SUBCELLULAR DISTRIBUTION OF SOME ACID HYDROLASES AND INTRAPARTICULATE HYDROLYSIS OF [^{131}I]ALBUMIN IN MOUSE PLACENTA

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Summary. Four conventional subcellular fractions were obtained from homogenates of mouse placenta on Day 17 of pregnancy. The activity of five acid hydrolases (cathepsin, deoxyribonuclease, ribonuclease, acid phosphatase and β -glucuronidase), as well as an alkaline esterase, were measured in the fractions and in mouse liver homogenates. The specific activities of the acid hydrolases were practically the same in both tissues and their distribution in placental homogenates was similar to that of the liver lysosomal enzymes. The acid hydrolases were mostly sedimentable. Placental esterase was found to be a soluble enzyme; its activity was one-fifth that of adult mouse liver.

After intravenous injection of formaldehyde-treated [^{131}I]albumin, the incorporation of radioactivity and the proteolytic activity of subcellular particles sedimenting at 27,000 *g* for 10 min on the incorporated labelled albumin were measured in placenta and other tissues of low (striated muscle) and high (liver and kidney) intracellular hydrolytic activity. Although the uptake in the placenta was much lower than in the liver and kidney, it was five times greater than in muscle.

This study shows that mouse placenta has a number of highly active lysosomal enzymes, and that it is able to incorporate and digest macromolecules to some degree. The intracellular hydrolytic activity of the placenta may play an important rôle in the defence and nutrition of the fetus.

INTRODUCTION

The view that the placental barrier, in addition to its rôle as a screen in materno-fetal exchanges, behaves as a transitory 'liver' is supported by a number of biochemical and histochemical studies (Ahmed & King, 1959). It is well known that high levels of acid hydrolytic activity are found in liver lysosomes, subcellular bodies which are involved in processes of auto- and heterophagia (De Duve, 1963). Heterophagia with concomitant hydrolysis of the incor-

porated macromolecules has been clearly demonstrated by Mego & McQueen (1965), who injected formaldehyde-treated [^{131}I]albumin into mice and separated, from liver homogenates, subcellular particles able to digest the engulfed albumin.

We therefore attempted to establish to what degree mouse placenta possesses 'hepatic' properties. The subcellular distribution of some acid hydrolases in the placenta has been studied, as well as the ability of the placenta to incorporate and hydrolyse the ^{131}I -labelled albumin. A comparison is drawn with data on liver and other tissues.

MATERIALS AND METHODS

Cell fractionation

The studies were carried out on female albino Swiss mice allowed free access to laboratory chow and water. One or two animals, i.e. the number sufficient to yield suitable amounts of tissue, were used in each experiment. They were killed by fracture of the neck on the 17th day of pregnancy. The placentae were pooled and homogenized in 10 vols of ice-cold 0.25 M-sucrose-tris-acetate buffer (pH 7.4) with a glass-Teflon homogenizer. About 1 g placental tissue was used in each experiment. The homogenates were spun at 600 g for 5 min; the sediments (Fraction N) were suspended in water; from the supernatants (Fraction C), particles sedimenting at 27,000 g for 10 min (Fraction M) and at 48,000 g for 60 min (Fraction P) were obtained, as well as a final supernatant (Fraction S). All pellets were suspended in water and sonicated for 15 sec to ensure that the enzymes had dissolved. All fractions were analysed for protein and for enzyme activity. The activity of acid phosphatase was tested in the presence of inhibitors as well.

Measurement of protein and enzyme activities

Protein was measured according to the method of Lowry, Rosebrough, Farr & Randall (1951). Acid phosphatase (pH 5.0) was assayed with Sigma p-nitrophenol as substrate (Bertini, Mego & McQueen, 1967). The catheptic activity (pH 3.5) was assayed using the substrate employed in the method described by Anson (1937). The substrate was labelled with ^{131}I according to an original radiochemical technique developed in our laboratory. The activities of the other hydrolytic enzymes were measured essentially according to the method described by De Duve, Pressman, Wattiaux, Gianetto & Appelman (1955); the substrates for deoxyribonuclease and ribonuclease were Sigma type III deoxyribonucleic acid and Schwartz ribonucleic acid, respectively (both at pH 5.0). For esterase determination at pH 7.4, β -naphthyl acetate was used as the substrate.

