DIFFUSIONAL PERMEABILITY OF HUMAN AND GUINEA PIG PLACENTAS TO CYANOCOBALAMIN

by

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Abstract

To examine the molecular size discrimination of the human placenta and establish if its size discrimination is more like the epitheliochorial placenta of the sheep or the histologically similar guinea pig placenta, we studied the diffusion permeability of cyanocobalamin in human placenta and compared this measurement to the diffusion permeability of the guinea pig placenta. The molecular weight of cyanocobalamin is well suited to distinguish between the two patterns of size discrimination found in the hemochorial and epitheliochorial placentas. The concentrations of cyanocobalamin that were used ensured that nondiffusional transport was negligible and experiments on guinea pigs confirmed that the diffusion permeabilities measured with cyanocobalamin were the same as those expected for inert hydrophilic substances of similar molecular weight. All human studies were performed on volunteers who were scheduled for elective cesarean section under spinal or epidural anesthesia. Endogenous plasma concentrations of cobalamin were measured in 10 pregnant volunteers and their newborns. Ten additional volunteers were given 1 mg of cyanocobalamin by i.m. injection 44 minutes prior to delivery. Placental permeability, calculated as the ratio of fetal uptake and the concentration time integral between maternal and fetal plasmas, was 14.3 µl/min per gram placental weight. The permeability of the human placenta parallels that of the histologically similar guinea pig placenta, at a slightly higher level, up to molecular weights of 1355.

Further experiments were performed to establish that the apparent permeability of cyanocobalamin measured in guinea pig placenta was not significantly invalidated by the transfer of cyanocobalamin by the yolk sac or by transfer of plasma bound cyanocobalamin by the placenta. The transfer of

^{57}[Co]-cyanocobalamin was assayed in the presence of excess unlabelled cyanocobalamin in four pregnant guinea pigs in which the vitelline vessels of two fetuses in each pregnancy were ligated. There was no difference in the clearance of cyanocobalamin per gram placental weight between fetuses with ligated vitelline vasculature and those with intact vitelline vessels. An increase in permeability per gram placental weight occurred as fetal weights increased.

To demonstrate that the maternofetal transfer of protein bound cyanocobalamin was a negligible fraction of the flux measured previously when a large excess of free cyanocobalamin was present in maternal plasma, the rate of transfer of protein bound cyanocobalamin to the fetus was measured *in vivo*. An intramuscular injection of a small amount of ^{57}[Co]-cyanocobalamin was given to nine pregnant guinea pigs at approximately 50 to 65 days gestation and fetal and placental content of ^{57}Co measured at either 2, 6, or 24 hours after injection. The majority of ^{57}Co was bound in the maternal plasma. A gradual transfer of radiolabelled cyanocobalamin to the fetus occurred over the 24 hours after injection. The rate of transfer to the fetus of bound cyanocobalamin exceeded the rate of transfer of an equal amount of unbound cyanocobalamin. The total amount of cyanocobalamin transferred to the fetus from the fraction bound in maternal plasma was only a small fraction of the transfer measured previously when a large excess of unbound cyanocobalamin was present.

Introduction:

The molecular size discrimination of the human placenta is not known. It has been assumed that the size discrimination in the human placenta is similar to the histologically similar guinea pig placenta, but this assumption was not based on experimental data (1). The purpose of the following studies was to measure the *in vivo* diffusional permeability of the human placenta to a large lipid insoluble molecule and to compare that permeability to the permeability of the histologically similar guinea pig placenta. Cyanocobalamin (vitamin B_{12}) was chosen because it is a nontoxic molecule which could be injected in quantities sufficient to overwhelm the endogenous carrier proteins. Further, it is slowly metabolized and has an appropriate molecular size to assess differences in placental types.

In the following studies the rate of maternofetal transfer of bound and unbound cyanocobalamin (vitamin B_{12}) was studied in the guinea pig (Cavia porcellus) and the clearance of cyanocobalamin was determined for the human placenta *in vivo* . Cyanocobalamin has a molecular weight of 1355 Daltons, which is well within the range of molecular weights of 60 to 5500 Daltons shown previously to cross the guinea pig placenta by diffusion, but much larger than would be expected to cross the ovine placenta by diffusion (2). Cyanocobalamin is thus an appropriate molecule for assessing differences in diffusional transfer between these two placental types. After maternal injection of an amount sufficient to cause a largely unbound concentration of cyanocobalamin in maternal plasma, the maternofetal clearance of cyanocobalamin was measured in humans at the time of delivery by cesarean section and in pregnant guinea pigs to determine if the placental weight normalized clearance across the human placenta is similar to the placental weight normalized clearance across the guinea pig placenta. To confirm that

these measurements of clearance are measurements of diffusional permeability, the flux of cyanocobalamin was measured across the guinea pig placenta with and without an intact yolk sac placenta, which has been shown to be responsible for transfer of proteins to the guinea pig fetus (2). The rate of transport of bound cyanocobalamin across the guinea pig placenta was measured to confirm that the active transport of the protein bound cyanocobalamin was a negligible amount of the maternofetal flux measured when a large excess of unbound cyanocobalamin was present in maternal plasma.

