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PROTEOLYTIC ENZYMES OF THE YOLK-SAC SPLANCHNOPLEUR

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SUMMARY

The yolk-sac splanchnopleur of the rabbit foetus is the site of a very active process of protein degradation.

Extracts of the yolk-sac splanchnopleur are able to hydrolyse a number of specific peptides and serum proteins indicating the presence of cathepsins A, B and C, leucine amino peptidase, carboxy-peptidase and dipeptidase.

The intracellular distribution of the proteolytic enzymes is with the lysosomal fraction sedimenting in isopycnic studies at a density of 1.22–1.23. The course of digestion of γ-globulin substrates has been followed and it emerges that intermediate molecular weight peaks are formed.

Comparison of proteolytic activities of various foetal tissues showed the amnion and chorion are both poor in this respect, while foetal liver has an activity lower than that of the splanchnopleur.

INTRODUCTION

The yolk-sac splanchnopleur of the rabbit foetus is the site of a very active process of immunoglobulin transport, from the uterine lumen into the foetal circulation. While it is clear that the first stage of this process, the adsorbtion into the entodermal cells of the splanchnopleur, is by pinocytosis and includes therefore any protein which is presented to the cells, the latter stages present a greater problem because the discharge into the foetal bloodstream is selective in that immunoglobulins of different kind, or of differing specific origin appear there in widely differing quantity[1,2]. At the same time, there is a rapid proteolysis of the ingested protein. It has been established that more than 80% of a dose of protein introduced into the uterine lumen is broken down after being absorbed by the cells of the yolk sac entoderm, and in the most favourable case the minor portion only is released from those cells into the foetal blood[3]. Concomitant with this observation, the crude saline extract of the yolk-sac was shown to posses strong proteolytic activity with a pH optimum of about 3.5. Clearly, the proteolytic activity of the cell is closely related to the function of transport of whole protein and may be competitive with it. It may or may not have to do with the selectivity of transport. Accordingly the work

reported here was undertaken to study further the acid protease activity of the yolk-sac entoderm.

MATERIALS AND METHODS

Collection of material

Yolk-sac splanchnopleurs were collected and freed of any contaminating embryonic tissue, from rabbits killed by an intravenous injection of nembutal between the 24th and 26th days of pregnancy. Enzyme extracts were prepared by homogenising the tissue with a Tenbrook grinder in 10 vol. of 0.9% saline. The homogenates were frozen and thawed three times to release any bound enzymes, centrifuged at 20 000 × *g* for 20 min and the supernatant retained for analyses.

Enzyme assays

Acid proteases

The proteolytic activities of various extracts were compared by a modification of the method of PRESS *et al.*[4], using acid-denatured albumin as substrate. Denaturation was achieved by incubating a 1.7% bovine albumin solution in 0.33 M citric acid, for 1 h at 37°. The resulting denatured albumin was adjusted to a concentration of 0.85% and the pH to 3.6 with 1 M NaOH. For the examination of proteolytic activity a volume of 1.2 ml of this solution was incubated for 20 min at 37° with 0.2 ml of enzyme solution. Any undigested protein was then precipitated with 2.5 ml of 0.6 M trichloroacetic acid and the absorbance of the clear filtrate after centrifugation was measured at 280 mμ. A reagent blank was carried out at the same time, by adding the trichloroacetic acid before the enzyme solution. The relationship between the absorption of the filtrate and the amount of enzyme used, was linear up to an extinction of 0.3, a linear relationship also existed between the enzyme activity and the time of incubation.

For samples separated by isopycnic centrifugation, the proteolytic activities were determined by using radioactive labelled bovine albumin as substrate. A 0.5% solution of bovine albumin ^{131}I was adjusted to pH 3.6 with 0.05 M citrate buffer and made 0.01 M with respect to cysteine. Volumes of 0.1 ml of this mixture were incubated with 0.02 ml of enzyme fractions at 37° for a given period of time. Controls containing distilled water in place of enzyme solution were run in parallel. After incubation, any undigested protein was precipitated with 1.0 ml trichloroacetic acid and removed. The radioactivity remaining was counted in a Panax scintillation counter and the amount of substrate digested was calculated. The amount of radioactivity in the trichloroacetic acid soluble fraction, increased linearly with time of incubation until 35% hydrolysis of the protein had occurred.

