

Inhibition of proteolysis in rat yolk sac as a cause of teratogenesis. Effects of leupeptin *in vitro* and *in vivo*

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SUMMARY

Conceptuses from 9.5-day pregnant rats were cultured for 48 h in heat-inactivated homologous serum to which leupeptin, a specific inhibitor of the lysosomal cysteine-proteinases, was added for the final or the penultimate 6 h.

The presence of leupeptin (25 µg/ml or above) increased the protein content of yolk sacs at harvesting to approximately twice the control value. The protein content of the embryo at harvesting was lower than that of controls.

When ¹²⁵I-labelled polyvinylpyrrolidone was added to the culture serum for the final 6 h of culture, radioactivity was found in the yolk sac at harvesting, but not in the embryo. The presence of leupeptin did not affect the rate of uptake of the radiolabelled macromolecule by the yolk sac, nor facilitate its entry into the embryo.

When formaldehyde-denatured ¹²⁵I-labelled bovine serum albumin was added to the culture medium for the final 6 h of culture, little radioactivity was found in the yolk sac at harvesting, and barely any was found in the embryo. Trichloroacetic acid-soluble radioactivity was found in the culture serum. The presence of leupeptin sharply increased the levels of radioactivity in the yolk sac (but not the embryo) and sharply decreased the acid-soluble radioactivity of the culture medium.

When rat serum whose proteins were labelled with [³H]leucine was used as culture medium, radioactivity was found in both yolk sac and embryo at harvesting. The presence of leupeptin increased the amount found in the yolk sac and decreased that found in the embryo.

These results are interpreted as follows. Leupeptin enters the lysosomes of the yolk sac, inhibiting their cysteine proteinases. The digestion of proteins pinocytosed by the yolk sac is consequently inhibited, resulting in the accumulation of protein by the yolk sac and a decreased flow of amino acids to the embryo.

Leupeptin (50 mg/kg), injected into pregnant rats at either 8.5 days or 9.5 days of gestation, induced congenital malformation in the offspring. It is proposed that leupeptin exerts its teratogenic action by inhibiting proteolysis in the lysosomes of the yolk sac, and so depriving the developing embryo of its supply of amino acids at a critical stage of development.

INTRODUCTION

Disturbance of yolk-sac-mediated embryotrophic (histiotrophic) nutrition during early organogenesis as a mechanism of teratogenesis in the rat was first

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proposed by Beck, Lloyd & Griffiths (1967). Recent experiments (Freeman, Beck & Lloyd, 1981; Freeman & Lloyd, 1983), have confirmed the existence and clarified the nature of this nutritional pathway, and its susceptibility to modification by a teratogen, anti-yolk sac antiserum (Freeman, Brent & Lloyd, 1982).

Briefly the nutritional pathway is as follows. The endodermal cells of the visceral yolk sac, a prominent extraembryonic membrane of rodents, pinocytically capture exogenous proteins and digest these within their lysosomes to provide a source of amino acids for utilization in protein synthesis by the rapidly growing and developing conceptus. Since the provision of amino acids by this route requires intralysosomal proteolysis within the yolk sac, we have sought to examine the effect of an inhibitor of several lysosomal proteinases, leupeptin. Leupeptin, a tripeptide of bacterial origin, inhibits cathepsins B, H and L and has recently been shown (Beck & Lowy, 1982) to induce embryopathy when added to the culture serum in which early organogenesis-stage rat conceptuses were growing. Earlier Umegawa & Aoyagi (1977) briefly reported teratogenic effects of leupeptin following chronic oral administration to pregnant rats during the second trimester of pregnancy. Using the isolated 17.5-day rat yolk sac in organ culture, Knowles, Ballard, Livesey & Williams (1981) demonstrated that leupeptin, added to the culture medium inhibited the degradation, but not the uptake, of exogenous ^{125}I -labelled bovine serum albumin.

In the present study, the effect of leupeptin on pinocytosis and lysosomal protein degradation by the cultured early organogenesis-stage rat conceptus has been assessed. In addition, we report observations on the teratogenicity of leupeptin following a single injection into pregnant rats at either 8.5 or 9.5 days of gestation.

