_2 stained intensely with NBS in areas where histological evidence of excessive cell death had been observed. The somites and the cranial nerve primordia appeared to be particularly susceptible, while other tissues such as the heart were relatively resistant. If one accepts the premise that chemical insult expands areas of normal PCD (Sulik et al., '88), then the resistance of the rat heart to cell death in the present study can be explained by the fact that normal PCD does not occur in this tissue until about GD 14 (Pexieder, '75). The GD 11 rat heart has also been noted to be resistant to heat shock-induced cell death (Mirkes, '85).

Although cell death is a common response of embryonic tissues to a variety of teratogenic insults, it has not previously been reported in conceptuses exposed to proteolytic enzyme inhibitors. Daston et al. ('91) observed a decrease in neural tube volume in GD 12 rat embryos exposed to leupeptin for 48 hours, but made no mention of cell death. This appears to contradict our observation that leupeptin treatment also increases NBS staining in GD 11 embryos. It has been noted, however, that because of the rapid removal of apoptotic bodies by neighboring cells, the histologic appearance of apoptosis is transitory and not always readily observable microscopically (Bursch et al., '90; Kerr and Harmon, '91).

Sulik et al. ('88) and others have proposed that damage of the cranial neural crest may be responsible for craniofacial abnormalities observed in children and animals exposed to ethanol and retanoic acid in utero. There is a remarkable similarity between the pattern of NBS staining observed in Z-Phe-Ala-CHN_2-treated embryos and the migratory pathways of cephalic neural crest cells as determined by fate mapping experiments (Noden, '75; Tan and Morris-Kay, '86; Serbedzija et al., '92). This supports previous suggestions that the cranial neural crest, in addition to the neuroepithelium, is particularly vulnerable to chemical insult near the time of neural tube closure. As pointed out by Sulik et al. ('87), however, cells derived from the ectodermal placodes of the visceral arches (ganglionic placodes) also populate the distal regions of the cranial nerves and may be the primary targets in cases where the exposure occurs near the end of neural crest migration (GD 9 in the mouse). Our exposure period appears to approximate GD 8.5–9.5 in the mouse, and might be predicted to affect both neural crest and ectodermal placode derivatives. The pattern of cell death observed in our embryos would seem to confirm this, since cells in both the proximal and distal portions of the trigeminal nerve were affected as well as cells in the visceral arch mesenchyme (which migrate earlier than the ganglionic placode cells).

At present, we can only speculate about how Z-Phe-Ala-CHN_2, a compound thought to be extremely selective in terms of its inhibitory effects and reactivity, might cause cell death in our embryos. One could easily envision that apoptosis might result from an interruption of histiotrophic nutrition, since physiological changes such as growth factor withdrawal or vitamin deficiency have previously been reported to induce apoptotic cell death in developing tissues (Menkes et al., '70; Barres et al., '92; Pesce et al., '93). A particularly interesting hypothesis is that disruption of normal nutritional pathways might lead to a decrease in trophic factors which normally suppress apoptosis. Certain cells, such as the migrating neural crest, may be particularly susceptible because of their dependence on environmental factors for survival and differentiation (reviewed by Stemple and Anderson, '93).