Cytochrome oxidase

This enzyme was assayed by the method of COOPERSTEIN AND LAZAROW[5],

Iodination of protein

Proteins were labelled with ^{131}I to a level of 0.5 to 1 atom of iodine per molecule by the jet mixing method of MACFARLANE[6]. Inorganic iodide was removed from the preparation by passage through an Amberlite I.R.A. 400 (OH) ion exchange column, followed by dialysis against 0.9% saline at 2°. The ion exchange column (6 cm ×

0.3 cm) was prepared for use by adjusting the resin to a pH of 6.5 with 20 ml of a 0.1 M histidine–NaOH buffer and then washing with 100 ml 0.9% NaCl. The radioactive protein passed through this column as a sharp peak, while the major proportion of the non-protein activity was retained on the column.

Cell fractionation

Differential centrifugation

For each experiment five splanchnopleurs were pooled and homogenised in 6.0 ml ice cold 0.25 M sucrose solution, using a Tenbrook tissue grinder, three up and down movements of the ground glass plunger being sufficient for homogenisation. The resulting homogenate was subjected to differential centrifugation at 2° in a Spinco Model L centrifuge, using an angle head rotor number 30.2, as described by DE DUVE[7] in his treatment of rat liver homogenates.

The nuclear fraction was sedimented by centrifuging the cell homogenate at 600 × *g* for 10 min, and the supernatant removed with a Pasteur pipette. The sediment was washed, rehomogenised and recentrifuged as above. Pooled washings and supernatant from the nuclear fractions were centrifuged at 25 000 × *g* for 10 min and the sedimented mitochondrial fraction was washed and recentrifuged under the same conditions. The microsomal fraction remaining in the final supernatant was sedimented as a small pellet at the bottom of the centrifuge tube by centrifugation at 100 000 × *g* for 30 min, this fraction was not washed, the supernatant which was decanted represented the cell sap.

Isopycnic centrifugation

Isopycnic centrifugation of the mitochondrial fractions was carried out as described by BEAUFAY *et al.*[8]. In this method, 0.2-ml samples were gently layered onto a sucrose gradient of specific gravity, ranging from 1.24–1.16, and centrifuged in a Model L Spinco Swinging Bucket Rotor S.W. 39 for 2.5 h at 100 000 × *g*. The rotor was maintained at a temperature of 1° during the run, violent acceleration and retardation were avoided so as to preserve the gradient.

After centrifugation the centrifuge tubes were firmly clamped and their contents fractionated into about 40 equal samples of about 0.15 ml with a fine bore glass siphon. These fractions were assayed for specific gravity[9], total protein[10], proteolytic activity, and cytochrome oxidase.

RESULTS

The detection of substrate specificity

The identity of some of the proteases occuring in the rabbit splanchnopleur tissue was investigated by the method of TODD AND TRIKOJUS[11]. Carbobenzoxy-glutamyl-L-tyrosine, benzoyl-L-argininamide, and glycyl-L-tyrosinamide acetate were used as specific substrates for the detection of cathepsin A, cathepsin B and cathepsin C (EC 3.4.4.9.)[12].

Peptidase activities were revealed by their action on L-leucinamide, carbobenzoxy-L-glycylphenylalanine and triglycine, these are specific substrates for the enzymes leucinaminopeptidase, (EC 3.4.1.1)[13], carboxy-peptidase A (EC 3.4.2.1)[14] and tripeptidase (EC 3.4.1.3)[15]. For catheptic activities the substrates were dissolved in sodium acetate buffer (0.1 M, pH 5) containing 0.02 M cysteine, whereas for

peptidase activities, the substrates were dissolved in 0.1 M Tris buffer (pH 7.8) and the cysteine was replaced by 0.005 M $MnSO_4$. Equal volumes (0.05 ml) of the above solutions and the tissue extract were incubated in tightly stoppered tubes for 16 h at 37°. Two sets of control tubes containing distilled water in place of the peptides or the enzymes were incubated under the same conditions. In each case the products of digestion were subjected to thin-layer chromatography on silica gel. Propanol–water (4:1, by vol.) was utilised as a solvent for the benzoyl-L-argininamide and L-leucinamide systems, and butanol–acetic acid–water (65:17:17, by vol.) was used for the other digests. The chromatograms were developed with ninhydrin solution, except for the separation containing benzoyl-L-argininamide, which was sprayed with Sakaguchi reagent[16]. Examination of the chromatograms showed that hydrolysis of all these substrates had occurred, indicating the presence of all the above mentioned enzymes.