Measurement of incorporation and intraparticulate hydrolysis of [^{131}I]albumin (digestion test)

A solution of [^{131}I]albumin was prepared according to the method of Mego & McQueen (1965) and 0.4 ml was injected into the tail vein. After 20 min, the mice were killed by fracture of the neck. The placentae were pooled

Table 1. Protein content and enzyme activities in the placenta and liver of mice

Component	No. of experiments	Placenta					Liver			
		Total activity /mg of tissue	Total activity /mg of protein	N	Percentage in the fraction M P S	Recovery				
Protein	5	111 ±14.9	—	26.6 ±2.5	15.3 ±2.2	7.8 ±1.8	54.3 ±6.0	103.3 ±4.5	160.6 ±19.0	—
Phosphatase	4	2.87 ±0.43	0.03	30.6 ±5.2	39.5 ±24.9	9.8 ±2.4	31.7 ±8.7	93.1 ±12.3	6.45 ±1.53	0.04
Glucuronidase	4	0.85 ±0.29	0.008	26.3 ±3.7	61.5 ±21.0	7.9 ±1.2	39.7 ±5.1	133.9 ±23.1	1.23 ±0.05	0.008
Deoxyribonuclease	4	257 ±10	2.32	22.7 ±2.1	49.8 ±2.6	6.4 ±1.2	26.1 ±2.6	104.9 ±1.8	422.9 ±37.9	2.31
Ribonuclease	4	770 ±232	6.9	20.5 ±5.1	31.1 ±8.6	2.6 ±3.0	24.0 ±5.07	76.7 ±3.9	1611 ±487	10.0
Cathepsin	4	41.1 ±9.4	0.37	22.4 ±4.3	29.7 ±14.6	7.9 ±5.6	31.3 ±8.9	100.6 ±18.7	58.7 ±4.4	0.37
Esterase	4	12.0 ±1.5	0.11	14.6 ±4.0	24.8 ±3.9	11.8 ±1.0	71.8 ±17.4	106.8 ±2.1	90.6 ±7.4	0.56

The units for total activity were: protein: mg; acid phosphatase: $\mu\text{mol p-nitrophenol utilized/min}$; β -glucuronidase: $\mu\text{mol phenolphthalein utilized/min}$; deoxyribonuclease and ribonuclease: increase of optical density/min; cathepsin: $\mu\text{g haemoglobin hydrolysed in 30 min}$; esterase: $\mu\text{mol } \beta\text{-naphthol utilized in 1 min}$.

and homogenized as described under subcellular fractionation. The kidneys, liver (0.5 g) and striated hind-leg muscle (0.5 g) were treated in the same way. After measuring the total radioactivity in an aliquot, the homogenates were centrifuged at 27,000 *g* for 10 min to obtain a sedimentable fraction (NM) and a supernatant fraction (PS). Fraction NM was used for the digestion test (Bertini, Bari & Mazzei, 1972). In essence, the digestion test is a measurement

TEXT-FIG. 1. Enzyme distribution patterns prepared from the data shown in Table 1. The bars represent the subcellular fractions from mouse placenta in the sequence in which they were obtained: N, M, P, S. The 'relative specific activity' values of the ordinate were calculated by dividing the percentage of total activity in the fraction by the percentage of the protein in the same fraction. C = cathepsin; D = deoxyribonuclease; R = ribonuclease; P = acid phosphatase; G = β -glucuronidase; E = esterase.

of the amount of [131 I]albumin hydrolysed during incubation for 60 min at 37°C in the '27,000-*g*' subcellular particles suspended in an iso-osmotic medium at an adequate pH. For this assay, the particles were gently suspended in 20 ml of 0.25 M-sucrose:0.05 M-acetate buffer (pH 5.0), and the acid-soluble radioactivity was measured at 0 min and after 60 min of incubation in aliquots treated with 25% trichloroacetic acid. The increase in acid-soluble radioactivity during incubation was finally expressed in μ g hydrolysed albumin/g tissue. Fraction PS was treated with 25% trichloroacetic acid to measure separately the acid-soluble and the acid-insoluble radioactivity.

RESULTS

Distribution of enzymatic activity in subcellular fractions

The distribution of enzymatic activity in the subcellular fractions of mouse placenta is shown in Table 1 and in Text-fig. 1. For comparison, Table 1 also shows the activities of the same enzymes in whole liver homogenates. The data clearly show that the specific activities of the acid hydrolases studied were nearly identical with those of the acid hydrolases of liver. These enzymes were mostly sedimentable in placenta homogenates; Fraction M contained the highest activity. By expressing the data of each enzymatic activity in terms of 'relative specific activity' (De Duve *et al.*, 1955), the distribution of acid hydrolases (Text-fig. 1) was found to resemble closely that of the same lysosomal enzymes in rat liver. Esterase activity, however, was found to be mostly confined to the supernatant fractions. The total esterase activity in the placenta was five times lower than in mouse liver.

