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Comparative effects of cathepsin inhibitors on rat embryonic development *in vitro*. Evidence that cathepsin D is unimportant in the proteolytic function of yolk sac.

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SUMMARY

The effects of two proteinase inhibitors, leupeptin and pepstatin on the development of 9·5-day rat conceptuses *in vitro* has been studied. All cultures were of 48 h duration and the inhibitors were present throughout the entire period. When pepstatin was added to the culture medium (5–25 μg/ml) conceptuses developed and grew to an extent that did not differ from untreated controls. However, leupeptin (1–4 μg/ml) caused severe growth retardation and abnormal development of conceptuses.

The effects of the two inhibitors on the hydrolysis of ^{125}I-labelled BSA and haemoglobin by homogenates of 10·5-day yolk sac indicated the biochemical basis for the differential toxic effects of the two inhibitors on development. Leupeptin was highly inhibitory of the degration of both substrates whereas pepstatin caused no inhibition of ^{125}I-labelled BSA hydrolysis, and only a slight inhibition of haemoglobin hydrolysis.

These observations demonstrate that cathepsin D, a lysosomal aspartic proteinase that is specifically inhibited by pepstatin is not involved in yolk-sac-mediated protein utilization by early organogenesis-phase conceptuses and that lysosomal cysteine proteinases, specifically inhibited by leupeptin, are of paramount importance in this yolk sac function.

INTRODUCTION

In a series of papers, Freeman and Lloyd have described quantitative experiments that demonstrated the nutritional function of the visceral yolk sac of the cultured early organogenesis-stage rat conceptus and its disturbance by several teratogens. Exogenous proteins are captured by pinocytosis by the endodermal cells of the yolk sac and digested within the lysosomes of these cells to provide a source of amino acids for the rapidly growing and differentiating conceptus (Freeman, Beck & Lloyd, 1981; Freeman & Lloyd, 1983*a*). Teratogenic anti-visceral yolk sac antiserum and suramin were shown to inhibit the uptake of protein by yolk sac (Freeman, Brent & Lloyd, 1982; Freeman & Lloyd, 1985) while two other teratogens, leupeptin and sodium aurothiomalate, were demonstrated to inhibit intralysosomal proteolysis in yolk sac (Freeman & Lloyd, 1983*b*; Freeman

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& Lloyd, 1985). The overall effect of these actions was to diminish the supply of nutritive amino acids to the conceptus and this was proposed as the primary mechanism of teratogenic action of these four agents.

In the present paper, the effects of the proteinase inhibitors pepstatin and leupeptin on the *in vitro* development of the early organogenesis-stage rat conceptus have been compared. Like leupeptin, which has previously been shown to be teratogenic to rat embryos in culture (Beck & Lowy, 1982), pepstatin is a microbial peptide but differs from the former in being an inhibitor of a group of proteolytic enzymes collectively known as aspartic proteinases and represented in the lysosome by cathepsin D. Leupeptin specifically inhibits cysteine proteinases, including lysosomal cathepsins B, H and L (Umezawa, 1976). The effects of the two inhibitors on development are correlated with their actions on proteolysis by yolk sac.

MATERIALS AND METHODS

Chemicals

Leupeptin and pepstatin, bovine serum albumin (Fraction V) and bovine haemoglobin (Type II) were obtained from Sigma (Poole, Dorset, U.K.). [^{125}I] Iodine (prep. IMS 40) was purchased from Amersham International (Amersham, Bucks, U.K.) and ultrogel AcA 202 was from LKB (Bromma, Sweden).

