The Steady State Concentration Gradients of an Electron-Dense Marker (Ferritin) in the Three-Layered Hemochorial Placenta of the Rabbit

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ABSTRACT Ferritin was injected into the fetal or the maternal circulation of 27–29-day-pregnant rabbits. After the occurrence of a quasi-steady state, the placentas were prepared for electron microscopy. Ferritin particles were counted in the electron micrographs in the fetal capillaries, in the maternal blood spaces, and in the two interstitial compartments of the three-layered placenta. Under the circumstances of the experiments (excessively elevated plasma ferritin concentrations), no evidence was found for nondiffusional transport of radiolabeled ferritin. Comparison of the standing concentration gradients in the placentas, recorded after maternal and after fetal injection, showed that the interstitial spaces "excluded" ferritin; the plasma-interstitial space ferritin partition coefficients were 10 in the basement membrane space and 3 in the space between the cyto- and syncytiotrophoblasts. 55% of the total concentration gradient across the rabbit placenta occurred across the fetal endothelium, about 45% across the cytotrophoblast, and less than 5% across the syncytiotrophoblast. These figures are believed to reflect the relative contributions of these three layers to the total diffusional resistance in the rabbit placenta. When compared to previous data on the relative contributions of these three layers for small ions and molecules, the present data lead to the conclusion that discrimination of molecular size is a function of the fetal capillary endothelium alone.

INTRODUCTION

Flexner and his co-workers studied placental permeability to hydrophilic materials in different species. This work of the early 1940's led to the generalization that the diffusion resistance of a placenta correlates roughly with the number of placental layers separating fetal from maternal blood (1). Recent electron microscopic studies have shown marked differences in ultrastructure in these layers, even between homonymous layers of placentas of related species (2, 3). It is unlikely, therefore, that further experiments comparing placental permeabilities in different species can bring out the specific properties of the individual histologic layers. It does seem possible, however, to proceed with an orderly characterization of placental permeability if one can compare the diffusion resistances of the individual layers in a given species. For this purpose, we chose the three layers of the hemodichorial placenta of the rabbit.

In many hemochorial placentas, a group of placentas including those of man, guinea pig, rabbit, mice, and rats, there are occasional very thin "epithelial plates" (2–4). The epithelial plates of the rabbit placenta, for instance, are less than 1.5 μm thick. They are further characterized by the presence of numerous pores (2) of about 80 nm diameter in one of the cell layers. The epithelial plates appear to be specialized areas for the diffusional transfer of materials whose transplacental pathways are restricted to extracellular spaces. It is the purpose of this study, therefore, to determine the relative contributions of each of the three placental layers in the epithelial plates to the total diffusion resistance in the placenta of the rabbit.

A previous attempt to separate placental diffusion resistance into its separate components was made by Faber and co-workers (5, 6), who used small hydrophilic materials. These studies showed that about 80% of the diffusion resistance of the three-layer rabbit placenta was localized in its middle layer, the cytotrophoblast, with the outer layers, i.e. the fetal capillary endo-

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thelium on the fetal side and the syncytiotrophoblast on the maternal side, contributing the remainder. In contrast to these previous studies, which relied on transient methods, the present study reports the steady-state concentration gradients across the entire placental barrier. We reasoned that 80% of the total concentration difference between fetal and maternal plasmas should occur across the cytotrophoblast if 80% of the diffusional resistance resides in this layer. The steady-state concentration differences across the individual histological layers were determined by counting electron-dense tracer molecules in the plasma compartments and interstitial spaces of the rabbit placenta. Ferritin was selected as the tracer since ferritin molecules show clearly and individually in electron micrographs. Ferritin is in the proper size range for such an experiment, since the rabbit placenta is permeable to molecules with diameters as large as 400 Å (7).

METHODS

Construction of a placental model. To determine the approximate proportion of the rabbit placental membrane that consists of thin epithelial plates, a portion of rabbit placental tissue previously embedded in Epon (Shell Chemical Co., New York) for electron microscopy was trimmed and serially sectioned into 2-μm-thick sections. These sections were arranged in order on a microscope slide and stained for light microscopy. The sections were photographed with a Zeiss photomicroscope (Carl Zeiss, Inc., New York) with a 40× objective and a magnification at the film plane of 400×. All negatives were printed at a final magnification of 2,700×. Outlines of fetal and maternal vessels of the sections were traced and painted on sheets of Plexiglas of appropriate thickness (Rohm and Haas Co., Philadelphia, Pa.). The positions of epithelial plates on the original sections were verified by light microscopy and were indicated on the Plexiglas sheets. The sheets were then stacked to make a transparent block model of the placenta, and this model was used by the medical illustrator to draw Fig. 1.

Preparation of radiolabeled ferritin with ^{59}Fe or ^{125}I for measurement of placental permeability. Pape et al. (8) dialyzed ferritin against a solution of the chelating agent nitrilotriacetic acid and showed that the iron in ferritin could be mobilized in its presence. Their Fig. 1 (8) suggested chemical equilibrium of ionic iron and ferritin iron after about 120 h.

Twice-crystallized horse spleen ferritin (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio) in the amount of 1 g in 10 ml was dialyzed in the cold against 100 ml of 0.1 M nitrilotriacetic acid in 5×10^{-3} M Tris/HCl and 0.2 M NaCl, pH 7.4, in the presence of 1 mCi $^{59}FeCl_3$, in carrier-free form (New England Nuclear, Boston, Mass.). After 3 days, the ferritin in the dialysis bag was separated from the nitrilotriacetic acid solution by repeated precipitation in half-saturated (final concentration) ammonium sulfate.

In a second labeling method, the outer protein shell of ferritin was labeled with ^{125}I by the chloramine-T method (9). 1 μCi of carrier-free $Na^{125}I$ (New England Nuclear) was used to label 100 mg of twice-crystallized horse spleen ferritin (Nutritional Biochemicals) in 50 ml 0.05 M phosphate buffer, pH 7.0, at 0°C. The minimum amount of chloramine-T necessary for proper labeling had been determined by preliminary titration of ferritin in the presence of starch iodide indicator (9). To remove unbound tracer, the labeled ferritin was washed and concentrated by repeated dialysis against a dextran solution, or was precipitated three times by addition of equal volumes of saturated ammonium sulfate and centrifuged.

Both labeled ferritins were indistinguishable from the original product upon inspection in the electron microscope. After labeling and washing, the ferritins were suspended in isotonic saline and frozen until used. Immediately before use, the ferritins were twice precipitated in ammonium sulfate, centrifuged, and resuspended in isotonic saline to remove label that might have detached from the parent protein during storage. No ferritin batch was doubly labeled, but ferritins from batches labeled with ^{59}Fe and ^{125}I were often used together in the same experiment, since the isotopes are easy to separate by scintillation spectrometry.

All samples were counted in a Packard Tricarb twin-channel scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) with a 3-inch crystal as previously described (7), except that the channels ratio techniques was used to separate ^{125}I and ^{59}Fe activities. Sample volumes were kept below 1 ml.

Determination of placental permeability for ferritin. Time bred New Zealand White rabbits of 27–29 days gestation were anesthetized with halothane in a gas mixture of 30% oxygen in nitrous oxide and catheters were placed in jugular veins of the does for injection and sampling. Each doe was artificially ventilated by means of a Harvard respirator (Harvard Apparatus Co., Inc., Millis, Mass.) with its intake port connected to the anesthesia machine.

In a first group of does no further preparations were made. A second group of does were partially eviscerated; the hepatic circulation was eliminated by ligation of the celiac, superior, and inferior mesenteric arteries. Ligatures of umbilical tape were tied around the esophagus just below the diaphragm, the duodenum, the hilus of the spleen, and the sigmoid colon; the intestine between duodenum and sigmoid colon was excised. Finally, the portal vein was ligated. The eviscerated preparations were given a total of 2.5 g of dextrose by i.v. infusion during the experiments.