METHODS

Cultures of 9.5-day rat conceptuses in the presence of radiolabelled macromolecules

All cultures were of 48 h duration. Procedures were as described by Freeman *et al.* (1981) with the following modifications.

(i) *^{125}I -Labelled polyvinylpyrrolidone (PVP) as substrate*

Radiolabelled substrate ($2.3\ \mu\text{g}/\text{ml}$) together with leupeptin (obtained from the Protein Research Foundation, Osaka, Japan), was added to the culture medium for the final 6 h of culture.

(ii) *Formaldehyde-denatured ^{125}I -labelled bovine serum albumin (BSA) as substrate*

Radiolabelled protein ($11\ \mu\text{g}/\text{ml}$) was present in culture for the final 6 h. Leupeptin was added to the culture medium either (together with radiolabel) for

the final 6 h, or for the penultimate 6 h. In the latter case conceptuses were transferred to fresh medium for the final 6 h of culture, a procedure that did not affect embryonic development or the pinocytic capacity of the yolk sac.

(iii) [^3H]Leucine-labelled serum proteins as substrates

Conceptuses were transferred from a medium of whole rat serum to one of vitamin- and glucose-supplemented dialysed [^3H]leucine-labelled rat serum for the final 6 h of culture. Leupeptin was present in culture either for the final 6 h (in dialysed serum), or for the penultimate 6 h of culture (in whole serum). Culture in dialysed serum does not alter the rate of pinocytosis by conceptuses.

Teratogenesis testing of leupeptin

Pregnant Wistar rats received a single subcutaneous injection of leupeptin (50 mg/Kg body weight) contained within 0.1 ml of 0.9% saline. At 20.5 days, uteri were removed, and the number of resorption sites and live foetuses recorded. Each foetus was weighed and examined for evidence of external malformation. Out of every three foetuses, two were fixed in Bouin's fluid and subsequently sectioned freehand with a razor blade to detect internal malformations, while the third was cleared with 1% KOH and stained with alizarin red-S to reveal skeletal anomalies, after the method of Wilson (1965).

RESULTS

Culture experiments

Conceptuses were exposed to radiolabelled substrates for the final 6 h only of a 48 h culture period. This is because, owing to the growth curve of the conceptus, radiolabel accumulated at an earlier stage is quantitatively insignificant at harvesting (Freeman *et al.* 1981).

The presence of leupeptin in the culture medium for either the final or penultimate 6 h of culture neither induced developmental abnormalities of conceptuses, nor increased the incidence of embryonic death. However, the protein contents of yolk sacs and embryos at harvesting were markedly affected (Fig. 1): yolk-sac protein content was increased, and embryo protein content was decreased. The increase in yolk-sac protein levels was independent of the concentration of leupeptin, while embryo protein levels were decreased by an amount apparently proportional to the leupeptin concentration in the culture medium. These observations are consistent with our proposal (Freeman & Lloyd, 1983) that protein taken into the yolk sac yields amino acids, which are in part transmitted to the embryo for protein synthesis and in part retained within the yolk sac for that tissue's own synthetic needs.