Whole embryo culture experiments

Female rats of the Wistar-Porton strain from the laboratory colony were mated overnight with males of the same strain. The morning on which a vaginal plug was observed was designated day 1 of gestation. On gestation day 10 (embryonic age 9·5 days), conceptuses were explanted into Hanks balanced salt solution and early-to mid-headfold-stage embryos selected for culture by the roller culture technique of New (1978). All cultures were maintained for 48 h in sterilized 40 ml bottles in a medium comprising, per bottle, 3 ml immediately centrifuged heat-inactivated homologous serum and 1 ml Eagles minimal essential medium (MEM). Proteinase inhibitors were added to the culture in the MEM and were present for the entire 48 h culture period; pepstatin is water-insoluble and was therefore added as a methanolic solution, methanol being preferred over dimethylsulphoxide as the vehicle owing to the toxic nature of the latter in embryo culture (Kitchin & Ebron, 1984). Control cultures either contained added methanol (at a concentration, 25 μl/bottle, equivalent to that used in those cultures to which 25 μg/ml pepstatin had been added), or were untreated. At harvesting, the morphological development of conceptuses was analysed by the criteria of Brown & Fabro (1981) and the protein content of embryos determined (Lowry, Rosebrough, Farr & Randall, 1951).

Assays of proteolysis by yolk sac

Preparation of substrates

Two substrates were chosen to determine the inhibitory effects of pepstatin and leupeptin on proteolysis by yolk sac: ^{125}I-labelled bovine serum albumin (BSA) and bovine haemoglobin. BSA was radioiodinated by the chloramine-T procedure (Bolton, 1977). Radiolabelled protein was separated from free [^{125}I] iodine by chromatography on a column of Ultrogel AcA 202 in 0·9 % saline. Labelled protein was recovered in the void volume and less than 4 % of this radioactivity was soluble in 6·7 % (w/v) trichloroacetic acid (TCA). Specific activity of the labelled protein was approximately 450 μCi/mg.

Bovine haemoglobin was mixed to a paste with distilled water and dialysed overnight against distilled water. Following dialysis the haemoglobin solution was diluted with distilled water to a

final concentration of 8 % (w/v). Both substrates were stored at − 20 °C and remained stable over the 2 month period during which the assays were performed.

Preparation of yolk sacs

Yolk sacs were removed from explanted 10·5-day conceptuses. All the yolk sacs obtained from an experiment were pooled in ice-cold 0·9 % saline in batches of 10 yolk sacs/ml saline. Yolk sacs were then homogenized by pulse sonication using a Dawe Ultrasonic generator at a duty cycle of 30 % and an output of 2 W, and the homogenate centrifuged for 10 min using a microcentrifuge. Supernatants from all batches were pooled and used directly in the assay.

Assay procedure

The hydrolysis of ^{125}I-labelled BSA was determined at both pH 4·0 and pH 5·5. Assays were performed at 37 °C in plastic 1·5 ml vials, each of which contained 75 μl yolk sac homogenate (10–15 μg protein), 50 μl assay buffer (0·2 M-sodium acetate adjusted to desired pH with 0·2 M-acetic acid and containing 0·8 % Triton X–100 and 5mM-dithiothreitol), 50 μl of 2 % BSA containing a trace (50 000 c.p.m.) of ^{125}I-labelled BSA, and 25 μl distilled water in which inhibitors were added. Pepstatin was added as a methanolic solution (5 μl maximum per assay vial) and appropriate methanol-containing control assays were performed concurrently. Non-enzymic substrate hydrolysis was determined by including with each assay a vial containing all the reaction components except yolk sac homogenate which was added immediately before stopping the reaction. Reactions were stopped by the addition of 20 μl foetal calf serum and 1·0 ml 20 % (w/v) trichloroacetic acid (TCA) to each vial. After mixing, TCA-insoluble material was precipitated by centrifugation and, after removal of the supernatant, solubilized in 1·0 ml 20 % (w/v) KOH. The dissolved pellet (TCA-insoluble) and the supernatant (TCA-soluble) were then counted separately and the counts of each combined to give the total radioactivity. The extent of substrate hydrolysis was estimated by calculating the increase above the blank value in the percentage of the total radioactivity that was TCA-soluble.