At time zero, the does received an intravenous injection of one or two radiolabeled ferritins. A maternal plasma sample was taken a few minutes later and three or more maternal plasma samples were taken at regular intervals in the next hour, after which the experiment was terminated, and the fetuses and placentas removed. Only fetuses that were plainly alive (pulsating umbilical arteries, respiring, or kicking) were kept for analysis. These were all killed and aliquots of the well-homogenized pooled fetuses were used for counting. A total of 15 does yielded living fetuses at the end of the experiment; the numbers of does used in each group are listed in Table I.

The maternal plasma concentration as a function of time was calculated and the average maternal plasma concentration, $\bar{C}^M$ was determined for each of the isotopes. Since fetal plasma concentrations remain close to zero, as do the arteriovenous differences, the mean transplacental concentration difference was approximately by $\bar{C}^M$ (7). The amount that diffused across the placental membrane was determined by the total fetal content (N^F) of the isotope. The placental permeability (P), normalized per gram placental weight (Wt) was therefore calculated from $P = N^F/(\bar{C}^M \cdot$

TABLE I

Placental Permeability Measured in Intact Does and Eviscerated Does with Ferritins Labeled with ^{59}Fe and ^{125}I

Preparations	Label	Permeability Mean	Permeability SEM	n*	P value	P value	P value
		$\mu l \cdot min^{-1} \cdot g^{-1}$					
Intact does	^{59}Fe	2.9	±0.6	6	<0.05	<0.01	≪0.01
Intact does	^{125}I	1.1	±0.3	5			
Eviscerated does	^{59}Fe	1.0	±0.1	7	<0.05		
Eviscerated does	^{125}I	0.5	±0.1	4			

* Number of experiments (does).

$Wt \cdot \Delta t$), in milliliters per minute per gram, where Δt was the duration of the experiment.

Electron microscopy. Pregnant New Zealand White rabbits of 27–29 days gestation were anesthetized with pentobarbital or with halothane in a nitrous oxide-oxygen mixture. Commercial cadmium-free ferritin (Nutritional Biochemicals) was injected into the fetal or maternal circulation to create a measurable steady-state ferritin gradient across each layer of the placenta. We either injected 0.05–0.1 g of ferritin (0.1 g/ml saline) into the fetal vitelline vessels, visible through the uterine wall and exposed by a small uterine incision, or we injected 2–4 g into a maternal ear vein.

After a predetermined period, the animals were killed. To prevent capillary washout, the umbilical cord was clamped and the placenta was quickly excised and cut into slices 4 mm thick while submerged in excess fixative. The placenta was fixed in 2.5% gluteraldehyde in 0.1 M phosphate buffer or in a mixture of 2.5% gluteraldehyde and 2% formalin in 0.1 M phosphate buffer. After about 45 min the slices were cut into thinner slabs and allowed to fix for an additional 30 min. Finally, the tissues were cut to embedding size and fixed for 30 min before being placed in a 0.1 M phosphate buffer, pH 7.3, at 4°C overnight. The tissue blocks were postfixed for 90 min in buffered 2% osmium tetroxide and dehydrated in an ethanol series. The tissues were embedded in either Epon-Araldite (Ciba Products Co., Summit, N. J.) or vinyl cyclohexene dioxide, as recommended by Spurr (10).

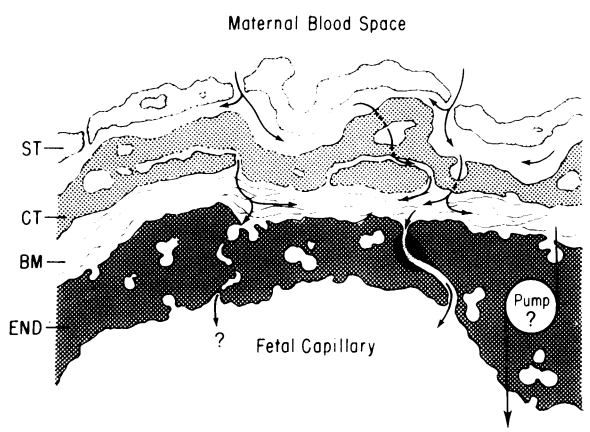

FIGURE 1 Diagrammatic representation of the placental barrier. END, endothelium of fetal capillary; BM, basement membrane; CT, cytotrophoblast; ST, syncytiotrophoblast. The relative resistance to ferritin transfer, found in this study, is indicated for each of the cell layers by its darkness. Hypothetical pathways across barriers are indicated by arrows, which are reversible, except for the direction of the possible pumped transfer across the endothelium.

Thin "plastic" sections were cut on a LKB Ultratome III ultramicrotome (LKB Instruments, Inc., Rockville, Md.) after inspection of 1–2-μm-thick sections under the light microscope. Electron micrographs were made on an RCA 3G (RCA Special Products Div., Camden, N. J.), a Siemens Elmiskop I (Siemens Corp., Medical/Industrial Groups, Iselin, N. J.), or a Philips 200 electron microscope (Philips Electronic Instruments, Inc., Mount Vernon, N. Y.).

Determination of ferritin concentrations in blood spaces and interstitial spaces of placental sections examined by electron microscopy. Ferritin particles were counted by two independent observers only in electron micrographs of epithelial plates that met the following criteria: (*a*) The maternal and fetal blood proteins were of similar density. Plasma protein and the ferritin contained in it may be washed out of the blood spaces during fixation. (*b*) The final print magnification was at least 30,000 ×. (*c*) The cell membranes were intact. Broken cell membranes indicate poor fixation and possible movement of ferritin out of the extracellular spaces. (*d*) The epithelial plates were less than 1.5 μm thick.

Originally, 191 photographs were taken for ferritin counting.[1] 57 photographs from 7 experiments in which the fetus was injected and 63 photographs from 8 experiments in which the doe was injected were acceptable for counting by the above criteria.

The most frequent reason for rejection was poor fixation. In such cases, all Epon blocks were affected and had to be

[1] The rejection of 71 of 191 photographs raises the question whether the retained photographs were truly representative; the same question can be raised regarding the selection of electron microscopic material for photography and counting. A deliberate effort was made to base the decision to photograph an area seen in the electron microscope only on its anatomical aspects. This was made easier by the fact that the ferritin particles were only barely visible on the screen of the electronmicroscope because of their lack of contrast and small size. In the judgment of the observers, there was nothing in the rejected photographs to suggest any modifications of the conclusions reached in this paper.

discarded. In other experiments, several blocks were sectioned and photographed. All acceptable micrographs were counted by two observers with the aid of a 30-power dissecting microscope. Observer A was not aware of the conditions of the experiment. In each photograph, the entire interstitial and vascular areas were counted, with the exception of the vascular compartment (fetal capillary or maternal blood space) in which the tracer had been injected. The concentrations of tracer were so high in the injected compartments that three to six representative squares of 2.5×2.5 cm were counted only. The areas of the micrographs counted were (Fig. 1) the fetal capillary blood space (FC), the interstitial basement membrane space (BM) between fetal capillary endothelium and cytotrophoblast, containing a double basal lamina (2), the interstitial space between cyto- and syncytiotrophoblasts (C-S), and the maternal blood space (MBS). The areas were measured by planimetry, corrected for magnifications, and multiplied by the thickness of the electron-microscopic section to yield volumes. The numbers of molecules, divided by the volumes and divided by Avogadro's number, yielded the ferritin concentrations for each of the four extracellular compartments of the rabbit placenta.

RESULTS

Placental model. A three-dimensional representation of the microcirculation in the rabbit placenta, based on serial sections, is shown in Fig. 2. The figure is analogous to a photograph of a cast of fetal and maternal exchange vessels [2] after digestion of the placental tissues. In addition, it shows the positions of the epithelial plates.

The unexpectedly large size of the maternal exchange vessels was confirmed by repeated visual inspection of the original sections and many other sections of the rabbit placenta. We found plane sections to be deceptive indicators of vascular volume. It can be seen that the epithelial plates form only a small part of the total surface area of the placental barrier. Electron micrographs demonstrating epithelial plates are shown in Figs. 3, 6, and 7.

Is there active or facilitated transfer of ferritin? Placental permeability, as defined in Methods, was determined in both intact does and eviscerated does with the ferritins labeled with ^{59}Fe and ^{125}I. The results, listed in Table I, were all of the same order of magnitude, but nevertheless, there were clearly important and statistically significant differences between the four groups.