Uptakes of radiolabelled substrates into conceptuses are expressed as

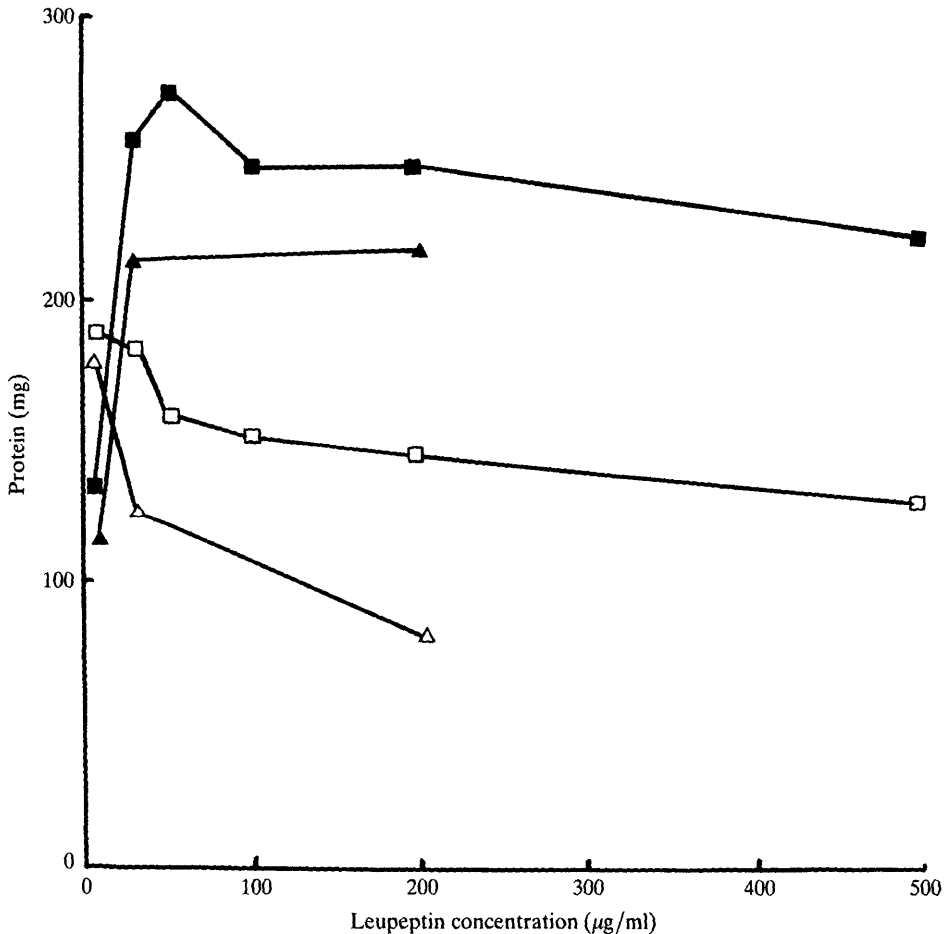


Fig. 1. Protein content of yolk sac (filled symbols) and embryo (open symbols) after incubation of conceptuses in the presence of leupeptin for either the final 6 h (squares) or the penultimate 6 h (triangles) of the culture period. Values are the means of at least six determinations.

clearances (μl of culture medium whose substrate content is captured, per mg of tissue protein), as explained by Freeman *et al.* (1981).

(i) ^{125}I -Labelled polyvinylpyrrolidone as substrate

^{125}I -Labelled polyvinylpyrrolidone is a non-metabolizable macromolecule that on pinocytosis by the yolk sac accumulates in the lysosomes of that tissue, with no uptake by or transfer to the embryo (Freeman *et al.* 1981). Fig. 2 shows that the uptake of this substrate into yolk sac was apparently decreased when leupeptin was present in the culture, together with the ^{125}I -labelled PVP, for the final 6 h. The extent of the decrease was independent of the leupeptin concentration. In agreement with previous observations (Freeman *et al.* 1981), little

radioactivity was detectable in the embryo. For subsequent experiments using protein substrates, two widely different concentrations of leupeptin (25 and 200 $\mu\text{g}/\text{ml}$) were used.

(ii) *Formaldehyde-denatured ^{125}I -labelled bovine serum albumin as substrate*

This substrate is pinocytically captured by the yolk sac and digested within the lysosomes to yield [^{125}I]iodotyrosine, which is then released into the culture serum (Freeman *et al.* 1981). The data of Fig. 3 clearly demonstrate that leupeptin, present in the culture for either the final or penultimate 6 h, caused an apparent decrease in the total uptake of radiolabel by conceptuses. Furthermore, a very much larger proportion of this radioactivity was retained, mostly in trichloroacetic acid (TCA)-insoluble form, in the yolk sac, and was not returned to the culture medium as TCA-soluble [^{125}I]iodotyrosine. The