The hydrolysis of haemoglobin was measured at 37 °C by spectrophotometric determination of tyrosine production according to the method of Barrett & Heath (1977). Each assay vial contained 75 μl yolk sac homogenate, 50 μl assay buffer (1·0 M-sodium formate-formic acid, pH 3·1, containing 0·8 % Triton X–100 and 5 mM-dithiothreitol), 50 μl 2·0 % haemoglobin and 25 μl distilled water, containing inhibitors. Reactions were stopped by the addition to each vial of 1·0 ml 3 % (w/v) TCA. The extent of haemoglobin hydrolysis was estimated by calculating the increase above blank values in the tyrosine concentration of the TCA-soluble portion. Initial assays of both ^{125}I-labelled BSA and haemoglobin hydrolysis were performed over a 2 h incubation period. Under the conditions described, product formation was linear over this time period.

RESULTS

Culture experiments

Table 1 compares the effects on embryonic development of the addition of either pepstatin or leupeptin to the culture medium. At all pepstatin concentrations tested, conceptuses grew and developed to an extent similar to that of conceptus cultured in the presence of the vehicle alone or in the absence of any additions. Measurements of crown–rump length, somite number, head length, yolk sac diameter and protein content of pepstatin-treated conceptuses showed no significant differences from either control groups ($P > 0·05$). Some abnormal embryos were observed in the groups treated with pepstatin/methanol or methanol alone but the numbers were very small (2 out of 15 in the methanol-treated group) and

Table 1. *Effects of added pepstatin or leupeptin on development of cultured embryos.*

	Pepstatin concentration				Leupeptin concentration		Untreated control (n=13)
	5 μg/ml (n=29)	10 μg/ml (n=19)	25 μg/ml (n=21)	MEOH control (n=15)	1 μg/ml (n=8)	4 μg/ml (n=9)	
Normal external appearance of embryo (%)	89·7	89·5	95·2	86·7	62·5	0	100
Yolk sac circulation (%)	93·1	94·7	100	86·7	87·5	11·11	100
Normal axial rotation (%)	89·7	89·5	95·2	86·7	75·0	0	100
Crown–rump length (mm)	4·02 ± 0·63	3·87 ± 0·47	3·91 ± 0·47	3·83 ± 0·62	3·49 ± 0·41†	1·98 ± 0·27*	4·22 ± 0·43
Somite Number	26·19 ± 1·79	24·60 ± 4·62	25·62 ± 2·16	25·86 ± 1·29	24·0 ± 2·56†	4·56 ± 4·16*	26·46 ± 0·97
Head length (mm)	2·22 ± 0·37	2·15 ± 0·31	2·18 ± 0·45	2·11 ± 0·37	1·91 ± 0·24*	0·88 ± 0·20*	2·35 ± 0·26
Yolk sac diameter (mm)	4·53 ± 0·69	4·45 ± 0·51	4·40 ± 0·58	4·81 ± 0·64	3·86 ± 0·33*	2·81 ± 0·34*	4·63 ± 0·40
Embryo protein content (μg)	232·40 ± 15·77	230·00 ± 22·01	257·33 ± 8·16	229·30 ± 24·72	124·38 ± 31·14*	39·44 ± 11·04*	206·86 ± 47·92

All crown–rump length, somite number, head length, yolk sac diameter and protein content were compared statistically with control values (right-hand column) by Student's t-test. Only those values indicated †($P<0·02$) and *($P<0·001$) were found to be significantly different from controls. Figures represent means ± standard deviation.

no dose–response relationships were apparent, suggesting that the anomalies were not attributable to the treatments.

In contrast with these observations, inclusion of leupeptin in cultures resulted in a high incidence of abnormally developed embryos. Measurements of all developmental parameters were significantly different from control values and the severity of the effects increased with an increased concentration of leupeptin. These effects of leupeptin treatment are very similar to those previously described (Beck & Lowy, 1982).