To review the possible means for transfer of cyanocobalamin across the placenta and the species differences and similarities between the guinea pig and human placentas, previous studies are discussed. The placental structure of the guinea pig and human placenta are compared and permeability studies of these two placentas are reviewed. Finally, current knowledge specific to the transfer of cyanocobalamin across the placenta is discussed.

Mechanisms of transfer across the placenta:

Lipid insoluble substances can cross the placenta by diffusion, carrier mediated (facilitated) diffusion, active transport or by endocytosis. Each of these methods of transport has been proposed for substances transferred from the maternal circulation to the fetus. The study of these transport properties is complicated by differences in the histology of the maternofetal barrier, differences in blood flow to the placenta, and changes in placental structure that occur during the course of gestation.

Diffusional exchange across the placenta for lipid insoluble nonelectrolyes is thought to occur by passage though water-filled channels. Measurements of placental permeability therefore can be made by application

of Fick's law of diffusion, and diffusional flux for the placenta is dependent on the concentration gradient across the water-filled channels dividing the maternal and fetal sides of the placenta. Diffusion is independent of energy metabolism and occurs at a rate proportional to the coefficient of free diffusion of water until steric hinderance or electrostatic forces between the molecule and the pore walls becomes significant or other forces interfere with the free molecules' availability for transfer (1).

Facilitated diffusion differs from simple diffusion in that the rate of transfer is faster than would be predicted by simple diffusion alone. The process depends on carrier proteins to assist diffusion through the cell and therefore can be saturated by excess concentration of the substance transferred or by the presence of other molecules with affinity for the binding site. Facilitated diffusion does not require energy or occur against an electrochemical gradient. Glucose and lactate are examples of molecules that may cross the hemochorial placenta by facilitated transport (3,4).

Active transport may occur against an electrochemical gradient and requires the expenditure of energy. Protein binding of the substrate, metabolic poisons, and competition for a transport site may slow this type of transport. Amino acids are thought to be actively transported across the placenta (5).

Endocytosis is a transfer process that requires incorporation of the molecule into a vesicle by the cell membrane and discharge of the vesicle onto the other side of the membrane. This process is generally slow, requires energy, and may be important for large molecules such as immunoglobulins and for the transport of iron (6-8).

Placental Structure:

The gross shapes of placentas are classified according to the distribution of the chorionic villi over the surface of the chorionic sac. These shapes are diffuse, coteledonary, zonary, incomplete zonary, discoid and double discoid (1). There is no obvious correlation between gross shape and physiologic function.

The histologic structure of the interhemal membrane, by contrast to the gross shape, seems to be related to permeability. Grosser's classification grouped placentas according to the number of maternal tissue layers that remained between the embryonic chorion and the maternal blood (9). These classifications have been modified by the greater detail provided by electron microscopy. The ultrastructual classification divides placentas into epitheliochorial, endotheliochorial, and hemochorial classes (1). The sheep has an epitheliochorial placenta with three maternal and three fetal barriers separating the maternal and fetal blood, while the guinea pig and human have hemochorial placentas, in which the maternal and fetal blood are separated by three layers of fetal origin: a single layer of trophoblast, a noncontinuous layer of fetal connective tissue, and the fetal capillary endothelium (1).

The vascular pattern within the placenta may also differ and is not considered in the above classification. Two vascular patterns are recognized, labyrinthine and villous (10). Complex capillary networks interconnect the fetal blood vessels in labyrinthine placentas. The maternal blood spaces intertwine with these networks. In the villous placenta, fetal blood vessels arise from stem vessels which form multiple branches with few interconnections and supply the surface area for diffusional exchange. The sheep and human have villous placentas, while the guinea pig has a labyrinthine placental vasculature. The

vascular arrangement of the placenta may be of great importance when studying the transfer of small or rapidly diffusing molecules where the delivery of the molecule to the placental barrier rather than the speed of diffusional transfer determines the rate of transfer. However, blood flows are not the rate determining factor of transfer of polar nonelectrolytes. The number of tissue layers separating the maternal and fetal circulation correlates better with the permeability differences between species than does the type of vascular arrangement of the placenta (1,11).