An alternative explanation for the observed cell death is that Z-Phe-Ala-CHN_2 alkylates indiscriminately, and is therefore directly cytotoxic to embryonic cells. This hypothesis seems unlikely because of the specificity of peptidyl diazomethylketones, which act as affinity labels for lysosomal cysteine proteinases and are relatively non-reactive toward nucleophiles other than the active site thiol of these enzymes. Other thiols (such as DTT and glutathione) do not react significantly with these compounds, and neither do the alcohols present in the active site of serine proteases (reviewed by Shaw, '90). One might also argue that the inhibitor does not reach target sites in the embryo proper, since Z-Phe-Ala-CHN_2 treatment did not inhibit cathepsin activity in embryonic tissues. Any inhibition of enzyme activity in embryonic tissues may be masked, however, by the adaptive response described in our previous study (Ambroso and Harris, '94). In that study, the embryo was found to be particularly responsive in terms of its ability to upregulate lysosomal cysteine proteinase activity. Thus, without measuring enzyme content, we cannot state with certainty that the inhibitor fails to reach embryonic tissues. Indeed, one of our speculations is that there *are* direct effects of the inhibitor on the embryo proper, in addition to the indirect effects mediated through the VYS. This is mainly a result of the observation of large, intensely stained eosinophilic vacuoles in cells associated with areas of cell death in inhibitor-treated embryos. While such an effect could be due to a normal increase in lysosomal enzymes seen in response to cell death (Ballard and Holt, '68), it may indicate that the lysosomal degradation of cell debris is inhibited by Z-Phe-Ala-CHN_2. If this is the case, apoptosis may be more apparent histologically because of slowed clearance. Clearly, further experimentation is required be-

fore the ultimate cause of embryonic cell death in Z-Phe-Ala-CHN_2-treated conceptuses is ascertained.

In summary, our results indicate that the peptidyl diazomethanes are potent in vitro embryotoxicants and strong candidates for in vivo teratogens. In addition to causing dramatic growth retardation and dysmorphogenesis in organogenesis-stage rat conceptuses grown in whole embryo culture, Z-Phe-Ala-CHN_2 treatment produces at least two characteristic histological changes that have frequently been associated with exposure to teratogenic agents: 1) an increase in the size and number of cytoplasmic vacuoles in the VYS endoderm epithelium (resembling lysosomal storage disease), and 2) selective cell death in the embryo proper. The peptidyl diazomethanes appear to act primarily by disrupting VYS proteolysis, which is rate-limited by the activity of lysosomal cysteine proteinases. Further experiments with the peptidyl diazomethanes may lead to more comprehensive characterization of these enzymes in early organogenesis-stage conceptuses. These findings also suggest that the therapeutic use of cysteine proteinase inhibitors should be critically evaluated with regard to their developmental toxicology.

ACKNOWLEDGMENTS

We are indebted to Sara Jane Carlson, Roongrudee Hiranruengchok, Bjorn Thorsrud, and Eunyoung Lee-Kim for technical assistance. Special thanks to the University of Michigan Reproductive Sciences Morphology Core for preparation of histological sections and to Dr. A. Kent Christensen, Linda Ambroso, and Joseph Randall for editing assistance. This work was part of a dissertation submitted by J.L.A. in partial fulfillment of the requirements for a Ph.D. in the Horace H. Rackham School of Graduate Studies in the University of Michigan and was supported by NIH grants ES 05235 and ES 07062, and the March of Dimes Birth Defects Foundation grant 15-179.

LITERATURE CITED

Alfieri, S.C., E.M. Pral, E. Shaw, C. Ramazeilles, and M. Rabinovitch (1991) *Leishmania amazonensis:* Specific labeling of amastigote cysteine proteinases by radioiodinated N-benzyloxycarbonyl-tyrosl-alanyl diazomethane. Exp. Parasitol., *73:*424–432.

Ambroso, J.L., and C. Harris (1994) Chloroquine accumulation and alterations in pinocytosis and proteolysis in the rat conceptus in vitro. Biochem. Pharmacol., *47:*679–688.

Ballard, K.J., and S.J. Holt (1968) Cytological and cytochemical studies on cell death and digestion in the fetal rat foot: The role of macrophages and hydrolytic enzymes. J. Cell Sci., *3:*245–262.

Barres, B.A., I.K. Hart, H.S.R. Coles, J.F. Burne, J.T. Voyvodic, W.D. Richardson, and M.C. Raff (1992) Cell death and control of cell survival in the oligodendrocyte lineage. Cell, *70:*31–46.

Barrett, A.J., and H. Kirschke (1981) Cathepsin B, cathepsin H, and cathepsin L. Methods Enzymol., *80:*535–561.

Beck, F., and A. Lowy (1982) The effect of cathepsin inhibitor on rat embryos grown in vitro. J. Embryol. Exp. Morphol., *71:*1–9.