FIGURE 2 Drawing of a "cast" of the fetal and maternal blood spaces in the placenta of the rabbit. The plexiglass reconstruction consisted of a stack of eight sections of a total thickness of 3.6 cm, a width of 16 cm, and a length of 18.7 cm. The drawing views the reconstruction from above. The total width represented by the model is about 75 μm. The system consisting of narrow channels is the fetal capillary system; the system consisting of wide, irregular channels is the maternal vessel system, which cannot strictly be called a capillary system since a maternal endothelial lining is absent (see Figs. 1 and 3). The maternal vessel system is referred to as the maternal blood space (MBS) in the text and figure legends. The 16 narrow white lines between the fetal and the maternal vessel systems indicate the positions of the epithelial plates. Everywhere else, the diffusion distances between the fetal and maternal vessels are very much larger (see Fig. 3).

Two additional experiments showed that the permeabilities of free ionic ^{125}I were 22.0 and 21.0 and those of free nitrilotriacetic acid-chelated ^{59}Fe were 8.6 and 2.2 $\mu l \cdot min^{-1} \cdot g^{-1}$. The high permeabilities of the unattached labels indicated that the presence of loose label in the ferritin experiments was a potential source of serious error and possibly the origin of the differences between the four groups of experiments.

Unfortunately, neither ferritin itself (11, 12), nor the labels, iodine and iron, are metabolically inert. It was

[2] Like the human placenta, the rabbit placenta is discoid and has a hemochorial histological structure, but the rabbit placenta is labyrinthine, without villi, and without an intervillous space. The maternal blood is contained in channels of capillary shape and dimensions, although the channels are not lined with endothelium. In the low-power electron micrograph of Fig. 3, there is a maternal blood space (MBS) just to the left of the fetal capillary (FC) in the center; the maternal blood space is like that capillary, except that it has a somewhat larger diameter.

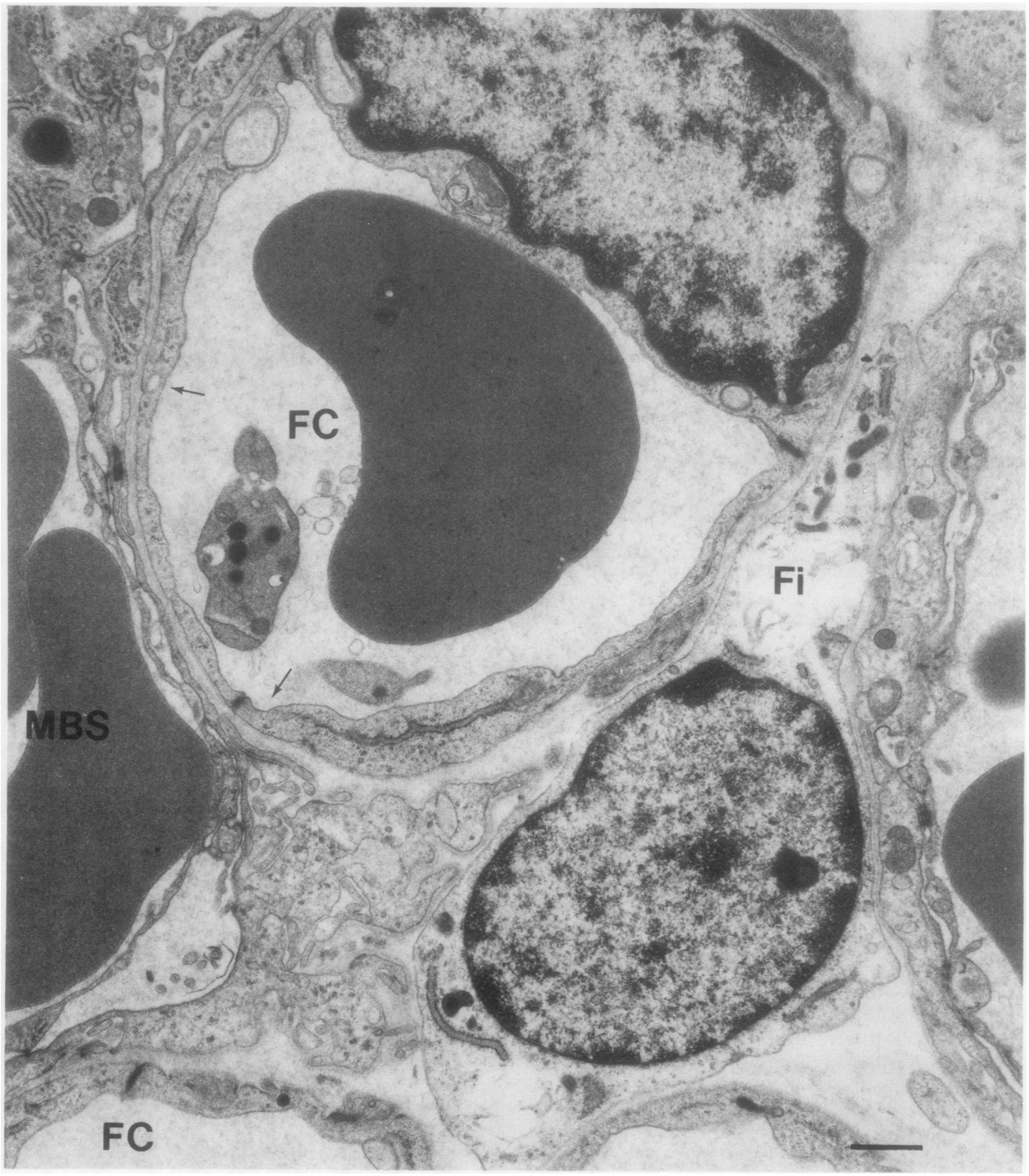

FIGURE 3 Rabbit placenta; a typical epithelial plate on the left side of the central fetal capillary is delineated by arrows. FC, fetal capillary; MBS, maternal blood space; Fi, fibroblast. The three-layered nature of the barrier (endothelium, cytotrophoblast, and syncytiotrophoblast) is clearly visible. The bar is 1 μm long.

not possible, therefore, to determine by precipitation of protein in the homogenized fetal tissues whether the fetal radioactivity had reached the fetus while still attached to ferritin. Although 20–75% of the radioactivity was precipitable, one cannot rule out that label that crossed the placenta while unattached to ferritin was

TABLE II

Ferritin Concentrations in the Extracellular Spaces of the Rabbit Placenta, Determined by Particle Counting by Observers A & B

	Observer	FC	BM	C-S	MBS
			μmol/liter		
Background, in noninjected material	A	0.010	0.058	0.107	*{0.010
	B	0.015	0.087	0.107	*{0.025
Mean in seven preparations in which fetus was injected with ferritin	A	7.73	0.34	0.21	0.25
	B	9.03	0.36	0.26	0.22
Mean in eight preparations in which mother was injected with ferritin	A	0.075	0.51	1.8	6.6
	B	0.063	0.37	1.6	5.8

FC, fetal capillary; BM, basement membrane space; C-S, space between cyto- and syncytiotrophoblast; MBS, maternal blood space. Data are raw data, not corrected for background.
* This is the only statistically significant difference between observers A and B ($P < 0.05$). Since this may easily happen by chance once when 12 differences are considered, we attached no importance to this P value.

subsequently incorporated into fetal protein molecules. Conversely, nonprecipitable label could have been removed from ferritin only after having reached the fetal circulation.