The guinea pig has a yolk sac placenta in addition to its chorioallantoic placenta. The yolk sac placenta is believed to be important in the transfer of macromolecules such as transferrin and immunoglobulins to the fetus (1,2,6-8).

Permeability:

Diffusion of most lipid insoluble molecules is not dependent on blood flows, but upon the pore size and barrier thickness of the placenta. These parameters have been studied by use of microscopic tracers and by *in vitro* perfusion and *in vivo* permeability experiments to determine the pore size of the placenta.

In the guinea pig placenta the tissue layers separating the maternal and fetal blood are the syncytiotrophoblast, connective tissue, and the fetal capillary endothelium (12,13). The two continuous layers separating the maternal and fetal blood are the syncytiotrophoblast, which lacks obvious pores, fenestrations, or water filled channels, and the nonfenestrated endothelium of the fetal capillaries (12,14,15). This is similar to the human placenta, but differs greatly from the epitheliochorial placenta of the sheep (16,17). The role the fetal endothelium plays in providing a barrier to large molecules is controversial. Some investigators have reported the transfer of small proteins

across the capillary epithelium, while others have reported the same molecules to be impermeable (18,19). Firth and others suggested that the capillary endothelium of the guinea pig placenta restricts the transfer of molecules to those with a radius less than 10nm and that all layers of the placenta must be considered in evaluating the resistance of the placenta to diffusion (20). Other studies have suggested that the syncytiotrophoblast produces the greatest restriction to transplacental diffusion (21-23).

There is little physical evidence that supports the existence of pores connecting the maternal and fetal blood. Perfusion of guinea pig placenta with either electron dense tracers or with peroxidases suggests that connections exist between the lateral intercellular spaces and the subendothelial space (15,18,19,24). These reports vary, however, in the amount of tracers that appeared on the maternal side of the placental barrier after perfusion of the umbilical vessels even when the same tracers were used in the studies. Kaufmann et al., explained these discrepancies (19). They showed that the application of higher than physiologic pressures in their *in vitro* preparation shifted perfusate from the fetal to maternal side of the placenta. These shifts were associated with buds and channels in the trophoblast . After the release of the higher pressure, the placental architecture seemed to return to its normal structure. Tracer proteins, such as ferritin, horseradish peroxidase, hemoglobin, and myoglobin did not cross the placental barrier under more physiologic pressures. In more recent studies lanthium hydroxide was chosen to demonstate pathways across the trophoblast (21). Entry into channels in the trophoblast was demonstrated from both the fetal and maternal side. Together these channels penetrated three quarters of the distance of the trophoblast, though a continuous channel was not demonstrated. The inability to

demonstrate a physical connection was thought to be due to the tortuosity of the channel.

Perfusion studies of the human placenta using electron dense markers have shown that horseradish peroxidase (40,000 Daltons) freely crosses the fetal capillary endothelium (25). A greater amount of resistance to diffusional flow of tracers was present at the syncytiotrophoblast layer (26).

Diffusional permeability of the placenta of several species, including human, was studied in the 1940's (27-29). By use of radioactive sodium ion (^{24}Na), these studies suggested that the placental resistance to diffusion correlates with the number of tissue layers separating maternal and fetal blood. The permeability of the human placenta to sodium ion was similar to the permeabilty of other hemochorial placentas, but markedly different from that in the epitheliochorial placenta of the sheep.

The search for intracellular channels through the trophoblast has been vigorously pursued because of permeabilty and clearance experiments performed on the *in vivo* guinea pig and perfused guinea pig placenta (2,31). Hedley and Bradbury suggested a pore with an equivalent pore radius of 10 nm in the guinea pig placenta as estimated by the increasing resistance shown to molecules as a function of their molecular weight (30,31). Other studies have also suggested a similar pore size in the hemochorial placentas of the rabbit and rat (32,33). Permeability studies of the epitheliochorial placenta of the sheep have shown a much smaller predicted pore size of 0.45 nm which would not permit passage of molecules of 400 Daltons (34).

Hedley and Bradbury found that the perfusion studies of the guinea pig placenta yielded permeabilities that were always greater than the *in vivo* measured permeabilities (30). This may be explained by the data of Leichweiss and Schröder who reported that leakage was a major problem in

the perfused placenta model as in their study only 20% of the placentas were without leaks (35).

Human experiments have largely dealt with *in vitro* perfusion of the placenta. These preparations may be subject to the same problems noted in other perfused placental preparations: leakage, hypoxia, acidosis and tissue degradation. Human placental preparations have been shown to have an ATP/ADP ratio to be only 50 to 70% of that observed in freshly obtained placentas from cesarean sections (36). Most perfusions have also been carried out with highly oxygenated perfusates, but hyperoxia has been shown to cause edematous changes in the placental villi (37). The utility of the *in vitro* human placental preparations in this type of study may thus be limited.