Beckman, D.A., J.E. Pugarelli, M. Jensen, T.R. Koszalka, R.L. Brent, and J.B. Lloyd (1990) Sources of amino acids for protein synthesis during early organogenesis in the rat. I. Relative contributions of free amino acids and of proteins. Placenta, *11:*109–121.

Beckman, D.A., J.E. Pugarelli, M. Jensen, T.R. Koszalka, R.L. Brent, and J.B. Lloyd (1991) Sources of amino acids for protein synthesis during early organogenesis in the rat. II. Exchange with amino acid and protein pools in embryo and yolk sac. Placenta, *12:*37–46.

Bradford, M.M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein using the principal of protein-dye binding. Anal. Biochem., *72:*248–254.

Brent, R.L., D.A. Beckman, M. Jensen, and T.R. Koszalka (1990) Experimental yolk sac dysfunction as a model for studying nutritional disturbances in the embryo during early organogenesis. Teratology, *41:*405–413.

Bursch, W., S. Paffe, B. Putz, G. Barthel, and R. Schulte-Hermann (1990) Determination of the length of the histological stages of apoptosis in normal liver and in altered hepatic foci of rats. Carcinogenesis, *11:*847–853.

Cazzulo, J.J., M.C.C. Franke, J. Martinez, and B.M. Franke de Cazzulo (1990) Some kinetic properties of cysteine proteinase (cruzipain) from *Trypanosoma cruzi.* Biochim. Biophys. Acta, *1037:*186–191.

Crawford, C.R., W. Mason, P. Wikstrom, and E. Shaw (1988) The design of peptidyl diazomethane inhibitors to distinguish between the cysteine proteinases calpain II, cathepsin L and cathepsin B. Biochem. J., *253:*751–758.

Daston, G.P., D. Baines, J.E. Yonker, and L.D. Lehman-Mckeeman (1991) Effects of lysosomal proteinase inhibition on the development of the rat embryo in vitro. Teratology, *43:*253–261.

Fantel, A.G., J.C. Greenaway, M.R. Juchau, and T.H. Shepard (1979) Teratogenic bioactivation of cyclophosphamide in vitro. Life Sci., *25:*67–72.

Freeman, S.J., and N.A. Brown (1985) Comparative effects of cathepsin inhibitors on rat embryonic development in vitro. Evidence that cathepsin D is unimportant in the proteolytic function of yolk sac. J. Embryol. Exp. Morphol., *86:*271–281.

Freeman, S.J., and J.B. Lloyd (1983a) Evidence that protein ingested by the rat visceral yolk sac yields amino acids for synthesis of embryonic protein. J. Embryol. Exp. Morphol., *73:*307–315.

Freeman, S.J., and J.B. Lloyd (1983b) Inhibition of proteolysis in rat yolk sac as a cause of teratogenesis. Effects of leupeptin in vitro and in vivo. J. Embryol. Exp. Morphol., *78:*183–193.

Freeman, S.J., and J.B. Lloyd (1986) Evidence that suramin and sodium aurothiomalate are teratogenic in rat by disturbing yolk sac-mediated embryonic protein nutrition. Chem. Biol. Interact., *58:* 149–160.

Freeman, S.J., F. Beck, and J.B. Lloyd (1981) The role of the visceral yolk sac in mediating protein utilization by rat embryos cultured in vitro. J. Embryol. Exp. Morphol., *66:*223–234.

Green, G.D.J., and E. Shaw (1981) Peptidyl diazomethyl ketones are specific inactivators of thiol proteinases. J. Biol. Chem., *256:*1923–1928.

Grubb, J.D., T.R. Koszalk, J.J. Drabick, and R.M. Metrione (1991) The activities of thiol proteases in the rat visceral yolk sac increase during late gestation. Placenta, *12:*143–151.

Hanada, K., M. Tamai, M. Yamagishi, S. Ohmura, J. Sawada, and I. Tanaka (1978) Studies on thiol protease inhibitors. I. Isolation and characterization of E-64, a new thiol proteinase inhibitor. Agric. Biol. Chem., *42:*523–528.