Proteolysis assays

In order to understand the biochemical basis for the differential effects of pepstatin and leupeptin on embryonic development, homogenates of yolk sac taken directly from the mother were examined for the presence or absence of pepstatin- and leupeptin-inhibitable proteolytic activity. Since the precise pH of the acidic lysosomal interior is not known, assays using ^{125}I-labelled BSA as substrate were

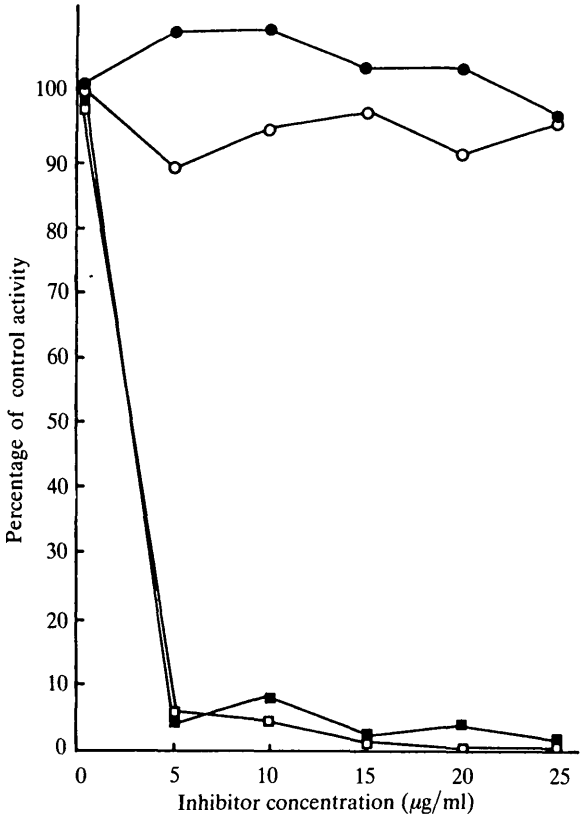

Fig. 1. Effect of pepstatin (circles) and leupeptin (squares) on the hydrolysis ^{125}I-labelled BSA by yolk sac at pH 4·0 (open symbols) and pH 5·5 (closed symbols). Results are expressed as a percentage of control values, which for pepstatin experiments were obtained by incubation in the presence of the methanol vehicle. Each point represents the mean of two separate experiments.

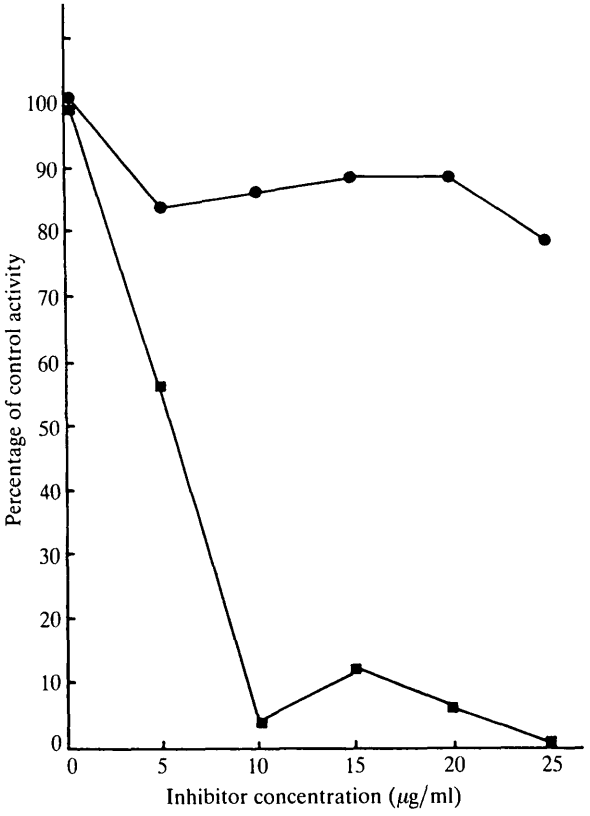

Fig. 2. Effect of pepstatin (●) and leupeptin (■) on the hydrolysis of haemoglobin by yolk sac at pH 3·1. Expression of results follows the same form as in Fig. 1. Each point represents the mean of two separate experiments.

conducted at pHs 4·0 and 5·5. Fig. 1 shows the effects of various concentrations of the inhibitors on the hydrolysis of ^{125}I-labelled BSA. At both pHs the results were qualitatively and quantitatively similar; leupeptin strongly inhibited substrate hydrolysis whereas pepstatin, over the concentration range tested, had no appreciable effect on proteolysis. At the lowest leupeptin concentration (5 μg/ml), less than 10 % of control activity was observed, and activity diminished further with increased leupeptin concentration.