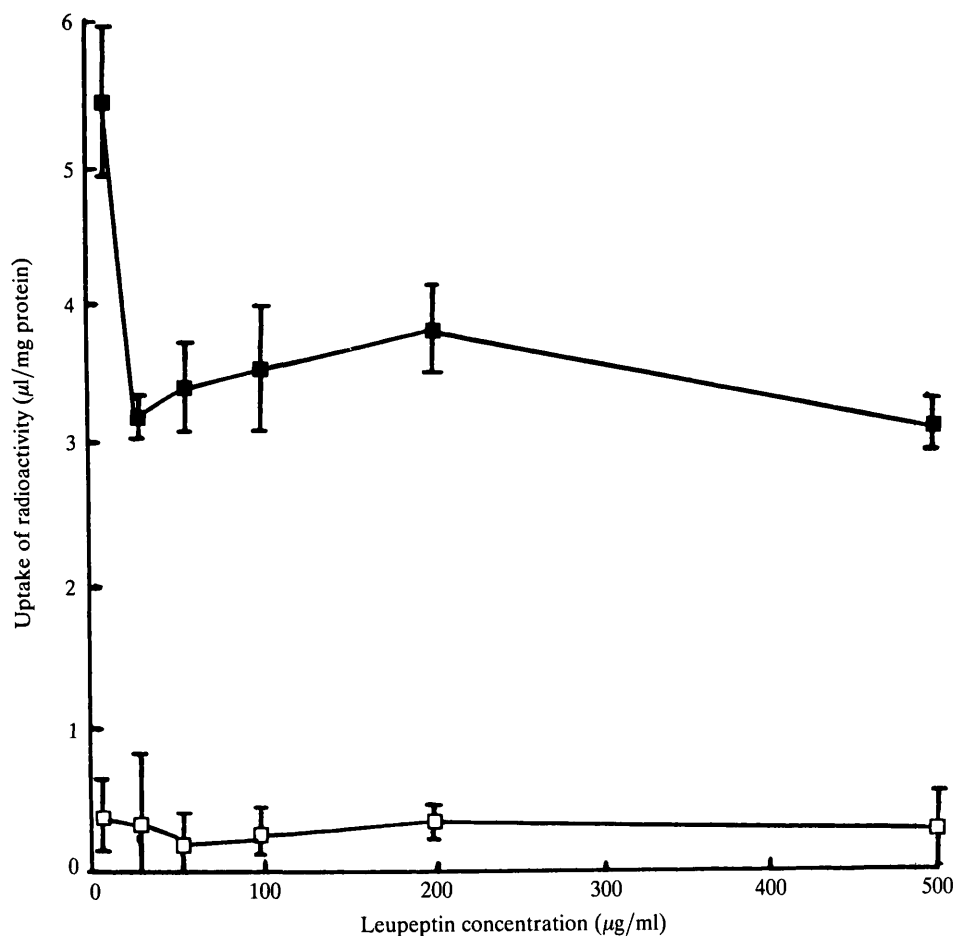


Fig. 2. Radioactivity associated with yolk sac (■) and embryo (□) after incubation of conceptuses in the presence of ^{125}I -labelled PVP (2.3 $\mu\text{g}/\text{ml}$) and leupeptin for the final 6 h of the culture period. Values are the means (\pm s.d.) of at least six determinations.

proportion of yolk-sac-associated radioactivity that was TCA-insoluble was slightly higher with the higher concentration of leupeptin used. No marked differences were observed between the data from those cultures in which leupeptin was present for the penultimate 6 h and those in which leupeptin was present for the final 6 h. As previously found (Freeman *et al.* 1981), small amounts of radioactivity, all of which was TCA-soluble, were detectable in the embryo.