TABLE I

TOTAL PROTEIN NITROGEN OF CELL FRACTIONS

The total protein nitrogen of cell fractions, prepared from rabbit splanchnopleurs, expressed as percentages of the total protein nitrogen of the starting material.

	Cell fraction					*Means*
	1	*2*	*3*	*4*	*5*	
Homogenate	100	100	100	100	100	100
Nuclear	24.7	23.4	21.0	28.4	23.6	24.2
Mitochondrial	15.5	17.7	18.8	15.3	16.7	16.8
Microsomal	9.0	9.3	10.5	8.9	7.6	9.0
Supernatant	48.5	46.0	52.2	46.1	39.5	46.5
% recovery	97.7	96.4	102.5	98.7	87.4	96.5

Study of intracellular distribution of splanchnopleur proteases

Five experiments were carried out to examine the intracellular distribution of the splanchnopleur proteases. Following differential centrifugation of the tissue homogenate, each cell fraction was diluted with 0.9% NaCl containing 0.1% of a non-ionic detergent (Triton X-100) so as to release all the bound enzymes into solution. The fractions were then examined for their total nitrogen[17] and acid protease activity[4]. The protein contents expressed as percentages of the total protein nitrogen of the starting material are given in Table I.

From the results of the total catheptic activities, in Table II, it is observed that the recoveries were nearly complete and although some proteolytic activity is associated with all of the separated fractions, the major proportion is found in the mitochondrial fraction. This is most evident when the specific activities (Total units of protease activity per mg of protein) of the cell fractions are plotted (Fig. 1). Further examination of this fraction by the technique of isopycnic ultracentrifugation resulted in the proteolytic activity sedimenting at a higher equilibrium density 1.22–1.23 than that of the cytochrome oxidase and the bulk of protein 1.20–1.21. As cytochrome oxidase is a known mitochondrial enzyme, one concludes that the cathepsins in the yolk-sac splanchnopleur are present in particles which are denser than mitochondria (Fig. 2).

TABLE II

TOTAL CATHEPTIC ACTIVITIES OF CELL FRACTIONS

The total catheptic activities of cell fractions prepared by differential centrifugation of rabbit splanchnopleurs. Catheptic activities were determined by a modification of the method of PRESS *et al.*[4].

	Cell fraction				
	1	*2*	*3*	*4*	*5*
Homogenate	9.32	7.50	9.37	7.40	5.07
Nuclear	1.39	1.68	2.62	1.79	1.62
Mitochondrial	3.95	3.19	3.70	2.81	1.65
Microsomal	0.39	0.60	0.23	0.19	0.24
Supernatant	3.31	2.40	3.65	2.47	1.54
% recovery	97	105	109	98	99

Digestive action of splanchnopleur acid proteases on γ-globulins

Samples of 20 mg of isotopically labelled bovine and rabbit γ-globulins in 0.05 M acetate buffer (pH 3.6) and containing 0.01 M cysteine were digested in turn for varying intervals of time with 0.1 ml of the enzyme extract. The reactions were stopped by adjusting the pH to neutrality with 0.1 M NaOH and the resulting digests fractionated on a (50 cm × 3.3 cm) column of Sephadex G-100 with phosphate buffered saline (0.01 M sodium phosphate buffer pH 7.4, 0.1 M NaCl).

After 5 h, 40% proteolysis of the bovine γ-globulins (Fig. 3) had occured as measured by the release of radioactive trichloroacetic acid soluble material. The Sephadex eluate at this stage, showed no intact globulin but two groups of protein fragments with molecular weights approximating to 100 000 and 40 000 were separated.