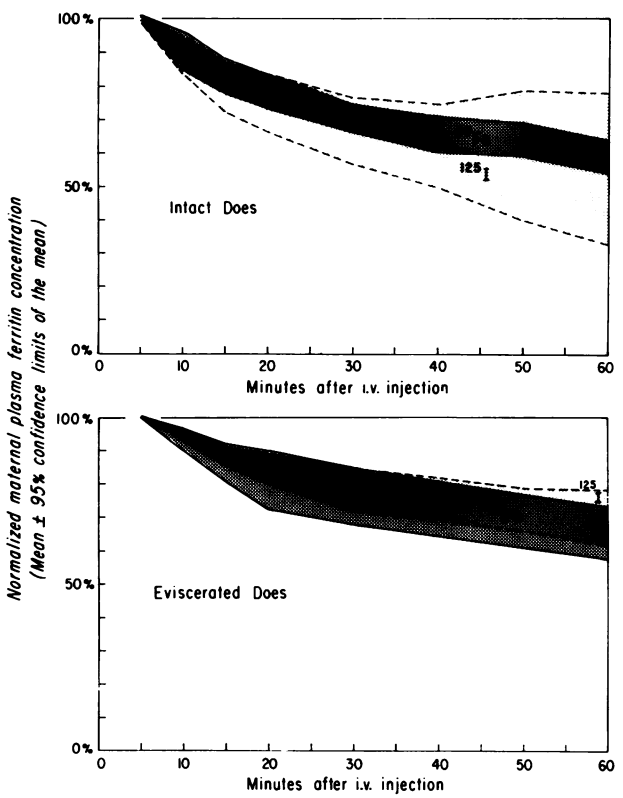

FIGURE 4 Disappearance of radioferritin from the maternal plasma after injection at time zero. Data are normalized by setting the concentrations at time of the first samples equal to 100%. Shaded areas are ±95% confidence limits of mean values. The half disappearance times of both ^{59}Fe- and ^{125}I-labeled ferritin are longer in the partially eviscerated does than in the intact does, but there is no great difference between the disappearance rates of the two isotopes.

Circumstantial evidence that the presence of loose label in the maternal circulation partially accounted for the differences between the four groups in Table I was found in the following: (*a*) Labeled ferritin disappeared faster from the maternal plasma in intact does than in eviscerated does (Fig. 4), suggesting that maternal hepatocytes removed the labeled ferritin and released free label into the maternal blood (11), (*b*) the ^{59}Fe label was more loosely bound to the ferritin core than the ^{125}I label to the apoferritin shell, since the former but not the latter was dissociated from the ferritin molecule by in vitro precipitation in 10% trichloroacetic acid. These differences between does and ferritins corresponded to the differences in apparent permeabilities listed in Table I.

The presence of loose label in the maternal circulation, however, could not cause an underestimate of the true permeability of the placenta for ferritin. Thus, we considered the lowest value in Table I (0.5 $\mu l \cdot min^{-1} \cdot g^{-1}$) the best approximation of the placental permeability for ferritin, although it might still be a substantial overestimate.

Previous results with the macromolecule polyvinyl pyrrolidone predicted that the diffusional permeability of a molecule as large as ferritin, with a coefficient of free diffusion of 3.6×10^{-7} cm^2/s in water of 20°C (13), is about 0.2 $\mu l \cdot min^{-1} \cdot g^{-1}$ (ref. 7, p. 691). In view of the probability that the lowest experimental value of 0.5 was still an overestimate because of the presence of loose label, we conclude that ferritin was probably transferred by a diffusional process. Fortunately, it can be shown (Appendix II) that the remaining uncertainty about the existence of active transfer of ferritin is without consequence for the main conclusions drawn from this study.

TABLE III

Normalized Ferritin Concentrations Calculated from Actual Particle Counts Corrected for Background Counts

Time after injection	Concentrations				Concentration
	FC	BM	C-S	MBS	
min		%*			*μM*
After injection of ferritin into the fetal circulation					FC
65	100	6.7	0.1	0.8	8.4
70	100	0.6	0.5	0.3	16
120	100	2.4	2.9	4.6	16
180	100	3.1	3.8	1.0	6.5
280	100	9.2	4.5	13.2	2.7
500	100	4.6	2.2	3.0	7.4
720	100	5.7	4.2	3.7	2.1
Mean	100	4.6	2.6	3.8	
SEM	—	1.1	0.7	1.7	
After injection into the maternal circulation					MBS
5	1.1	6.2	32.7	100	5.6
40	2.3	3.8	14.1	100	2.9
42	0.8	4.7	30.0	100	4.4
60	0.7	6.0	15.7	100	7.7
75	2.3	9.7	27.3	100	7.6
90	0.3	2.3	22.6	100	12
220	0.2	8.3	55.3	100	7.1
323	0.7	20.6	63.0	100	1.8
Mean	1.1	7.7	32.6	100	
SEM	0.3	2.0	6.3	—	

FC, fetal capillary; BM, interstitial space between fetal capillary endothelium and cytotrophoblast, containing the basement membranes; C-S, interstitial space between cyto- and syncytiotrophoblasts; MBS, maternal blood space.
* Injected compartment normalized to 100%.

The standing gradients of ferritin in the placenta of the rabbit. Ferritin particles can be confused with other specks in electron micrographs. Micrographs obtained from animals not injected with ferritin were, therefore, analyzed and the computed "background" concentrations were subtracted from all other measurements.

Table II lists the background concentrations obtained by each of the observers, and also the concentrations obtained by them in injected material before the subtraction of background. Except for the background concentrations in the maternal blood space, none of the differences between the concentrations in Table II were statistically different between the two observers at the 5% level. The results of the two observers were, therefore averaged. These backgrounds probably do not represent endogenous ferritin, since "normal" serum ferritin concentrations (12) are two orders of magnitude less than the backgrounds in Table II.

Ferritin counts were made in 57 electron micrographs from seven placentas after ferritin injections into the fetal circulation (Table III). The times between injection of the ferritin and the fixation of the placentas varied from 65 to 720 min. In each experiment the calculated concentrations were normalized by setting the concentration in the injected fetal capillary compartment equal to 100%. Table III shows that these normalized concentrations did not vary systematically with time. Thus, the transplacental concentration gradient may be considered a standing gradient. Fig. 5A shows the mean normalized concentrations in each compartment with their 95% confidence limits. Plainly, the greatest concentration drop occurred across the fetal capillary endothelium that separates the fetal capillary blood from the

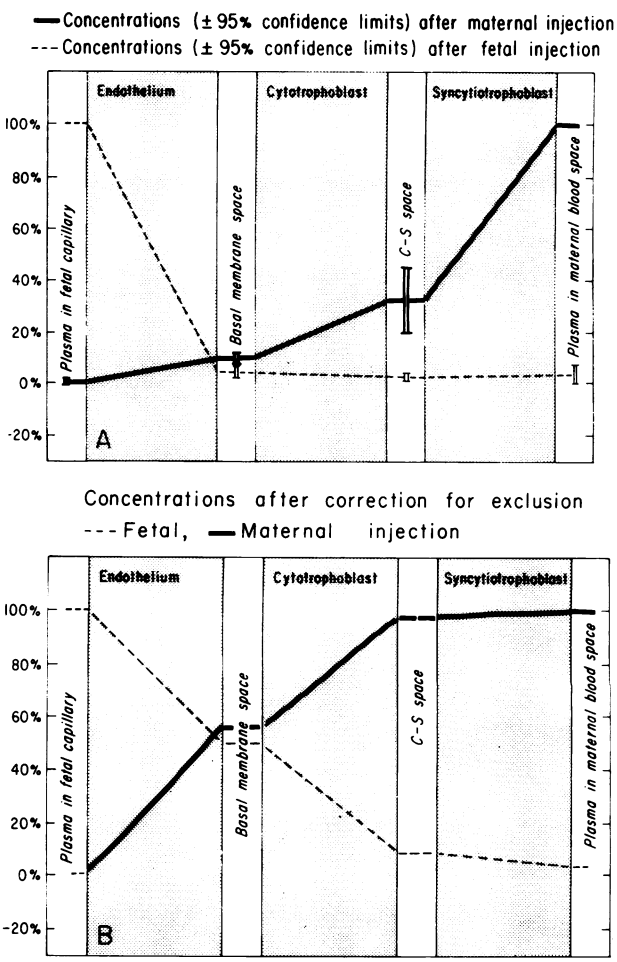

FIGURE 5 A. Graphical representation of the ferritin gradients across the rabbit placenta after fetal or maternal injection of ferritin. The concentrations are based on counts of ferritin particles in electron micrographs and calculations of the volumes counted. For comparison all concentrations are normalized by division by the concentration found in the injected compartment and expressed as percent, ±95% confidence limits (Data in Table III). B. Graphical representation of the ferritin gradients after the interstitial concentrations in the basement membrane space and the C-S space were corrected for exclusion. Data from Table IV.