Harris, C., K.L. Stark, and M.R. Juchau (1988) Glutathione status and the incidence of neural tube defects elicited by direct acting teratogens in vitro. Teratology, *37:*577–590.

Huet, G., R.M. Flipo, C. Richet, C. Thiebaut, D. Demeyer, M. Balduyck, B. Duquesnoy, and P. Degand (1992) Measurement of elastase and cysteine proteinases in synovial fluid of patients with rheumatoid arthritis, sero-negative spondylarthropathies, and osteoarthritis. Clin. Chem., *38:*1694–1697.

Hunter, E.S., III, L.S. Phillips, S. Goldstein, and T.W. Sadler (1991) Altered visceral yolk sac function produced by a low-molecular-weight somatomedin inhibitor. Teratology, *43:*331–340.

Ibbotson, G.E., and K.E. Williams (1979) Rate of pinocytic capture of macromolecular substrates by rat yolk sac incubated in serum-free culture medium. Biochem. J., *178:*785–792.

Ishii, S., and K. Kasai (1981) Affinity methods using argininal derivatives. Methods Enzymol., *80:*842–848.

Kerr, J.F.R., and B.V. Harmon (1991) Definition and incidence of apoptosis: An historical perspective. In: Apoptosis: The Molecular Basis of Cell Death. D. Tomei and F. Cope, eds. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 5–29.

Kirschke, H., and A.J. Barrett (1987) Chemistry of lysosomal proteases. In: Lysosomes: Their Role in Protein Breakdown. H. Glaumann and F.J. Ballard, eds. Academic Press, London, pp. 193–238.

Kirschke, H., and E. Shaw (1981) Rapid inactivation of cathepsin L by Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2. Biochem. Biophys. Res. Commun., *101:*454–458.

Kirschke, H., J. Langner, S. Riemann, B. Wiederanders, S. Ansorge, and P. Bohley (1980) Lysosomal cysteine proteinases. In: Protein Degradation in Health and Disease. D. Evered and J. Whelan, eds. Ciba Foundation Symposium. Excerpta Medica, Amsterdam, Vol. 75, pp. 15–35.

Knowles, L.E., and F.J. Ballard (1981) Effects of microbial proteinase inhibitors on the degradation of endogenous and internalized proteins by rat yolk sacs. Biochem. J., *196:*41–48.

Labarca, L., and K. Paigen (1980) A simple, rapid and sensitive DNA assay procedure. Anal. Biochem., *102:*344–352.

Lah, T.T., M. Kokalj-Kunovar, M. Drobnic-Kosorok, J. Babnik, R. Golouh, I. Vrhovec, and V. Turk (1992) Cystatins and cathepsins in breast carcinoma. Biol. Chem. Hoppe-Seyler, *373:*595–604.

Leary, R., D. Larsen, H. Wantanabe, and E. Shaw (1977) Diazomethyl ketone substrate derivatives as active-site-directed inhibitors of thiol proteases. Papain. Biochemistry, *16:*5857–5861.

Mason, R.W., D. Wilcox, P. Wikstrom, and E. Shaw (1989) The identification of active forms of cysteine proteinases in Kirsten-virus-transformed mouse fibroblasts by use of a specific radiolabelled inhibitor. Biochem. J., *257:*125–129.

Menkes, B., S. Sandor, and A. Ilies (1970) Cell death in teratogenesis. In: Advances in Teratogenesis. D.H. Woolham, ed. Academic Press, New York, Vol. 5, pp. 169–215.

Mirkes, P.E. (1985) Effects of acute exposures to elavated temperatures on rat embryo growth and development in vitro. Teratology, *32:*259–266.

Noden, D. (1975) An analysis of the migratory behavior of avian cephalic neural crest cells. Dev. Biol., *42:*106–130.

Parkes, C., A.A. Kembhavi, and A.J. Barrett (1985) Calpain inhibition by peptide epoxides. Biochem. J., *230:*509–516.