Permeability measurements of a variety of hydrophilic substances in perfused human placentas demonstrates permeabilities similar to those measured *in vivo* in guinea pig placenta and similar to more recent *in vivo* permeabilities measured for cyanocobalamin, inulin and mannitol in pregnant women undergoing cesarean section (29,38-43). The molecular size of the substances studied in these *in vivo* experiments was too small to demonstrate sufficient steric hindrance for calculation of an estimated pore diameter for the human placenta. Sneider and others have suggested the human placenta has a pore radius of 10nm from their studies of the dually perfused *in vitro* placenta, in which the rinsed maternal surface is perfused in a sealed, fluid filled chamber in addition to perfusion of the fetal side of the placenta through the umbilical vessels (40). Clearance of tracers from both the fetal and maternal side of the placenta can thus be studied.

The permeability of the guinea pig placenta has been shown to increase as a function of fetal weight (27,44-46). These changes in permeability correlate with observations of an increase in carbon monoxide diffusion

capacity of the guinea pig placenta with fetal weight (47). Carbon monoxide transverses the cell membrane with ease and, therefore, represents a change in the membrane diffusing area or distance and not a change in pore area. The permeability of lipid insoluble molecules increases with fetal weight as well and suggests that the barrier to their passage also changes with placental maturation. Firth and Farr provided a structural description of the changes in the guinea pig placental membrane over the last third of gestation and noted several alterations that would favor a more efficient exchange of material between the fetal and maternal circulation (14). They reported an increase in surface area available for placental exchange as fetal vessels become larger and microvilli proliferate. The effective diffusion distance between the two circulations thus decreases. Anatomic studies earlier in gestation in the guinea pig also suggest that there are physical changes that may increase permeability as the placenta undergoes morphologic differentiation prior to 36 to 45 days of gestation and as the placental labyrinth becomes a larger percent of placental volume (12). Human placentas also show an increase in branching of chorionic villi prior to the third trimester and a thinning of cytotrophoblast as the placenta ages (16,48,49).

Cyanocobalamin:

Cyanocobalamin (vitamin B_{12}) has been shown to cross the placenta in many mammalian species. Cyancobalamin has been shown to cross the human placenta when a large dose (1 mg) was administered intravenously to the mother before delivery (50). The recommended daily allowance for vitamin B_{12} in adult humans is 3 µg/day and increases to 4 µg/day in pregnancy (51). Most of the vitamin B_{12} absorbed during pregnancy is transferred to the

placenta and fetus (52-54). The means of transfer of cyanocobalamin across the placenta has been the subject of many investigations.

Cyanocobalamin normally circulates in the blood tightly bound to protein carriers, transcobalamin I, II, and III. Seventy to 90% of the circulating cyanocobalamin is bound to transcobalamin I (55). The half life of circulating transcobalamin I is 9 -12 days while the half life of transcobalamin II of 1.3 hours (56). No physiologic function other than serum binding has been demonstrated for transcobalamin I, but transcobalamin II has been shown to be important for entry of cyanocobalamin into cells. Transcobalamin II accounts for 40% to 90% of the unsaturated binding capacity for cyanocobalamin in plasma and, therefore, any unbound cyanocobalamin entering the circulation largely becomes bound to transcobalamin II (56,57).

Entry of cyanocobalamin into cells has been shown to occur by binding of the transcobalamin II-cyanocobalamin complex to a cell surface receptor (58). The transcobalamin II-cyanocobalamin complex then enters a subcellular compartment and the transcobalamin II is released in an altered form back into the circulation (55,59,60). A measurable portion of the tissue bound cyanocobalamin reenters the circulation bound to transcobalamin I or as free cyanocobalamin (55,58).

A receptor for transcobalamin II has been identified on human placenta (61-63). This receptor binds the transcobalamin II-cyanocobalamin complex with higher affinity than apo-transcobalamin II. From analysis of discordant transcobalamin phenotypes in maternal serum and cord serum, it has been determined that the human fetal transcobalamin II is of fetal origin (64).

Uptake of the transcobalamin II-cyanocobalamin complex has been shown in human placental tissue slices from early gestation (65,66). Binding of the transcobalamin II-cyanocobalamin complex was greater than binding of

transcobalamin II alone and binding of free cyanocobalamin was negligible. The fate of the complex after binding was not determined, however, radiolabelled cyanocobalamin reappeared in the incubation solution after binding, but in association with a different circulating protein. This is similar to the binding of the transcobalamin complex and cyanocobalamin uptake in nonplacental tissues.