Effect of inhibitors on acid phosphatase activity

The effect of fluoride or of molybdate on acid phosphatase activity was not the same for all the fractions studied (Table 2). In the presence of these inhibitors,

Table 2. Effect of inhibitors on acid phosphatase activity in subcellular fractions of mouse placenta

Inhibitor	Concentration (mM)	Subcellular fractions			
		N	M	P	S
Fluoride	50	57	75	69	31
Molybdate	1	91	100	100	73
Citrate	20	0	0	0	0

The data under subcellular fractions represent the percentage of activity lost in the presence of the inhibitor.

the activity of the particulate fractions decreased to a greater extent than that of the supernatant fractions. Citrate had no effect on any fraction.

Radioactivity incorporation and digestion test

The data of radioactivity incorporation in the placenta and other tissues, as well as the results of the digestion tests, are shown in Table 3. The highest incorporation was found to take place in liver and the lowest in striated muscle. The uptake by the placenta was five times greater than that of muscle, but about one-tenth that of the liver.

Incubation of the '27,000-g' particles (digestion test) resulted in the formation of soluble radioactivity. The amount of solubilized radioactivity depends on (a) the amount of [^{131}I]albumin trapped by the NM particles, and (b) the hydrolysis rate of the labelled albumin in these particles. Both parameters were high in liver and kidney, and low in placenta and muscle. Since incorporation of acid-insoluble radioactivity in the placenta was higher than in muscle, however, the formation of acid-soluble radioactivity in the placental subcellular particles was six times that of the muscle particles.

Table 3. Incorporation and intraparticulate hydrolysis of [^{131}I]albumin in mouse placenta and other tissues

<i>Tissue</i>	<i>Total radioactivity in the tissue (ct $\times$ 1000/min/g)</i>	<i>Radioactivity in supernatants (% of total radioactivity in the tissue)</i>	<i>Acid-soluble radioactivity in supernatants (% of total radioactivity in the fraction)</i>	<i>Radioactivity in 27 000-g¹ pellets (% of total radioactivity in the tissue)</i>	<i>Acid-soluble radioactivity formed in pellets during incubation (ct $\times$ 1000/min/g)</i>	<i>[^{131}I]-albumin hydrolysed during pellet incubation (μg)</i>	<i>Relative hydrolytic activity (placenta = 1)</i>
Placenta	43.1	86.9	29.1	13.1	0.38	0.13	1.0
Muscle	9.5	88.4	48.4	11.6	0.07	0.02	0.15
Liver	415.3	33.9	41.2	66.1	49.0	16.6	127.0
Kidney	102.8	56.0	51.3	44.0	8.5	2.9	22.3

The data for radioactivity incorporation and the digestion test were obtained from the tissues of animals killed 20 min after the injection of form-aldehyde-treated [^{131}I]albumin, and represent the average of six experiments.

DISCUSSION

The enzymes

The enzymology of the placenta appears to be no less complex than that of liver. The enzymes of human placenta, especially at term, have been extensively studied (see the review by Hagerman, 1969).

Proteolytic enzymes were studied by Arinstein (1926); the activity of other enzymes was examined by Abe (1932) using placentae at various stages of pregnancy. More recently, two nucleases were measured in human placental homogenates (Brody, 1953). In a study by Stark & Jung (1955), alkaline phosphatase activity was found to be confined to supernatant fractions and to a 'microsomal' fraction. This can be explained by the fact that this enzyme is seldom linked to cell membranes; acid phosphatase, on the other hand, might be expected to be contained in particulate fractions prepared at lower centrifugal forces. Instead, Stark & Jung (1955) and Ahmed & King (1959) found it to be mostly soluble in human placental homogenates. In the mouse placental homogenates studied here, acid phosphatase was sedimentable. The reasons for this discrepancy appear principally to be due not to species characteristics, but to the difficulty in obtaining a good homogenate of human placenta, which is much harder to homogenize than mouse placenta. This enzyme was studied in detail by Di Pietro & Zengerle (1967), who demonstrated the existence of at least three acid phosphatases in human placenta, with different molecular weights and different behaviour in the presence of inhibitors.

The activity of β -glucuronidase was studied histochemically by Conte (1965) in the chorionic villi of human placentae at two stages of pregnancy, and by Bulmer (1962) in rat placentae. More recently, the enzyme was purified by Contractor & Shane (1972). Corash & Gross (1972) prepared a lysosomal fraction from human placental trophoblast by density gradient centrifugation. The fraction showed a tenfold increase in acid phosphatase and neuraminidase activity over the total homogenate; the activity was located at a gradient density which differed from that of an enzyme used as a marker for mitochondria.