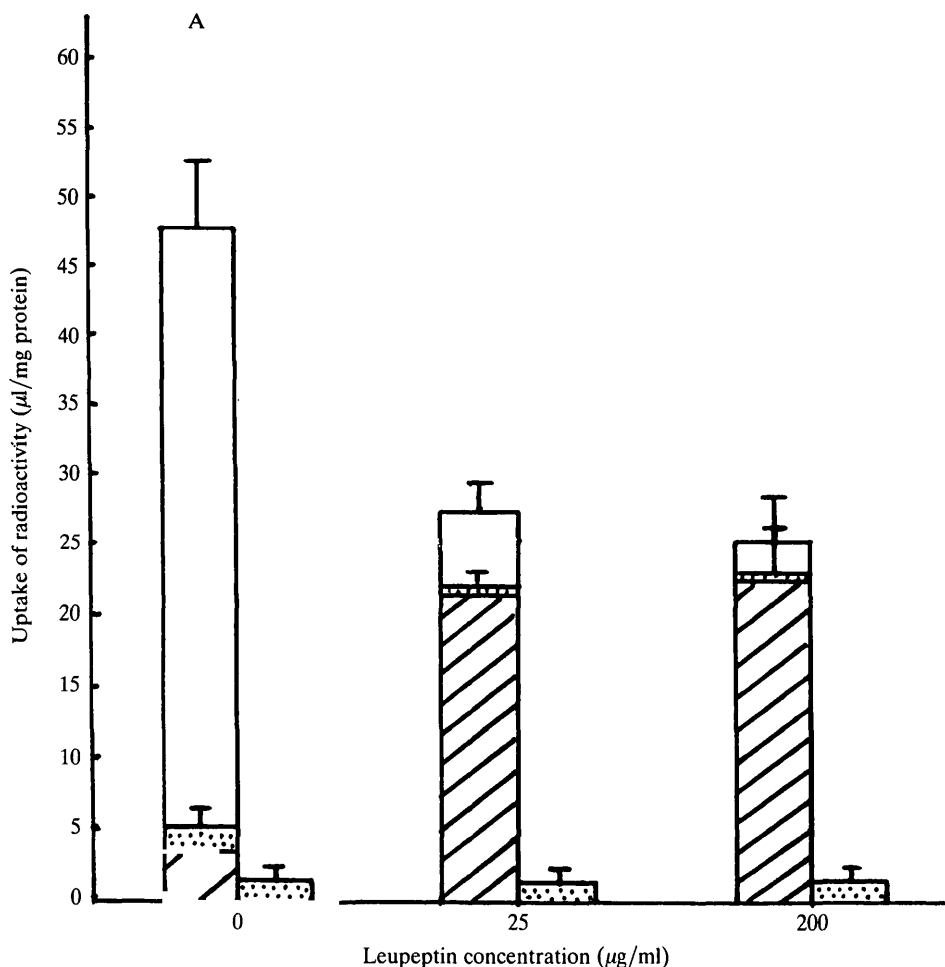
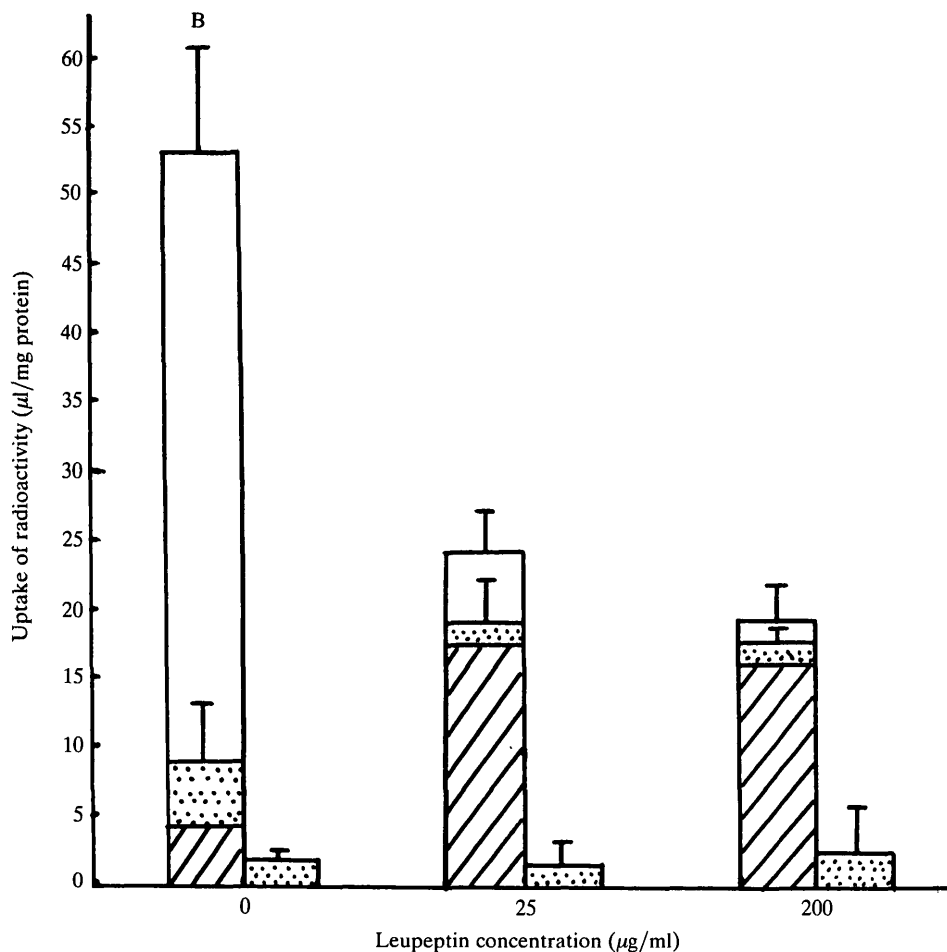