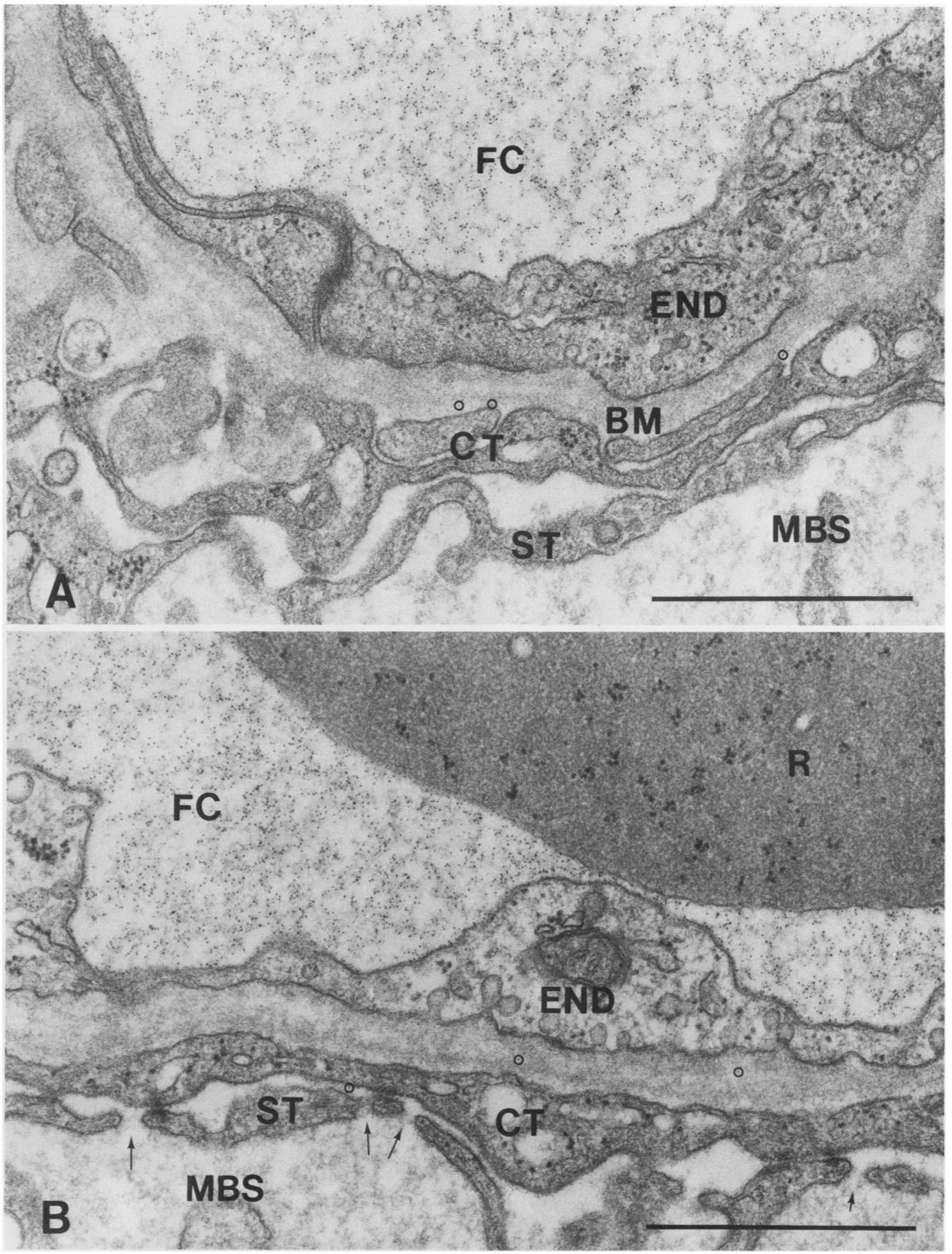

FIGURE 6 Ferritin injections into the fetal circulation. Ferritin particles in the basement membrane (BM) have been circled. A shows an endothelial cell junction, common in epithelial plates. B shows a great abundance of ferritin-filled vesicles in the endothelium (END), some seemingly arranged in chains, and a fetal reticulocyte (R). Both figures show the invaginations in the cytotrophoblast (CT), and B shows the pores (arrows) in the syncytiotrophoblast (ST). Basement membrane matrix material may partially fill the endothelial junctions. Bars are 1 μm.

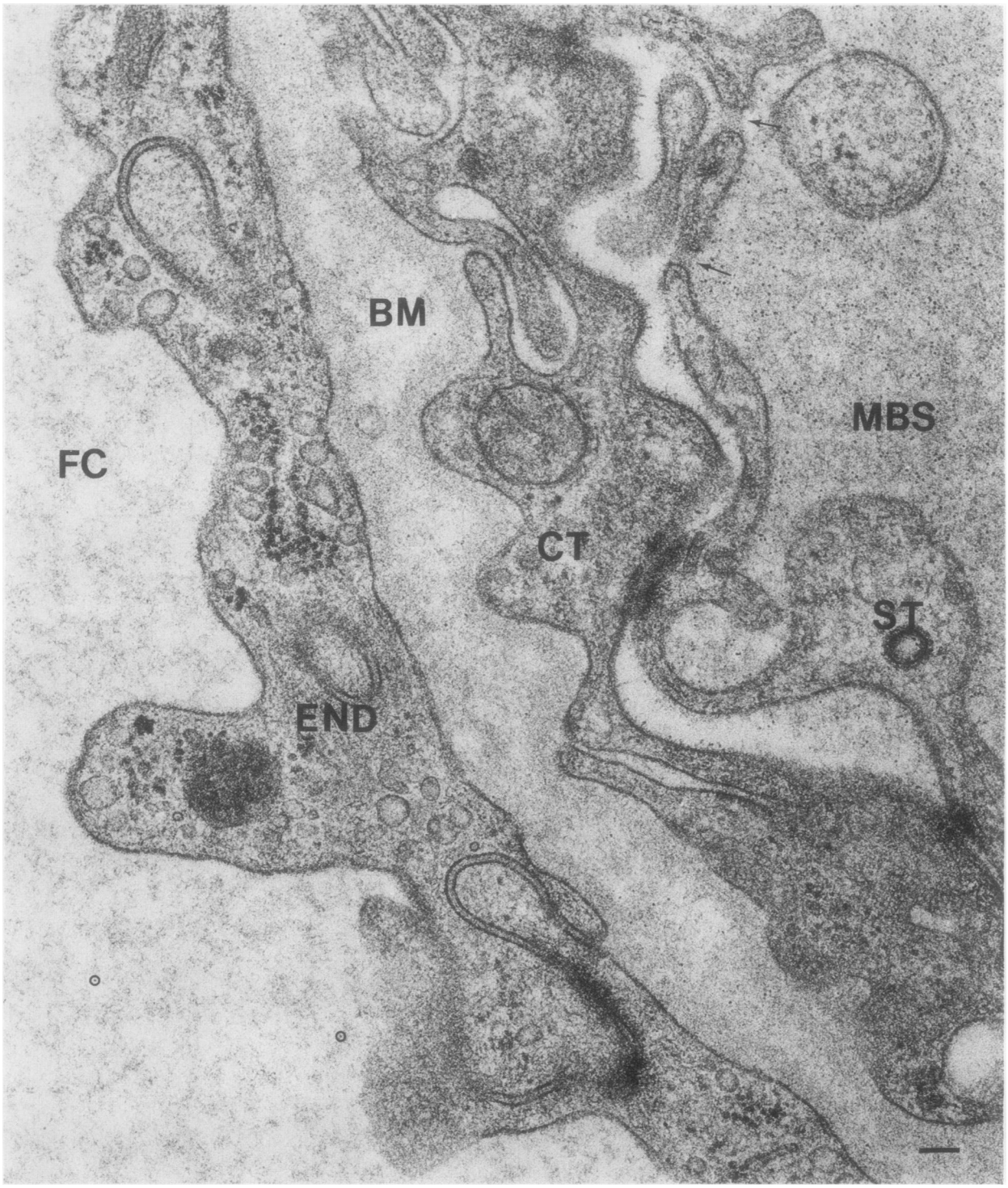

FIGURE 7 Ferritin injected into the maternal circulation. Ferritin and (plasma) protein readily enter the C-S space through large pores (arrows) in the syncytiotrophoblast (ST). Unlike micrographs obtained after fetal injection of ferritin, these micrographs show appreciable concentrations of ferritin in the basement membranes (BM). Ferritin particles in fetal capillary (FC) are circled. The pathway across the cytotrophoblast is not apparent in electron micrographs. END = endothelium. Bar is 0.1 μm.