The *in vivo* transfer of cyanocobalamin across the placenta has been studied by intravenous injection of small amounts of radiolabelled cyanocobalamin into the pregnant mouse (52-54). A larger percentage of labelled cyanocobalamin was retained in dams, fetuses, and placentas when 20 ng was injected (95%) than when 200 μg was given (4.7%). Urinary excretion of the excess cyanocobalamin occurred mostly in the first 3-4 hours after injection. Autoradiography of whole sectioned mouse and fetuses was performed after injection of 50 ng of radiolabelled cyanocobalamin. Most of the initial binding occurred at the placenta and was transferred over hours to the fetus. At 1.5 hours the fetal concentration exceeded maternal concentration. At 24 hours the fetal concentration exceeded placental concentration (53).

In the following studies cyanocobalamin transfer across the placenta of the guinea pig and human was investigated. To do this, first the diffusional permeability of exogenous cyanocobalamin was studied across the human and guinea pig placenta after maternal injection of an amount of cyanocobalamin sufficient to cause a large free circulating concentration. This provided the first *in vivo* permeability measurement across the human placenta for a molecule larger than sodium. Next the effect that elimination of the yolk sac placenta by vitelline vessel ligation on the diffusional uptake of the guinea pig fetus was investigated. Finally, the rate of accumulation of cyanocobalamin by the guinea pig fetus was studied when the injected

radiolabelled cyanocobalamin in maternal serum was largely protein bound. These measurements permit calculation of the effect of bound cyanocobalamin on the measurement of cyanocobalamin flux across the guinea pig placenta when a large excess of unbound cyanocobalamin was present in maternal serum.

Paper 1

Diffusion Permeability of Cyanocobalamin in Human Placenta†

† Previously published,

Willis DM, O'Grady JP, Faber JJ, Thornburg KL. Diffusion permeability of cyanocobalamin in human placenta. Am J Physiol. 1986;250:R459-64.

Abstract

The molecular weight of cyanocobalamin is well suited to distinguish the two patterns of size discrimination found in the hemochorial and epitheliochorial placentas. The concentrations of cyanocobalamin that were used ensured that nondiffusional transport was negligible and experiments on guinea pigs confirmed that the diffusion permeabilities measured with cyanocobalamin were the same as those expected for inert hydrophilic substances of similar molecular weight. All human studies were performed on volunteers who were scheduled for elective cesarean section under spinal or epidural anesthesia. Endogenous plasma concentrations of cobalamin were measured in 10 pregnant volunteers and their newborns. Ten additional volunteers were given 1 mg of cyanocobalamin by i.m. injection 44 minutes prior to delivery. Placental permeability, calculated as the ratio of fetal uptake and the concentration time integral between maternal and fetal plasmas, was 14.3 μl/min per gram placental weight. The permeability of the human placenta parallels that of the histologically similar guinea pig placenta, at a slightly higher level, up to molecular weights of 1355.

Introduction:

The barrier that separates maternal and fetal bloods in the human placenta is classified as hemomonochorial (4). It is composed of two primary tissue layers, the syncytial trophoblast and the fetal capillary endothelium. this histological arrangement is similar to that of the guinea pig placenta, which is also hemomonochorial. However, the arrangement is quite different from that of the three layered endotheliochorial placenta (e.g. dog) or that of the four layered epitheliochorial placenta [e.g. sheep, (6)].

Hydrophilic solutes, although restricted to water filled channels, are known to traverse the placentas of all studied species. The can diffuse across the epitheliochorial placenta of the sheep only when their molecular weight is below 400 (2). However, the hemomonochorial placentas of the rabbit (5, 6) and the guinea pig (3, 8, 13) permit diffusion of hydrophilic solutes of molecular weights above 5000. Controversy surrounds the molecular size discrimination of the human placenta. It has been assumed (6) that size discrimination in the human placenta is like that in the histologically similar placentas of the rabbit and the guinea pig. However, this assumption is not based on experimental data and others have argued on theoretic grounds that the human placenta behaves more like the sheep placenta than like the placentas of the rabbit or the guinea pig (14).

The molecular weight of 1355 of cyanocobalamin (vitamin B_{12}) is ideal for an experimental study of the permeability of the human placenta. The diffusional permeability of cyanocobalamin, when compared to the known permeability of sodium ion (7), would clearly distinguish a size selectivity like that of the guinea pig placenta from a selectivity like that of the placenta of the

sheep. Cyanocobalamin also has a low toxicity (10), is only slowly metabolized and is readily assayed by a radiobinding assay.