Fig. 3. Total radioactivity taken up by yolk sac (each left-hand column) and embryo (each right-hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of formaldehyde-denatured ^{125}I -labelled bovine serum albumin for the final 6 h of the culture period. Leupeptin was present for either the final (3A) or the penultimate (3B) 6 h. The proportions of yolk sac-associated and embryo-associated radioactivity that were TCA-soluble (▨) and TCA-insoluble (▤) are indicated. Values are the means (\pm s.d.) of at least 6 determinations.



(iii) [^3H]Leucine-labelled serum proteins as substrate

The radiolabelled digestion product of this substrate is [^3H]leucine which, in contrast to [^{125}I]iodotyrosine, can be utilized for protein synthesis in yolk sac and embryo. In previous experiments using this substrate (Freeman *et al.* 1981) it was shown that the radioactivity accumulated by the yolk sac was all retained within the conceptus; none was released back into the medium in TCA-soluble form.

The inclusion of leupeptin in the culture medium, whether for the final or the penultimate 6 h, grossly altered the distribution of [^3H]radioactivity within the conceptus (Table 1). Much more remained in the yolk sac and much less found its way into the embryo. These effects were even more pronounced at the higher than at the lower concentration of leupeptin. Leupeptin also increased the proportion of the radioactivity in yolk sac and embryo that was in a form insoluble in TCA (results not shown). When leupeptin (at either concentration) was present in cultures for the penultimate 6 h, all the radioactivity in the embryo was TCA-insoluble.

Table 1. *Distribution of radioactivity between yolk sac and embryo at harvesting, after incubation of conceptuses in dialysed and vitamin-supplemented [³H]leucine-labelled serum for the final 6 h, and leupeptin for the penultimate or the final 6 h of culture*

Concentration of leupeptin (µg/ml)	Leupeptin present for penultimate 6 h		Leupeptin present for final 6 h	
	Percent radioactivity in		Percent radioactivity in	
	yolk sac	embryo	yolk sac	embryo
0	45.0	55.0	36.3	63.7
25	87.5	12.5	81.1	18.9
200	96.1	3.9	90.6	9.4

Teratogenicity of leupeptin

Injection of leupeptin (50 mg/Kg) into pregnant rats at either 8.5 or 9.5 days of gestation resulted in the production of malformed foetuses (Table 2). Following injection at 8.5 days, the resorption rate at term was 24%. Of the surviving foetuses, more than 50% were visibly externally malformed, and the proportion of foetuses subsequently sectioned that showed malformation was 73%. The most common defects observed in this group (Table 3) were anophthalmia, microphthalmia, hydrocephalus and kidney malformations. After injection at 9.5 days, the resorption rate doubled, though a much lower incidence of malformation was observed (Table 2). Only 2 of the 25 surviving foetuses were externally malformed, while none of those examined displayed internal malformations. In contrast to the report of Umezawa & Aoyagi (1977), no skeletal malformations were detected in the present study. The average weight of foetuses from rats injected at either 8.5 or 9.5 days of gestation was significantly ($P < 0.001$) lower than that of saline-treated controls.

DISCUSSION

The observation that leupeptin decreased the uptake by yolk sac of the radiolabelled macromolecules ¹²⁵I-labelled PVP and ¹²⁵I-labelled albumin is seemingly at variance with previous reports (Berg, Ose & Tolleshaug, 1981; Knowles, Ballard, Livesey & Williams, 1981) that the peptide, although markedly inhibiting the degradation of pinocytosed protein by rat hepatocytes and 17.5-day rat yolk sac in culture, did not affect pinocytosis itself. However, on closer examination, the present data clearly show that leupeptin does not inhibit pinocytosis. The values presented for pinocytic uptake are normalized to the