TABLE IV

The Variables in Eqs. 4 and 5 (Appendix I) as Calculated from the Experimental Results, and after Correction of C^{BM} and C^{C-S} by Multiplying These Concentrations by K^{BM} and K^{C-S}, Respectively

		$\left(\frac{C^{FC}}{C^{FC}-C^{MBS}}\right)^{F\to M}$	$\left(\frac{C^{BM}}{C^{FC}-C^{MBS}}\right)^{F\to M}$	$\left(\frac{C^{C-S}}{C^{FC}-C^{MBS}}\right)^{F\to M}$	$\left(\frac{C^{MBS}}{C^{FC}-C^{MBS}}\right)^{F\to M}$
For diffusion from the fetus to the mother, seven experiments:	Mean	1.042	0.049	0.027	0.042
	SEM	±0.020	±0.012	±0.007	±0.020
After correction of C^{BM} and C^{C-S} for exclusion/dilation:	Mean	1.042	0.496	0.080	0.042
	SEM	±0.020	±0.122	±0.021	±0.020
		$\left(\frac{C^{FC}}{C^{MBS}-C^{FC}}\right)^{M\to F}$	$\left(\frac{C^{BM}}{C^{MBS}-C^{FC}}\right)^{M\to F}$	$\left(\frac{C^{C-S}}{C^{MBS}-C^{FC}}\right)^{M\to F}$	$\left(\frac{C^{MBS}}{C^{MBS}-C^{FC}}\right)^{M\to F}$
For diffusion from the mother to the fetus, eight experiments:	Mean	0.011	0.055	0.329	1.011
	SEM	0.003	0.009	0.063	0.003
After correction of C^{BM} and C^{C-S} for exclusion/dilation:	Mean	0.011	0.557	0.974	1.011
	SEM	±0.003	±0.091	±0.186	±0.003

interstitial space containing the basement membranes. In fact, the ferritin densities in the basement membrane spaces were so low that it appeared at first as if ferritin did not cross the capillary endothelium at all. Upon close inspection, however, unquestionable ferritin particles were found in the basement membranes, as reflected in the concentrations reported in Table III. Fig. 6A and B exemplify ferritin concentration gradients in the placenta after fetal injection of the material.

Ferritin counts were made in 63 electron micrographs from eight placentas after ferritin injections into the maternal circulation (Fig. 7). The times between injection of the ferritin and the fixation of the placentas varied from 5 to 323 min. In each experiment, the calculated concentrations were normalized by setting the concentrations in the maternal blood vessels equal to 100% (Table III).[3] These mean normalized concentrations and their 95% confidence limits are also plotted in Fig. 5A.

In an additional doe, ferritin was injected simultaneously into the maternal circulation and into two of the fetuses. The placentas were prepared for electron microscopy 38 min after the injections. The concentrations of ferritin in the maternal and fetal plasma spaces were similar, but the concentrations in the basement membrane spaces and in the spaces between the cyto- and syncytiotrophoblasts were much lower than those in the plasmas.

Calculations. As shown in Fig. 5A, the fractional concentration drop across the fetal endothelium depends on whether the tracer is injected into the fetal or into the maternal circulations. The same is true for the fractional concentration drops across the remaining cell layers. This finding can be explained by plasma-interstitial fluid partition coefficients for ferritin that deviate from unity or by the existence of active transport in the barrier.

Consider the first alternative. The plasma-interstitial fluid partition coefficient, K, is defined by $K = C^{plasma}/C^{interstitial\ fluid}$ under conditions of equilibrium, e.g. when both fetal and maternal circulations are injected. The diffusionally effective concentrations of ferritin in the interstitial fluid compartments are K times the observed concentrations. This use of K is analogous to the use of "corresponding quantities" in a theoretic treatment of multi-layer membranes (14). Appendix I demonstrates that in the basement membrane space $K^{BM} = 10.13$ and that in the space between the cyto- and syncytiotrophoblasts $K^{C-S} = 2.96$. Fig 5B shows that when the observed interstitial concentrations of ferritin are corrected for their respective partition coefficients, the diffusionally effective concentration differences across each of the cell layers are the same, irrespective of the direction of net transfer across the barrier. A fraction of 0.546 of the total concentration drop occurs between the fetal capillary and the basement membrane space, 0.416 between the basement membrane space and the space between the cyto- and syncytiotrophoblasts, and 0.038 from the latter space to the maternal blood space (Fig. 5B, Table IV). Thus, the fetal capillary endothelium contributes 54.6% of the total resistance to diffusion of the placenta, the cytotrophoblast 41.6% and the syncytiotrophoblast 3.8% only.

[3] It can be seen in Table III that after maternal injection of ferritin in the two experiments of 220 and 323 min duration, the concentrations in the cyto-syncytiotrophoblastic space were abnormally high. The reason for this is not certain. It is possible, however, that these spaces dilated during fixation (see Discussion) and it is possible that they dilated less than usual in these last two experiments because of unappreciated improvements in technique. However, incorporation in the results or rejection of these two experiments would not appreciably affect the conclusions of this paper.

Appendix II considers the alternative that the observed concentration gradients in Fig. 5A must be explained by the existence of active transfer of ferritin. In this case also, the endothelial layer contributes over one-half of the total diffusional resistance of the rabbit placenta and in this case also, we may conclude that most of the placental resistance to the diffusion of ferritin resides in the endothelial layer of the fetal capillary.

DISCUSSION

There is reason for believing that a quasi-steady state existed in these experiments and that the recorded interstitial concentrations approximated a standing gradient. The root mean square distance ($\bar{x}$) travelled by a molecule in a time interval t is given by $\bar{x} = (2 \cdot D \cdot t)^{\frac{1}{2}}$ (ref. 15, p. 163), where D is the coefficient of diffusion. The epithelial plate is about 1 μm thick. For a root mean square distance of that magnitude, t needs to be only $\sim 10^{-2}$ s if the coefficient of diffusion of ferritin is 3.6×10^{-7} cm^2/s, as it is in water (13). Although the coefficient of diffusion in the interstitial space is undoubtedly less than that in water, there is a factor 10^6 in hand before the necessary time approaches the mean duration of the experiments in Table III.

Measurements of placental permeability with radiolabeled ferritin did not convince us of the presence of active or carrier-mediated transport of ferritin under the conditions of our experiments.[4] If nondiffusional transfer does not occur, we must accept that the gradients observed with the electron microscope are diffusional gradients and that the interstitial partition coefficients are less than one. This could well be due to exclusion of large molecules by interstitial polysaccharides, a process reviewed by Laurent (17). For instance, in a 1.5% hyaluronic acid gel a molecule with an equivalent radius of about 61 Å, such as ferritin (18), would be excluded from all but 5% of the water volume of the gel (17). It seems that the "exclusion phenomenon" could be a quantitatively satisfactory explanation for the ferritin concentrations observed in the interstitial spaces of the rabbit placenta and for the calculated partition coefficients K.

A similar exclusion phenomenon is strikingly illustrated by Figs. 14 and 15 in ref. 19, which show abundant concentrations of 32,000-mol-wt dextran in the capillary lumen and in the urinary spaces of the glomeruli a few minutes after injection, but almost none in the intervening basement membranes. Exclusion is also compatible with our observations that when both fetal and maternal compartments were injected, the interstitial concentrations still did not approach the plasma concentrations.