To determine whether the lack of inhibition by pepstatin was confined to ^{125}I-labelled BSA as substrate, similar experiments were performed using haemoglobin as substrate. Haemoglobin hydrolysis was measured at the optimum pH of 3·1 (Barrett, 1967) only. Fig. 2 shows that a similar effect of the inhibitors on proteolysis was observed. Leupeptin induced a marked, concentration dependent, inhibition of substrate hydrolysis; pepstatin caused only a slight decrease in the extent of haemoglobin degradation, relative to controls.

In a subsequent experiment, using ^{125}I-labelled BSA as substrate, the assay period was extended to 24 h. It was considered that the 2 h incubation period employed initially might be of insufficient length to permit inhibition of proteolysis

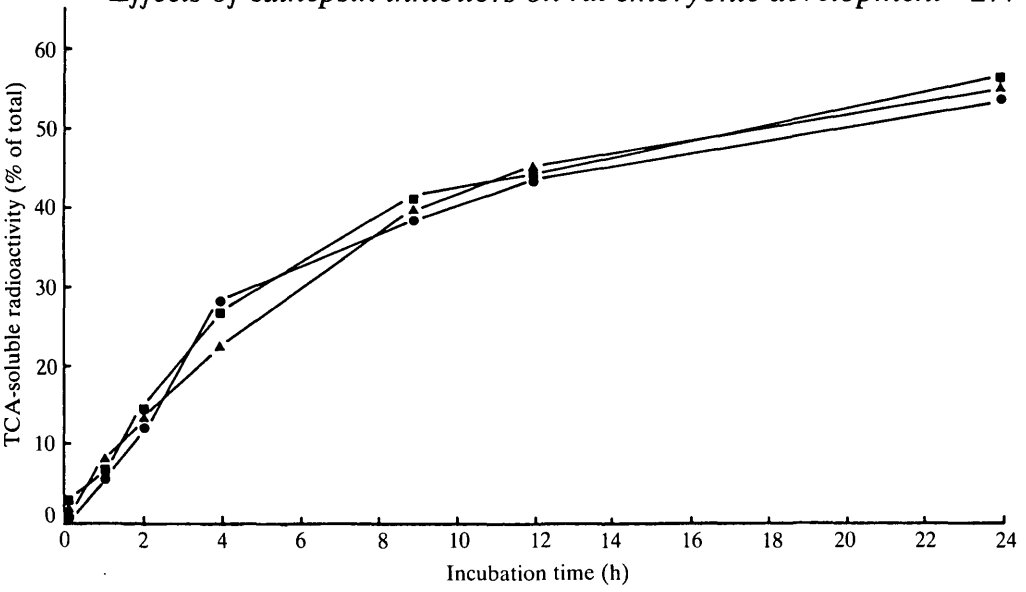

Fig. 3. Time course of the hydrolysis of ^{125}I-labelled BSA by yolk sac at pH 5·5 in the presence of pepstatin (●), methanol vehicle only (▲) and in the absence of either pepstatin or methanol (■). Results are expressed as the percentage of the total radioactivity (c.p.m.) that was converted into TCA-soluble form. Each point is the mean of two separate determinations.