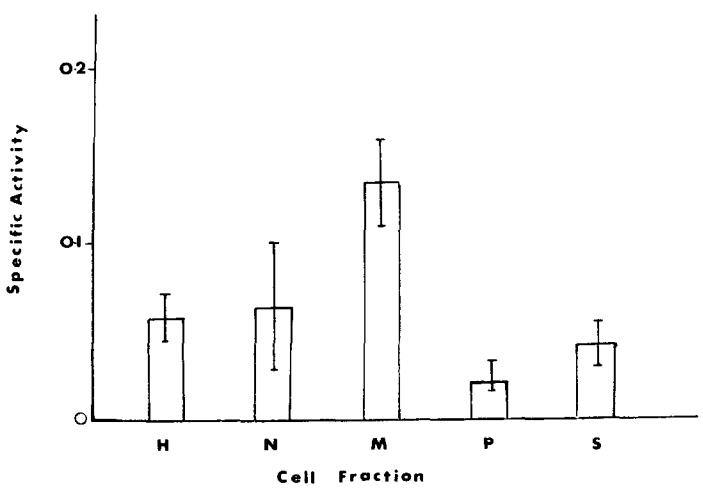

Fig. 1. The specific activities (Total enzyme units in fraction/Total mg protein in fraction) of the acid proteases found in the cell fractions, prepared from rabbit splanchnopleurs by differential centrifugation. Five experiments were conducted, the vertical lines on the tops of the columns show the range of individual values. H, homogenate; N, nuclear; M, mitochondrial; P, microsomal; S, supernatant, fraction.

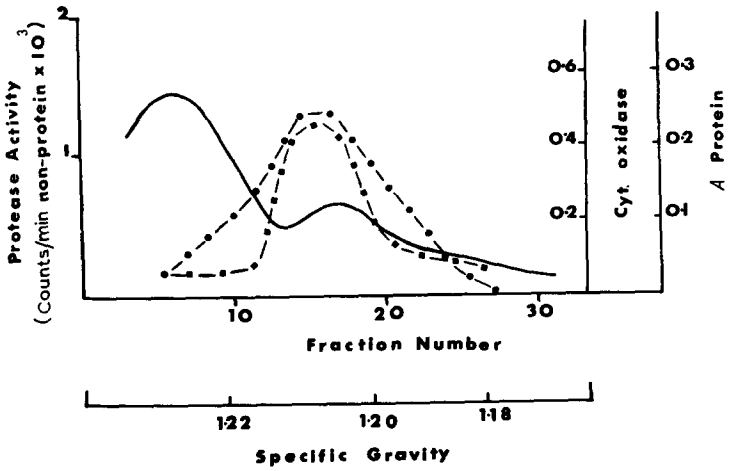

Fig. 2. The isopycnic centrifugation of the washed mitochondrial fraction. ———, protease activity. This was determined by incubating 0.1 mg of a 0.5% labelled bovine albumin solution with 0.1 ml of sample, precipitating with 1.0 ml of 0.6 M trichloroacetic acid and measuring the release of the radioactive soluble material. —■—■—, cytochrome oxidase. Assayed by following the rate of oxidation of reduced cytochrome *c* at 550 nm[5]. The units are expressed as log (ferrocytochrome *c*) per min. —●—●—, protein concentration. The absorbance of the protein at 550 nm was measured by the method of LOWRY *et al.*[10]. The specific gravity of the fractions were determined by gravity equilibration in a calibrated linear xylene–bromobenzene gradient[9].

A 30% rabbit γ-globulin digest is shown (Fig. 4). Three zones of radioactive protein are seen, the first attributable to undigested protein, and the other two being eluted at the same volumes as the bovine γ-globulin fragments.

A comparison of the protease activities of other rabbit foetal membranes

The pH optima for the digestive action of the other foetal membranes namely the amnion and chorion, as well as the foetal liver, were examined and found to be 3.6. Saline extracts of these tissues were adjusted to a protein concentration of 1% and their proteolytic activities compared with a similar concentration of splanchnopleur extract at the same pH.

The results are presented in Fig. 5, only the liver and splanchnopleur extracts show any appreciable proteolytic activity and while the liver is known to be an organ rich in lysosomal enzymes the higher concentration of proteases is found in the yolk-sac splanchnopleur.

DISCUSSION

The yolk-sac splanchnopleur of the 24 day pregnant rabbit, contains proteases which are capable of digesting a number of synthetic peptides as well as serum proteins. HEMMINGS[3] demonstrated that these proteases have a pH optimum of 3.5 in their reaction with rabbit γ-globulin, but it must be emphasised that this optimum pH is not necessarily the same for all protein substrates. PRESS *et al.*[4] have shown that bovine cathepsin D hydrolysed haemoglobulin optimally at pH 3 but bovine albumin at pH 4.