Exclusion is not, however, the only possible explanation for the observed interstitial concentrations. It can be seen in Figs. 3 and 7 that the spaces between cyto- and syncytiotrophoblasts contain a grey material of the same density as plasma protein. It can also be seen that parts of these spaces are often devoid of protein, as if the spaces had dilated during fixation for electron microscopy (Fig. 7). When dilation factors are defined, equations identical to eqs. 1–5 (Appendix I) are derived (20), except that K is the dilation factor. The experimental results cannot definitely distinguish between exclusion and dilation. But it is not necessary to do so to correct for their occurrence; exclusion and dilation give rise to identical correction formulas.

This study focusses on the standing concentration gradients across the epithelial plates (3, 4) of the rabbit placenta. The reconstructed model in Fig. 1 shows that such plates occupy at most a small fraction of the total contact area between fetal and maternal vessels. The belief that the epithelial plates are the specialized structures that serve as the permeable part of the placental barrier for hydrophilic solutes is based on their extreme thinness (Figs. 3, 6, and 7). The syncytiotrophoblast in the epithelial plates also is frequently punctured with holes (2) large enough to permit the passage of large molecules such as ferritin (Figs. 6 and 7).

The finding in the present study that the endothelial layer contributes more than half of the total diffusion resistance to ferritin contrasts with our previous findings (5, 6) that it contributes only some 5–10% of the total diffusion resistance to smaller molecules. Large molecules are less permeable than small ones because large molecules have relatively smaller coefficients of free diffusion in water (15, 16, 21–23), but if the three layers of the rabbit placenta do not discriminate molecular size, there would be no reason to expect that the relative contribution of any one of these layers changes with the molecular size of the diffusing solute.

However, in membranes with water-filled passages of diameters comparable to the diameters of diffusing water-soluble molecules, the diffusion resistance to large molecules may be much greater than expected from the smallness of their coefficients of free diffusion (21, 22). The resistance of such membranes becomes infinite for molecules with diameters equal to or greater than the diameter of the passages. It was previously shown (7) that the rabbit placenta discriminates molecular size in such a fashion, but it was not known in which of the three layers the discriminatory function was located. The fact

[4] This conclusion is subject to this important restriction since it may be valid only for the high ferritin concentrations used in the tracer studies and the even higher concentrations used in the electron microscopic studies. Normal plasma ferritin concentrations are some 10^4 times lower (11) and it is possible that hypothetical active or carrier transport mechanisms exist, but cease to contribute measurably when faced with such excessive concentrations of substrate (16).

that the proportion of the diffusional resistance contributed by the endothelium is much greater for ferritin than for small molecules indicates that the size-discriminating function is located in this layer. Fig. 8 (note logarithmic scales) shows quantitatively that the small resistance of the syncytiotrophoblast and the larger resistance of the cytotrophoblast increase linearly with the diameter of the permeating molecules. The resistance of the endothelium, however, rises disproportionately compared to the increase in molecular diameter, depending somewhat on the assumed diameter of the passages in this layer. We propose that it is because of the narrowness of the intercellular passages through the endothelium, in comparison to the widths of the passages in the other layers, that the relative contribution of this layer to the total resistance to diffusion is so much greater for ferritin than for smaller molecules.

The possible existence of exclusion in the basement membranes implies the existence of molecular size discrimination in this interstitial space, due to differences in the partition coefficients K related to molecular size. Inspection of all electron micrographs taken, however, did not show the existence of ferritin concentration gradients in the extracellular spaces. Probably, the diffusion resistance in the basement membranes (and cytosyncytiotrophoblast spaces) is size dependent in excess of the size dependence of the coefficients of free diffusion, but is so small in comparison to the diffusion resistances across the placental cell layers that it plays no role. In this respect, the placenta appears quite different from the glomerular membrane (19), not because of inherent differences in basement membrane properties, but because of differences in endothelial ultrastructure.

It is possible, however, that the basement membrane matrix invades the diffusion channels in the endothelium and is thus nevertheless responsible for the discrimination of molecular size, a discrimination apparently localized in this cell layer. Unfortunately, we do not know whether the transendothelial pathway is through the cell junctions or through a pinocytotic system (Fig. 1). Several times we saw ferritin particles in the cell junctions, always after fetal injection of the tracer, and never beyond the junctional complex more than halfway towards the basement membrane. These observations would be in agreement with the possible invasion of these junctions by basement membrane matrix and partial exclusion of ferritin, or with obstruction by the junctional complex. But we also saw ferritin in pinocytotic vesicles, as in Fig. 6.

The pathway across the middle layer is enigmatic. Numerous invaginations of this layer give it a Swiss-cheese appearance, suggesting possible transcellular continuity, but continuity has never been actually seen in electron micrographs taken by us. An alternative pathway consists of great lengths of interstitial spaces until

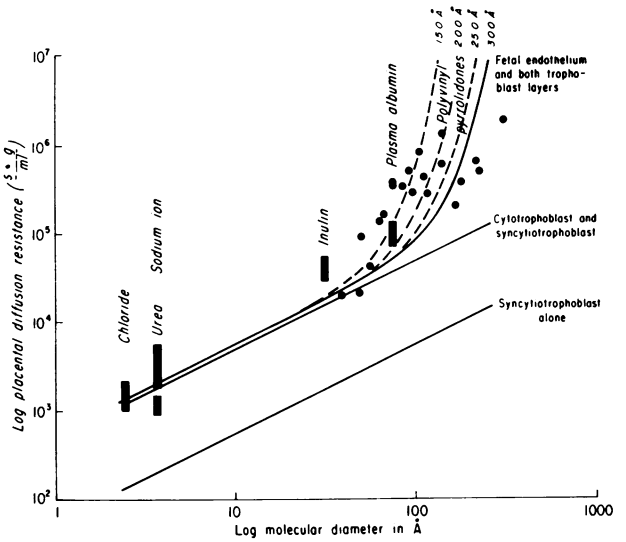

FIGURE 8 Molecular size discrimination in the rabbit placenta. Note logarithmic scales. Bars indicate mean±2 SEM. The average placental resistance is taken from previously published data for urea, chloride, and sodium ion (23) and the two outer layers of the placenta are each assumed to contribute 10% of the diffusion resistance, leaving 80% for the cytotrophoblast. The left-hand sides of the curves are anchored by these considerations. It is seen that for small ions (Na^+ and Cl^-), the rabbit placenta does not discriminate electric charge. The diffusion resistances of the syncytio- and cytotrophoblasts are assumed to increase in proportion to molecular diameter because, according to the Einstein-Stokes equation (15), the products of molecular diameters ($\emptyset$) and coefficients of free diffusion (D) are constant. The value of ($\emptyset \cdot D$) calculated from experimental data on ferritin (13, 18) is 3.393×10^{-13} cm/s, which agrees within 6% with its Einstein-Stokes value at 37°C. Thus, all molecular diameters are calculated from this product ($\emptyset D = 3.393 \times 10^{-13}$) with appropriate values for coefficients of free diffusion. These considerations establish the abscissa and the right-hand sides of the straight lines for the syncytio- and cytotrophoblasts. The diffusion resistance of the capillary endothelium is only about 10% at small molecular diameters (5) but is 54% at the molecular diameter of ferritin. Application of the Renkin formula (21, 22) shows that this increase can be accounted for by the presence of hypothetical cylindrical passages in this layer of 300 Å diameter. The resistance of the endothelium is calculated from the Renkin formula based on a homogeneous population of "pores" of 300 Å, and added to the resistances of the other layers (solid curved line). The right-hand side of the line for the total resistance to diffusion (solid curved line) may therefore be compared with previously published data for the diffusion resistance of inulin, albumin (23), and polyvinylpyrrolidones of different molecular diameters (7). It should be stressed that the value of 300 Å is very sensitive to experimental error. For instance, the resistance of the endothelium calculated from the Renkin formula on the basis of pore diameter of 200 Å (broken line) might give a better fit to the previous data than the resistance based on a 300 Å value. Each broken line is derived from the Renkin formula and represents the resistance to diffusion based on a homogeneous population of pores of the specified diameter.

joined by intercellular clefts of this nonsyncytial membrane. Such a path would account for the high resistance of this layer.

The outer layer on the maternal side, the syncytiotrophoblast, contributes only a few percent of the total resistance for molecules of all sizes (6), an observation easily reconciled with the ultrastructurally evident pore system in this layer (Figs. 6 and 7) and its extreme thinness.