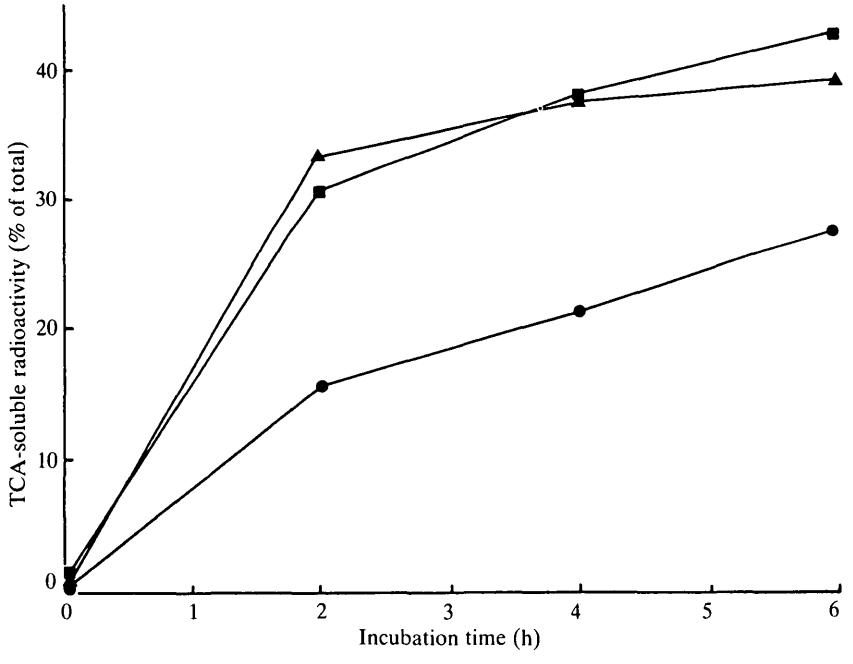

Fig. 4. Time course of hydrolysis of ^{125}I-labelled BSA at pH 5·5 by rat liver fraction in the presence of pepstatin (●), methanol vehicle only (▲) and in the absence of either pepstatin or methanol (■). Expression of results follows the same form as in Fig. 3. Each point is the mean of two separate determinations.

by pepstatin. Fig. 3 indicates that even over the extended assay time, pepstatin (25 μg/ml) did not decrease the extent to which the substrate was degraded. Incubations containing pepstatin, or the methanol vehicle alone, or without any additions, all showed similar levels of proteolysis.

Finally, to determine whether the preparation of pepstatin used in the experiments was able to inhibit proteolyis under the conditions of the assay, the hydrolysis of ^{125}I-labelled BSA by rat liver was studied. A 'light mitochondrial' fraction, enriched in lysosomes, was purified from fresh liver by differential centrifugation (Dean, 1977). The particles from this fraction were resuspended in hyptonic saline and pulse sonicated as described above for yolk sac. The residual solid material was precipitated by centrifugation for 10 min at 4 °C in a microcentrifuge and the supernatant was used in the assay.

Fig. 4 shows that hydrolysis of ^{125}I-labelled BSA by rat liver was inhibited by pepstatin (25 μg/ml). The extent of inhibition was between 40 % and 50 % over the period of the assay, relative to control values. The inclusion of methanol alone in the incubation mixture did not decrease substrate hydrolysis compared to control incubations containing no additions.

DISCUSSION

The embryopathic effect of the cysteine proteinase inhibitor leupeptin on rat conceptuses cultured *in vitro* has been reported previously (Beck & Lowy, 1982). In that study, abnormal embryogenesis was associated with a severe yolk sac pathology: vacuolation of the endodermal cells indicative of a lysosomal storage disorder. In a separate biochemical study (Freeman & Lloyd, 1983*b*), the nature of the stored material was identified as undigested protein that had been pinocytically captured by the yolk sac. The present experiments confirm the embryotoxic property of leupeptin in culture and demonstrate, by direct *in vitro* assay, an inhibition by leupeptin of the hydrolysis of ^{125}I-labelled BSA and haemoglobin by yolk sac homogenates.

From all of these observations, two facts clearly emerge. The first is that degradation of pinocytically captured protein within the lysosomes of the visceral yolk sac cells is an essential nutritional process in the normal development of the early organogenesis-stage conceptus; the second that lysosomal cysteine proteinases are important in the process. Using the specific inhibitor pepstatin, the present study has sought to determine to what extent, if any, the lysosomal aspartic proteinase cathepsin D is involved in the nutritional pathway.