The work of the latter authors and the data on acid hydrolases presented here demonstrate that a lysosomal system, which can be separated with proper methods, exists in both human and mouse placentae.

There is also reason to believe that placental lysosomes are mainly related to the trophoblast, as might be inferred from the reports of Conte (1965) and Corash & Gross (1972). The electron microscope study by Burgos & Rodríguez (1966) showed that two zones (α and β) can be differentiated in human placental syncytium. The β -zone appears to be mainly involved in synthesis, as indicated by the highly developed ergastoplasm of its cells. The α -zone contains lysosome-like bodies in addition to microvilli, pinocytosis vesicles and canaliculi. These authors also found morphological evidence of transport of material by vacuoles. Their ultrastructural description, therefore, strongly suggests that this zone is involved in active transport and intracellular hydrolysis.

If the syncytium is regarded as the lysosome-rich layer of the placenta, its content of acid hydrolases would be notably higher than that of liver, since the

syncytium represents only a small fraction of the entire mass of placenta which, as a whole, possesses the same enzyme activity as the liver.

Our data on the recovery of some enzymatic activities were remarkably high (β -glucuronidase) or low (ribonuclease). Such apparent increase (or loss) of activity during cell fractionation could be due to the fact that this procedure removes inhibitors (or activators) from particulate fractions. The inhibitors or activators would only act on Fractions C and S. A similarly high recovery for catheptic activity in rat liver subcellular fractions was reported by De Duve *et al.* (1955), and about 160% recovery for cathepsin was found in a rat prostate subcellular distribution study of some acid hydrolases by Bertini & Brandes (1965).

Since the acid phosphatase activity in the supernatant fraction was less affected than that of the subcellular particles, it seems that at least two acid phosphatases are present in mouse placenta: a soluble one and a sedimentable one. The localization of the latter may be ascribed to the lysosomes, but there is no evidence for the cytological site of the former.

Esterase, which was studied here as an example of a non-lysosomal enzyme, has a distribution pattern in the placenta which differs from that of the acid hydrolases; this is in agreement with the esterase distribution in rat liver (De Duve *et al.*, 1955).

Incorporation and intracellular hydrolysis of labelled albumin

The meaning of the digestion test following [^{131}I]albumin injection has been explained elsewhere by us and by other authors working on liver and kidney of the mouse and the toad (Mego & McQueen, 1965; Mego, Bertini & McQueen, 1967; Bertini *et al.*, 1972). The sedimentable amount of radioactivity found in tissue homogenates would indicate the ability of the tissue to incorporate macromolecules. The soluble radioactivity formed during incubation would be the chemical expression of the formation of secondary lysosomes involved in hydrolysis of the incorporated albumin, and it is taken as a measure of the capacity of the tissue to degrade the foreign molecule. Mouse liver and kidney have high activities in this respect (Mego & McQueen, 1965; Bertini *et al.*, 1972). The data presented here for the placenta and other active (liver and kidney) and non-active (striated muscle) tissues show that the placenta is capable of incorporating and degrading a measurable amount of foreign molecules. This activity, though much lower than in liver and kidney, becomes relevant if compared with that of muscle.

Whereas placental tissue presents a level of enzymatic activity equal to that of liver, its ability to incorporate and degrade macromolecules is much lower; this indicates that the acid hydrolytic activity of a given tissue does not necessarily bear a relationship to its ability to incorporate and degrade macromolecules. Toad kidney, in spite of its high content of lysosomal enzymes, is also incapable of an intracellular digestion rate as high as that of toad liver or mouse kidney (Bertini *et al.*, 1972). Not all tissues with high catheptic and acid phosphatase activity should, therefore, be expected to be able to engulf and digest large amounts of foreign macromolecules, though it may be assumed that every tissue or cell able to do so has high levels of these hydrolytic enzymes.

The ability of the placenta to incorporate and digest maternal proteins compared to its ability to incorporate and digest a foreign protein such as [^{131}I]albumin is at present being studied in experiments with labelled maternal serum proteins in this laboratory. Krauss (1970) found a concentration of aminoacids in human retroplacental blood which was higher than that in the peripheral venous blood of the mother. She suggested that at least one acid protease in the human placenta is responsible for this difference, and that degraded maternal proteins, as well as aminoacids, may be utilized by the fetus as a source of nitrogen-rich compounds.

Another hypothesis concerning the high activity of acid hydrolases in the placenta is that this may imply autophagic activity in view of the need for continuous and rapid rearrangement, renewal and repair of cell structures in this tissue.

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