Cobalamin is normally found in the plasma at very low concentrations. It is bound to specific protein carriers and it is likely that the carrier proteins are involved in fetal uptake (1,9). In this study, cobalamin uptake by the fetus was measured while maternal plasma concentration was purposely maintained at a level that was several orders of magnitude above normal. The animal experiments of this study corroborate that any co-existing nondiffusional transplacental transport was inconsequential in comparison to transport by diffusion alone.

Methods:

Animal Studies

Sources of animals:

Pregnant guinea pigs were obtained from the breeding colony of the Department of Animal Care of the Oregon Health Sciences University. Pregnancy and the approximate lengths of gestation were determined by palpation and the animals were used when the fetuses were estimated to be 45 to 55 days old. Term in the guinea pigs is about 65 days. All fetuses were weighed at the end of the experiment.

Control cobalamin levels in the guinea pigs:

The dam was anesthetized by administration of ≈2% halothane in a mixture of 80% oxygen and 20% nitrous oxide. This procedure also produces fetal anesthesia. Fetal blood samples and a maternal blood sample were taken by cardiac puncture. All samples were immediately mixed with EDTA for

anticoagulation and centrifuged in a refrigerated centrifuge. Plasma samples were stored at -40^{0}C.

Permeability of guinea pig placenta to radiocyanocobalamin:

An indwelling catheter was placed in a carotid artery of an anesthetized pregnant sow after which the sow was allowed about two hours to recover from anesthesia. A control sample of maternal blood was taken and the sow was given an intramuscular injection of 2.5 to 4 μCi of ^{57}Co labelled cyanocobalamin (10 μCi/mg, Medical Products Division of Amersham Corporation, Arlington Heights, IL 60005). The sow was also given a dose of 1.4 mg/kg maternal body weight of nonlabelled cyanocobalamin (except that in the first sow the dose of unlabelled cyanocobalamin was 15 μg/kg).

About 7 maternal blood samples were taken over the next 90 minutes after which the sow was reanesthetized. The vasculature of the uterus was clamped with hemostats, the time of clamping was noted and a final maternal blood sample was taken. Fetal blood samples were taken and samples of amniotic fluid were collected when possible. All fetuses were alive at the end of the experiment. Fetuses and placentas were weighed. The fetuses were ashed and total fetal radioactivity was determined by counting the ash.

Permeability of the guinea pig placenta to nonlabelled cyanocobalamin:

An indwelling carotid artery catheter was placed as described above and a control maternal blood sample was taken. The sow then received an i.m. injection of 1.4 mg/kg maternal weight nonlabelled cyanocobalamin (Cyanocobalamin injection, U.S.P., I mg/ml, Elkin-Sinn, Inc., Cherry Hill, NJ 08002). Subsequent maternal and fetal samples were obtained as described

in the preceding paragraph and all samples were treated like the control samples.

Fetal volume of distribution of cyanocobalamin in guinea pigs:

Guinea pig sows were anesthetized. An extra amniotic fetal vein, the vitelline vein, was located and catheterized without damage to the amnion (11). When possible two fetuses, in separate horns of the uterus, were used. A known amount of nonradioactive cyanocobalamin was injected into the vitelline catheter from a weighed syringe (average approximately 10 μg per fetus) and flushed into the fetal circulation with 0.2 ml of sterile saline. A fetal blood sample was taken 2 to 30 minutes later by direct puncture of the umbilical cord; the sample was treated as described above. A measured aliquot of the injectate was stored to be analyzed at the same time as the fetal blood samples for the calculation of the injected amount of cyanocobalamin and its fetal volume of distribution. The fetuses and placentas were weighed.

Human Studies

Informed consent was obtained from patients of 20 to 37 years of age who were scheduled for a repeat cesarean section, except for one patient who underwent a primary section because of a prior myomectomy. Patients included in the study were normal pregnant women at term, as evidenced by interview, review of the history, physical examination and ultrasound evaluation. Patients with hypertension, gestational diabetes, renal disease, systemic lupus erythematosus, blood isoantibodies, or labor were excluded.

All patients were given an i.v. infusion of Ringer's solution (USP) before delivery; the usual volume infused was 1.5 to 2.0 liters. Two groups of patients were studied. In the first group, only the control maternal and fetal level of

cobalamin was measured. In these patients, control samples from the maternal vein were taken before the intravenous fluid infusions were started and again at the time of delivery. Cesarean sections on these patients were performed under spinal (1 patient), epidural (8 patients), or general (1 patient) anesthesia. Eight of the ten patients were nonsmokers.