In contrast with leupeptin, pepstatin does not interfere with the development of conceptuses in culture (Table 1) – the highest concentration of pepstatin (25 μg/ml) used in these experiments is almost four times the molar concentration of leupeptin required to completely abolish embryonic development. Several explanations for pepstatin's lack of toxicity can be advanced. Firstly, the yolk sac lysosomes do not possess aspartic proteinase activity, or that it is present but in a modified embryonic

form which is not pepstatin-inhibitable. Secondly, pepstatin may not attain a high enough concentration within the lysosome to inhibit proteolysis. And thirdly, aspartic proteinase activity may be present but is physiologically unimportant in the nutritional process. In order to test these hypotheses directly, the effect of pepstatin on the hydrolysis of protein by yolk sac homogenates was determined.

Using an initial 2 h incubation period, pepstatin did not interfere with the ability of the yolk sac to degrade ^{125}I-labelled BSA, and only slightly decreased the extent to which haemoglobin was hydrolysed. This observation contrasts sharply with the effect of leupeptin which completely inhibited the degradation of both substrates. The lack of effect of pepstatin on the degradation of haemoglobin is perhaps the more surprising since this substrate is popularly used to measure cathepsin D activity in cells and tissues (Barrett, 1980). When the incubation period was extended to 24 h, once again no inhibition of ^{125}I-labelled BSA degradation by pepstatin was observed (Fig. 3). Pepstatin's ineffectiveness in inhibiting proteolysis suggests the near-absence of cathepsin D activity from the lysosomes of the early organogenesis-stage yolk sac endoderm. That the enzyme is present in this tissue later in gestation (17·5 days) has been demonstrated (Knowles, Ballard, Livesey & Williams, 1981) by the direct *in vitro* inhibition by pepstatin, albeit to an extent somewhat less than by leupeptin, of ^{125}I-labelled BSA degradation. Thus the expression of cathepsin D activity in yolk sac would seem to vary with gestational age.

The catalytic activity of cathepsin D depends on a considerable degree of interaction of the enzyme with hydrophobic residues of the substrate protein (Barrett, 1980). Therefore as used in the present study in its native conformation in which hydrophobic residues are largely unexposed, albumin degradation by cathepsin D might be expected to be neglible. and certain studies (Huisman *et al*; 1974; Griffiths & Lloyd, 1979) confirm this idea. However, a more recent report (Kooistra, Millard & Lloyd, 1982) has shown that native albumin is degraded by cathepsin D in the presence of thiols which act by reducing disulphide bonds in the albumin molecule, thereby opening up its structure and rendering it more susceptible to attack by cathepsin D. Since all the assays in the present study were conducted in the presence of the thiol dithiothreitol, hydrolysis of native albumin by cathepsin D would be expected and incubations using a light mitochondrial fraction of rat liver as a source of proteinases clearly indicate the presence of pepstatin-inhibitable activity towards this substrate (Fig. 4). Therefore, the absence of observable cathepsin D activity in yolk sac cannot be ascribed to the assay conditions. Another, less-plausible, explanation for the inability of pepstatin to inhibit proteolysis in the yolk sac is the existence of an embryonic form of cathepsin D whose activity is unaffected by pepstatin. Although various isoforms of cathepsin D occur in most species (Barrett, 1980) all of these bind to pepstatin. The presence of an endogenous inhibitor of cathepsin D in the yolk sac might mask the effect of pepstatin on the enzyme. While this possibility has not been addressed in the present paper, the existence of physiologically important inhibitors of this type would, of course, imply that the enzyme, though present, had no role in the nutritional function of the yolk sac.

CONCLUDING REMARKS

The evidence presented here indicates strongly that proteolysis by cathepsin D, as judged by the action of the specific inhibitor pepstatin, plays little or no part in the overall action of the yolk sac in supplying amino acids to the conceptus through intralysosomal digestion of protein.

The contrasting effects of the cysteine proteinase inhibitor leupeptin, which is toxic to embryos in culture and completely inhibits proteolysis *in vitro*, indicate that this group of proteolytic enzymes are entirely responsible, or at least rate limiting, for the digestion of protein to amino acids in the yolk sac during early organogenesis.

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