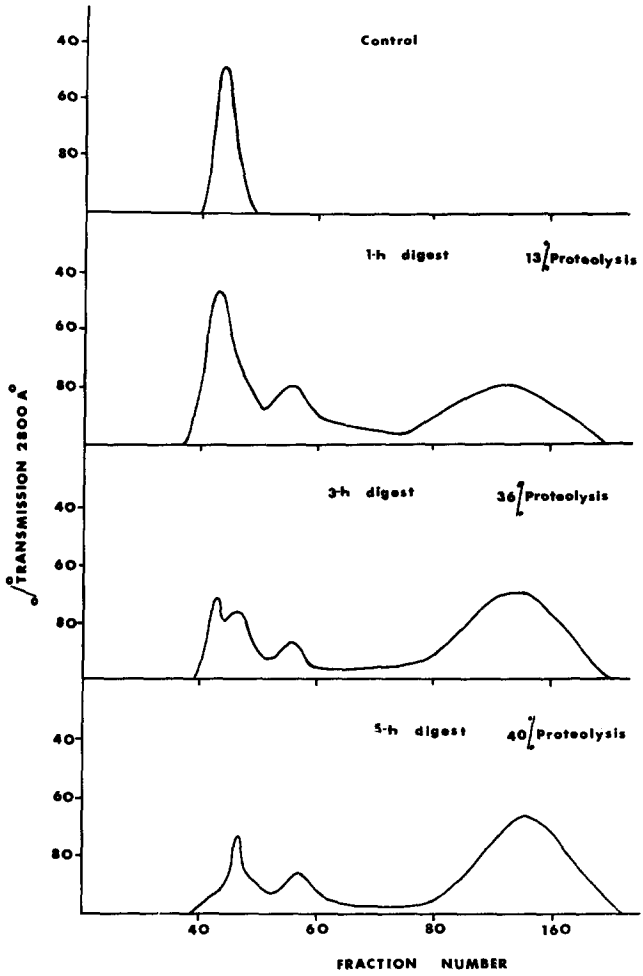

Fig. 3. Sephadex G-100 separations of 20-mg samples of labelled bovine γ-globulin which had been digested with 0.1 ml of splanchnopleur extracts for various periods of time. % proteolysis was determined by measuring the release of radioactive soluble material on precipitating 0.05-ml aliquots of the digests with 1.0 ml of 0.6 M trichloroacetic acid.

Benzoxy-glutamyl-L-tyrosine, benzoyl-L-argininamide and glycyl-L-tyrosine amide acetate are among a number of peptides which are hydrolysed by a saline extract of splanchnopleur tissue. These are specific substrates for the enzymes of the gastrointestinal secretions and for cathepsins A, B and C. As the above mentioned cathepsins are recognised as having no significant activity on intact proteins, one is led to the conclusion that the proteolytic activity, which is the major point of this paper, is undoubtedly due to cathepsin D (EC 3.4.4.23)[4]. This is the main protease of many animal tissues, but its presence in the rabbit splanchnopleur was not categorically proven as there are no specific low molecular weight substrates available for this enzyme[18].