The patients in the second group received exogenous cyanocobalamin. A control blood sample was taken from an indwelling heparin lock catheter in a superficial vein. The patients than received an intramuscular injection of 1 mg of cyanocobalamin about 30 minutes prior to the anticipated time of delivery of the baby. After injection, maternal blood samples were taken at ten minute intervals until delivery. Cesarean sections on these patients were performed under spinal (4 patients) or epidural (6 patients) anesthesia. All of these patients were nonsmokers. At delivery, an attempt was made to collect a sample of amniotic fluid through the exposed membranes but this was not always successful. A section of the cord was clamped between two hemostats. Fetal venous and, if possible, arterial blood samples were drawn from the cord vessels between hemostats. A final maternal venous blood sample was taken also. All samples were collected in EDTA, protected from light exposure, and immediately taken to the laboratory for processing.

Analytical procedures

Separation of protein bound and free cobalamin:

A sephadex G-50 gel filtration column maintained at 4^0C was calibrated with blue dextran, ovalbumin, IgG, and cyanocobalamin. Typical fractionation runs consisted of 90 tubes of 1.5 ml each. About 30 tubes separated the emerging protein peaks from the peak of free cyanocobalamin.

Half a milliliter of plasma in 1 g/ml sucrose was placed on the column. Cobalamin concentration in each fraction was determined by radioassay; when radiolabelled cyanocobalamin was used fractions were counted directly.

Radioassay of cobalamin:

Cobalamin concentrations were determined by means of a commercial Vitamin B_{12} radioassay kit (Becton & Dickinson, Orangeburg, NY 10962).

In this assay, cobalamin in plasma competes with added radiolabelled cobalamin for sites on a standard concentration of binder. Nonbound cobalamin is precipitated with charcoal and bound label is counted in the supernatant. Unknown cobalamin concentrations are read from the standard curve that is generated as part of every assay. We determined "true" cobalamin concentrations by blocking nonspecific binding sites by means of a solution of cobalamin analogues. This blocking solution is a standard part of the commercial kit.

A sample from a frozen pool of plasma was processed with each batch. In our laboratory, the coefficient of interassay variation with this assay was 10% (N = 14) with a first pool and 5.2% (N = 10) with a second pool of plasma. The absolute levels of cobalamin reported here depend on standards supplied by the manufacturer of the assay. However, the correctness of the values to be reported for volumes of distribution and placental permeabilities are independent of the accuracy of the standards used in the assay since the same scale factor appears in the numerator and denominator of the equations used to calculate these parameters.

^{57}Co activity was determined in a Nuclear Data ND600 system with a Packard Auto-gamma detector and sample changer.

Results:

Animal Studies

Endogenous cobalamin levels:

The mean plasma concentrations of cobalamin measured in 11 pregnant sows was 1.53 ± 0.16 (SEM) ng/ml and the mean concentrations in the plasma of 49 fetuses was 11.3 ± 0.16 SEM ng/ml. Mean fetal weight of these fetuses was 75 ± 19 (SD) grams.

Volume of distribution of exogenous cyanocobalamin:

Transfer of radiolabelled cyanocobalamin was measured in 19 fetuses carried by 5 sows. Placental permeability was calculated from maternal and fetal plasma concentrations and fetal content. Figures 1 and 2 show that maternal plasma concentrations in between measurement points were approximated by straight line segments. Fetal plasma concentrations of labelled cyanocobalamin were assumed to be zero at the beginning of the experiment and fetal concentration was, therefore, approximated as a straight line segment between zero at the time of injection and the measured concentration at the time of termination of the experiment. Any errors introduced by neglecting the curvatures in the concentration-time lines are insignificant in comparison to the biologic variation in placental permeability.

By definition,

$$PS = N/(Wt^P \times \int[C^M - C^F]dt), \mu l/(min\ g) \quad (1),$$

where PS is the placental permeability in µl/min per gram placental weight, where N is the total content of labelled cyanocobalamin of the conceptus and Wt^P is placental weight (grams). C^M and C^F are the maternal and fetal plasma concentrations, integrated over the period of time between injection of the

radiolabelled cyanocobalamin into the sow and the termination of the experiment.

The conceptual content of radiolabelled cyanocobalamin is the sum of the fetal content and the amniotic fluid content. Fetal placental content could not be measured since it was dwarfed by the content of radiolabelled cyanocobalamin in maternal placental blood, the fetal plasma concentrations at the end of the experiment being much less than maternal plasma concentrations. The content of radiolabelled cyanocobalamin in the blood on the fetal side of the placenta was, therefore, ignored.

Table 1 shows the placental permeabilities measured with radiolabelled cyanocobalamin in guinea pigs.