Table 2. *Teratogenic effects of subcutaneous injection of leupeptin (50 mg/kg) or saline (0.9%)*

Nature and timing of injection	No. of pregnant rats used	No. of implantations	Resorptions	No. of surviving foetuses	Average weight of surviving foetuses (g)	Foetuses with external abnormalities		No. of foetuses sectioned	Foetuses sectioned showing internal/external abnormalities	No. of foetuses stained with alizarin red S
						No.	% of total surviving implantations			
Leupeptin 8.5 days	4	51	12 (24%)	39	3.45 ± 1.1*	21	54	41	19 (73%)	13
Leupeptin 9.5 days	4	48	23 (48%)	25	3.32 ± 0.42*	2	8	4	2 (12%)	8
Saline 8.5 days	4	45	2 (4%)	43	4.19 ± 0.42	0	0	0	0	15
Saline 9.5 days	4	44	2 (5%)	42	4.40 ± 0.60	0	0	0	0	12

*Significantly different ($P < 0.001$) from saline-injected controls as judged by Student's t-test.

Table 3. *Abnormalities detected after leupeptin treatment of pregnant rats*

Abnormality	Day of injection	
	8.5	9.5
Anophthalmia	22 (39)	2 (25)
Hydrocephalus	9 (26)	0 (17)
Kidney (deformed)	9 (26)	0 (17)
Facial defects	1 (39)	0 (25)
Limb defects	0 (39)	1 (25)
Tail defects	0 (39)	1 (25)

Figures in brackets indicate the size of the population examined for the relevant malformation.

quantity of tissue protein. Fig. 1 indicates that yolk-sac protein content at harvesting is increased by the inclusion of leupeptin in the culture medium. Since leupeptin is known not to increase protein synthesis (Seglen, 1978; Libby, Ingwall & Goldberg, 1979), the most plausible explanation for this observation is that pinocytosed protein from the culture serum, its intralysosomal digestion inhibited by leupeptin, accumulates within the lysosomes, thus markedly increasing the tissue's protein content. This increase will itself result in a decrease in the normalized values for pinocytic uptake of radiolabelled markers. This interpretation is supported by the fact that the yolk-sac protein content (the numerical value of which appears in the denominator of the expression used to calculate uptake) is approximately twice the control value at all leupeptin concentrations studied, while the uptake of radiolabel is approximately halved.

Although leupeptin does not affect pinocytosis by the yolk sac, it decreases severely the extent to which pinocytosed proteins are degraded within the yolk-sac lysosomes. Fig. 3 and Table 1 show that nearly all the radioactivity taken into the yolk sac remains within that tissue; and most of it is in a TCA-precipitable form.

Our previous experiments (Freeman *et al.* 1981; Freeman & Lloyd, 1983) have shown that [³H]leucine, generated by degradation of radiolabelled proteins in the yolk sac, is incorporated into freshly synthesized embryonic proteins. The data of Table 1 afford further, powerful evidence that this is so. Leupeptin, by inhibiting the proteolytic mechanism in the yolk sac, causes a marked reduction in the amount of radioactivity transmitted to the embryonic tissues, once more underscoring the importance of the visceral yolk sac in the nutritional mechanism of the early organogenesis embryo.

Leupeptin is a specific inhibitor of lysosomal cysteine proteinases (Huisman *et al.* 1974; Kirschke *et al.* 1977) and presumably it is these enzymes that are being inhibited in the yolk sac. Cathepsin L is currently believed to be quantitatively the most significant cysteine proteinase of the lysosomes.

The data recorded in Tables 2 and 3 indicate that leupeptin is teratogenic. Typical of teratogens acting during early organogenesis, leupeptin induces defects of the eyes and the cerebral ventricular system, and is more teratogenic following administration at 8.5 days of gestation than at 9.5 days. Beck & Lowy (1982) reported a teratogenic effect of leupeptin *in vitro*, following longer periods of exposure of conceptuses to the peptide than were used in the present study, and hypothesized that the mechanism of teratogenic action was an inhibition of proteolysis in yolk-sac lysosomes which disturbed the histiotrophic nutritional pathway. Our direct biochemical data lend strong support to this theory and illustrate how susceptible yolk-sac-mediated nutrition of the early organogenesis-stage rodent embryo is to teratogenic insult.

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