From the results of differential centrifugation studies, it was found that al-

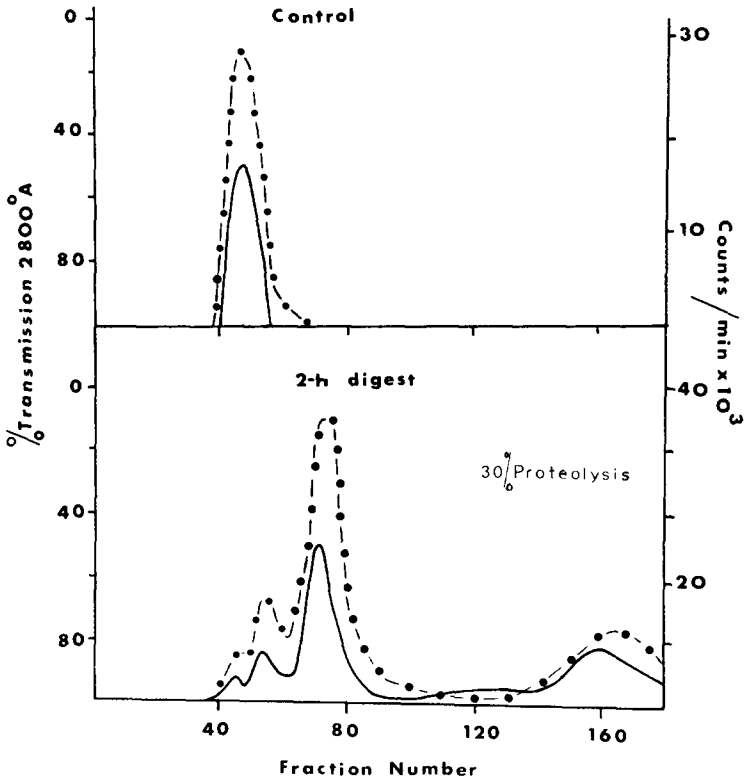

Fig. 4. The elution patterns on Sephadex G-100 of normal labelled rabbit γ-globulin and of a sample which had been digested with a rabbit splanchnopleur extract for 2 h. Proteolysis was determined by measuring the release of radioactive soluble material on precipitating an aliquot of 0.05 ml of the digest with 1.0 ml of 0.6 M trichloroacetic acid. ———, transmission at 2800 Å; —●—●—, counts/min radioactivity.

Fig. 5. The mean proteolytic activities of rabbit foetal tissues. Extracts of amnion, chorion, splanchnopleur and foetal liver, were adjusted to concentrations of 10 mg of protein per ml, and their proteolytic activities examined by incubating with denatured albumin and measuring the absorbance of the 0.6 M trichloroacetic acid soluble material at 280 nm[4]. Four differing extracts of each tissue were examined, the vertical lines on the tops of the columns represent the range of individual values.

though some proteolytic activity is associated with all the separated cell fractions, the major portion came down with the mitochondrial fraction. The distribution of proteolytic activity after isopycnic centrifugation of a washed mitochondrial fraction resembles that obtained by BEAUFAY[8] in his examination of a similar fraction of rat liver. The values of the sedimentation densities of the protease and cytochrome oxidase activities correspond exactly with those of rat liver lysosomes and mitochondria respectively. It seems reasonable to conclude therefore that a major part, possibly the whole of the acid protease activity of the splanchnopleur cell is associated with lysosomes. The indications of lysosomal particles in the homogenates of the rabbit yolk-sac splanchnopleur is important when it is considered that a property of lysosomes is the digestion of extracellular material which has been taken into the cell by endocytosis[7]. As well as this, BRAMBELL[2] and JACQUES[19], have reasoned that another function of the lysosomal system could be in the vesicular transport of material through the cells. The digestion of bovine and rabbit γ-globulins by extracts of the yolk-sac splanchnopleurs was followed at the optimum pH of 3.6, however the optimum pH for lysosomal digestion *in vivo* could be considerably higher than this, as COFFEY AND DE DUVE[20] have shown, the hydrolysis of some proteins by extracts of highly purified rat liver lysosomes occurs preferentially between a pH of 4.4–5.6. Examination of the digestion products of the bovine and rabbit globulins by molecular filtration, revealed an orderly breakdown of the molecules. Polypeptide fragments with molecular weights less than about 20 000 were not detected, leading to the conclusion that intermediate products below this size must be broken down very quickly to relatively small peptides and amino acids. This last stage of the reaction is probably accomplished very effectively by the peptidases which are known to be present in the enzyme extract. Both bovine and rabbit γ-globulins seem to pass through similar stages of breakdown at the same rates, there is therefore no evidence that *in vivo* the process of proteolysis could set up that selective differential in the passage of protein out of the cell which has such important implications in any overall hypothesis of protein transport. It must be presumed that molecules destined to be transported to the foetal circulation are withdrawn from contact with the lysosomal enzymes at an early stage, or never come in contact with them. BRAMBELL[2] puts forward the hypothesis that receptors exist on the wall of the vacuoles, attachment to which preserves the homologues molecules from cleavage so that they are carried through the cell and eventually discharged, intact molecules remaining free in the vacuolar sap, whether homologous or heterologous, are degraded while the vacuole is passing down the cell.

Comparison of the proteolytic activity of chorion and amnion showed that these tissues are relatively inert compared to the yolk-sac. Only foetal liver showed a value, which though lower, was comparable to that of the splanchnopleur. Clearly this picture confirms that the tissue predominantly involved in protein digestion at this stage of development is the splanchnopleur. BRAMBELL *et al.*[21] have shown that antibodies reach the stomach of the foetal rabbit across the chorion and amnion. The transmission of these proteins is not selective, in marked contrast to their entry into the foetal circulation by way of the splanchnopleur. The very low concentrations of cathepsins found in the chorion and amnion is in accord with these observations, since it is unlikely that any gross protein degradation can take place in these membranes.

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