APPENDIX I

Calculation of the interstitial partition coefficients. The flux of the injected tracer is denoted by J (in moles·s^{-1} cm^{-2}). The symbols $J^{F\rightarrow M}$ and $J^{F\leftarrow M}$ are used, depending on whether the injection was made in the fetal compartment (net transfer from fetus to mother) or in the maternal compartment. For brevity, the superscripts $\rightarrow$ and $\leftarrow$ will be used, corresponding to the above definitions and to the directions of net transfer used in Fig. 5. When used with concentrations, e.g. $C^{FC\rightarrow}$, the symbols refer to the concentrations reported in Table III after fetal or after maternal injection.

In a steady state, the flux across the endothelial cell layer of the placenta is the same as the flux across the entire barrier. Fick's law of diffusion may therefore be written as

$$J^{\rightarrow} = (C^{FC\rightarrow} - C^{BM\rightarrow})/R^{END} = (C^{FC\rightarrow} - C^{MBS\rightarrow})/R \quad (1a)$$

for net diffusional transfer from fetus to mother, and as

$$J^{\leftarrow} = (C^{BM\leftarrow} - C^{FC\leftarrow})/R^{END} = (C^{MBS\leftarrow} - C^{FC\leftarrow})/R \quad (1b)$$

for net diffusional transfer in the opposite direction. R is the diffusion resistance in the cell layer identified by the superscript, or the total diffusion resistance if not superscripted.

If the total concentration difference across the placenta is denoted by $\Delta C^{\rightarrow}$ or $\Delta C^{\leftarrow}$, and the J's are eliminated,

$$R^{END}/R = (C^{FC\rightarrow} - C^{BM\rightarrow})/\Delta C^{\rightarrow} \quad (2a)$$

and

$$E^{END}/R = (C^{BM\leftarrow} - C^{FC\leftarrow})/\Delta C^{\leftarrow} \quad (2b)$$

If the plasma/basement membrane space partition coefficient is K^{BM}, the diffusionally effective concentration of ferritin in the basement membrane space is not C^{BM} but $K^{BM}\cdot C^{BM}$. Eqs. 2 become:

$$R^{END}/R = (C^{FC\rightarrow} - K^{BM}\cdot C^{BM\rightarrow})/\Delta C^{\rightarrow}, \quad (3a)$$

and

$$R^{END}/R = (K^{BM}\cdot C^{BM\leftarrow} - C^{FC\leftarrow})/\Delta C^{\leftarrow}. \quad (3b)$$

The quantity R^{END}/R can now be eliminated, and the equations solved with the result:

$$K^{BM} = [(C^{FC\rightarrow}/\Delta C^{\rightarrow}) - (C^{FC\leftarrow}/\Delta C^{\leftarrow})]/[(C^{BM\rightarrow}/\Delta C^{\rightarrow}) - (C^{BM\leftarrow}/\Delta C^{\leftarrow})] \quad (4)$$

The values of all four variables on the right side of eq. 4 can be calculated from the recorded ferritin concentrations in Table III. The means and the standard errors of these variables are listed in Table IV. Their substitution into eq. 4 yields $K^{BM} = 10.13$.

The reasoning that the steady-state flux across one of the cell layers is equal to the flux across the entire barrier can also be applied to the syncytiotrophoblast. A sequence of equations analogous to eqs. 1 through 3 gives rise to:

$$K^{C-S} = [(C^{MBS\leftarrow}/\Delta C^{\leftarrow}) - (C^{MBS\rightarrow}/\Delta C^{\rightarrow})]/[(C^{C-S\leftarrow}/\Delta C^{\leftarrow}) - (C^{C-S\rightarrow}/\Delta C^{\rightarrow})] \quad (5)$$

Again the variables on the right side can be evaluated from the data tabulated in Table III (see Table IV) and substituted into eq. 5, with the result $K^{C-S} = 2.96$.

The lower rows of figures in Table IV top and bottom show the normalized interstitial concentrations after correction for the partition coefficients K^{BM} and K^{C-S}. In Table IV, the total concentration drops from mother to fetus and from fetus to mother, are both equal to 1.000. In each case, the same fraction of the total concentration difference is dropped across the endothelial layer (0.546), the cytotrophoblast (0.416), and the syncytiotrophoblast (0.038), regardless of the direction of net transfer. This requirement for a purely diffusional exchange is thus satisfied by the computed partition coefficients K^{BM} and K^{C-S} and the corrected interstitial concentrations. These results are graphically represented in Fig. 5B, which shows the correct diffusion gradients.

APPENDIX II

The active transport hypothesis. If active transport of ferritin across the placental barrier exists, the direction of transfer must be from mother to fetus, if it is to account for the fetal uptake of radioferritin at rates slightly in excess of those expected from pure diffusion of a molecule of comparable size (Results). The observed gradients in Fig. 5A make clear that such a pump directed from mother to fetus must be situated in the endothelial layer. No other localization could account for transport from mother to fetus across the endothelial layer in the presence of almost no diffusional gradient, nor for the much smaller fractional gradient across the endothelium after maternal injection than after fetal injection.

The alternative hypothesis of active transport is therefore explored on the assumptions that the pump operates from mother to fetus (Fig. 1), that it is located in the endothelial layer, that its activity is proportional to the concentration of ferritin at its inlet side in the basement membrane space, and that the pumping of ferritin may occur against a background of diffusional transfer. Thus, the total flux across the endothelium is the algebraic sum of the pump flux and the diffusional flux. In a steady state, the total flux across the endothelium is equal to the flux across the remaining two layers, whose combined resistance will be symbolized by R^{CS}. This consideration is expressed by a set of equations analogous to eqs. 1 in Appendix I:

$$J^{\rightarrow} = (C^{BM\rightarrow} - C^{MBS\rightarrow})/R^{CS} = (C^{FC\rightarrow} - C^{BM\rightarrow})/R^{END} - p\cdot C^{BM\rightarrow} \quad (6a)$$

$$J^{\leftarrow} = (C^{MBS\leftarrow} - C^{BM\leftarrow})/R^{CS} = (C^{BM\leftarrow} - C^{FC\leftarrow})/R^{END} + p\cdot C^{BM\leftarrow} \quad (6b)$$

where p is the proportionality constant of the pump. The negative sign of p in 6a indicates that the pump acts against transfer from fetus to mother; the values of J are always de-

fined positive. The J's are again eliminated, and the equations rewritten to yield the set:

$$[1 - (C^{BM}/C^{FC})^{\rightarrow}](R^{CS}/R^{END}) - (C^{BM}/C^{FC})^{\rightarrow}(R^{CS}\cdot p) = [(C^{BM}/C^{FC})^{\rightarrow} - (C^{MBS}/C^{FC})^{\rightarrow}] \quad (7a)$$

$$[(C^{BM}/C^{MBS})^{\leftarrow} - (C^{FC}/C^{MBS})^{\leftarrow}](R^{CS}/R^{END}) + (C^{BM}/C^{MBS})^{\leftarrow}\cdot(R^{CS}\cdot p) = [1 - (C^{BM}/C^{MBS})^{\leftarrow}] \quad (7b)$$

The concentration ratios are listed in Table III, and the two unknowns, (R^{CS}/R^{END}) and $(R^{CS}\cdot p)$ can therefore be solved; the result is $(R^{CS}/R^{END}) = 0.563$, and $(R^{CS}\cdot p) = 11.5$. From the ratio $R^{CS}/R^{END} = 0.563$, and the sum $R^{CS} + R^{END} = R$ (total resistance) it is readily apparent to the endothelial resistance to diffusion, R^{END} is 64% of the total resistance.

Whether the gradients in Fig. 5A must be explained by partition coefficients that deviate from unity in the interstitial spaces (Appendix I) or by the presence of a ferritin pump, one may conclude that the endothelial layer of the rabbit placenta contributes somewhat over one half of the total resistance to diffusion for molecules of the size of the ferritin molecule. The reasoning in the Discussion that the endothelial layer is the size-discriminating layer of the rabbit placenta is likewise unaffected by the existence or nonexistence of active transfer of ferritin.

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