Pesce, M., M.G. Farrace, M. Piacentini, S. Dolci, and M.D. Felici (1993) Stem cell factor and leukemia inhibitory factor promote primordial germ cell survival by suppressing programmed cell death (apoptosis). Development, *118:*1089–1094.

Pexieder, T. (1975) Cell death in the morphogenesis and teratogenesis of the heart. Adv. Anat. Embryol. Cell Biol., *51:*6–99.

Rich, D.H. (1986) Inhibitors of cysteine proteinases. In: Proteinase Inhibitors. A.J. Barrett and Salvesen, eds. Elsevier, Amsterdam, pp. 153–178.

Rosenthal, P.J., J.H. McKerrow, M. Aikawa, H. Nagasawa, and J.H. Leech (1988) A malarial cysteine proteinase is necessary for hemoglobin degradation by *Plasmodium falciparum*. J. Clin. Invest., *82:* 1560–1566.

Rosenthal, P.J., G.K. Lee, and R.E. Smith (1993) Inhibition of a *Plasmodium vinckei* cysteine proteinase cures murine malaria. J. Clin. Invest., *91:*1052–1056.

Rowe, P.B., and A. Kalaizis (1985) Serine metabolism in rat embryos undergoing organogenesis. J. Embryol. Exp. Morphol., *87:*137–144.

Saunders, J.W. (1966) Death in embryonic systems. Science, *154:*604–612.

Seegmiller, R.E., C. Harris, D.L. Luchtel, and M.R. Juchau (1991) Morphological differences elicited by two weak acids, retanoic and valproic, in rat embryos grown in vitro. Teratology, *43:*133–150.

Serbedzija, G.N., M. Bronner-Fraser, and S.E. Fraser (1992) Vital dye analysis of cranial neural crest cell migration in the mouse embryo. Development, *116:*297–307.

Shaw, E. (1990) Cysteinyl proteinases and their selective inactivation. Adv. Enzymol., *63:*271–347.

Sloane, B.F., J. Rozhin, K. Moin, G. Ziegler, D. Fong, and R.J. Muschel (1992) Cysteine endopeptidases and their inhibitors in malignant progression of rat embryo fibroblasts. Biol. Chem. Hoppe-Seyler, *373:*589–594.

Song, C.Y., and A.A. Rege (1991) Cysteine proteinase activity in various developmental stages of *Clonorchis sinensis:* A comparative analysis. Comp. Biochem. Physiol., *99B:*137–144.

Stemple, D.L., and D.J. Anderson (1993) Lineage diversification of the neural crest: In vitro investigations. Dev. Biol., *159:*12–23.

Sulik K.K., M.C. Johnston, S.J. Smiley, H.S. Speight, and B.E. Jarvis (1987) Mandibulofacial dystosis (Treacher Collins syndrome): A new proposal for its pathogenesis. Am. J. Med. Genet., *27:*359–372.

Sulik, K.K., C.S. Cook, and W.S. Webster (1988) Teratogens and craniofacial malformations: relationships to cell death. Development *103(Suppl.):*213–232.

Tan, S.S., and G.M. Morriss-Kay (1986) Analysis of cranial neural crest cell migration and early fates in postimplantation rat chimeras. J. Embryol. Exp. Morphol., *98:*21–58.

Umezawa, H. (1982) Low-molecular-weight enzyme inhibitors of microbial origin. Annu. Rev. Microbiol., *36:*75–99.

van der Stappen, J.W.J., C. Paraskeva, A.C. Williams, A. Hague, and R.A. Maciewicz (1991) Relationships between the secretion of cysteine proteinases and their inhibitors and malignant potential. Biochem. Soc. Trans., *19:*362S.

Van Noorden, C.J.F., R.E. Smith, and D. Rasnick (1988) Cysteine proteinase activity in arthritic rat knee joints and the effects of a selective systemic inhibitor, Z-Phe-Ala-CH_2F. J. Rheumatol., *15:* 1525–1535.