Placental permeability to exogenous nonlabelled cyanocobalamin:

Placental permeability was measured in 39 conceptuses carried by 10 sows. The total conceptual content of exogenous cyanocobalamin was calculated as the product of: fetal distribution volume (0.28 ml/g), the sum of fetal and placental weights, and the difference between the terminal fetal plasma concentration and 11.3 ng/ml. The value of 11.3 ng/ml was the mean fetal endogenous concentration (see first paragraph of results); it was used as an approximation of the fetal plasma concentration at the beginning of the experiment.

Mean amniotic fluid concentration was less than 10% of fetal plasma concentration. Amniotic fluid content, therefore, was negligible and was ignored.

Fetal plasma concentration was estimated as a straight line between the mean fetal endogenous concentration of 11.3 ng/ml and the measured fetal plasma concentration at the end of the experiment (Figure 1). Mean final fetal

plasma concentration was 379 ± 57 SEM ng/ml. The final fetal plasma concentration averaged 7.3% of maternal peak plasma concentration.

Table 1 shows the results. The two groups of guinea pigs in which permeabilities were measured with radiolabelled and with nonradioactive cyanocobalamin were of comparable gestational age, as indicated by fetal and placental weights, and the calculated placental permeabilities were not statistically significantly different (t = 0.52).

Protein binding of exogenous cyanocobalamin:

Three female guinea pigs received an injection of 1.4 mg/kg cyanocobalamin and blood samples were taken 30 minutes later. Gel filtration of the plasma showed that 99.94%, 99.91% and 98.40% of the cyanocobalamin was present in free form, the remainder eluting with plasma proteins.

Human experiments

Endogenous cobalamin levels:

Ten patients and their newborns were studied. Before the administration of i.v. Ringer's solution mean maternal venous plasma concentration was 0.37 ± 0.06 SEM ng/ml (N = 9). At the time of delivery it was 0.32 ± 0.05 SEM ng/ml (N = 10); the difference was statistically significant by paired t-test ($P<0.005$). Table 2 summarizes the results.

Umbilical venous blood taken at delivery showed a concentration of 0.41 ± 0.06 SEM ng/ml (N = 10), which was not statistically significantly different from either (pre- or postinfusion) value for maternal plasma. Umbilical artery concentrations were measured in 5 samples only; its mean value was not statistically significantly different from the umbilical vein value. Amniotic

fluid values were determined in only six samples; they were all lower than the corresponding cord blood values.

Human placental permeability to cyanocobalamin:

Placental permeabilities were measured in 10 patients (Table 3). Mean maternal plasma level before the injection of cyanocobalamin was 0.34 ± 0.03 SEM ng/ml, which was not significantly different from the mean maternal control levels in Table 2. The average of the highest maternal plasma concentration (which usually occurred at the end of the experimental period) was 27.4 ± 3.4 SEM ng/ml, or about 80 times above the control level.

The mean concentration in fetal cord vein plasma at the time of delivery was 4.1 ± 0.7 SEM ng/ml, which was 10 times higher than the control level in Table 2, but still well below maternal plasma concentration (Figure 2).

The average time between administration of exogenous cobalamin to the mother and the clamping of the cord at delivery was 44 minutes with a range of 27 to 110 minutes.

Placental permeability was calculated from equation 1. The conceptual content of exogenous cyanocobalamin was taken as a fetal distribution volume of 0.28 ml/(gram fetal + placental weight) multiplied by the rise in fetal plasma concentration. The rise in fetal plasma concentration was taken to be the difference between the concentration in umbilical cord plasma at the end of the experiment and the maternal plasma concentration before the injection of the cyanocobalamin. This was based on the assumption that initially fetal plasma concentration was approximately equal to maternal plasma concentration (Table 2). The concentration difference across the placental barrier was calculated as the difference between measured maternal plasma concentrations and the fetal plasma concentration approximated as a straight

line between the control value at the beginning of the experimental period and the cord plasma value at the end of that period (Figure 2). Amniotic fluid concentrations were low (Table 3) and amniotic fluid contents, therefore, were neglected.

A sample of maternal control plasma was mixed with ^{57}Co-cyanocobalamin and nonradioactive cyanocobalamin to a final concentration of 35 ng/ml plasma of exogenous cyanocobalamin. Upon gel filtration, less than 6% of the radioactivity emerged in elution volumes that normally contain macromolecules and 94% of the radioactivity emerged as free cyanocobalamin. The cyanocobalamin that eluted with the macromolecules was filtered again to determine whether bound cyanocobalamin dissociated from its parent molecules during gel filtration. It was found that 84% of the bound cyanocobalamin again eluted with the macromolecules. Figure 3 shows the elution of a maternal plasma sample from one of the pregnant patients who received an intramuscular injection of cyanocobalamin. We concluded that, at the maternal concentrations prevailing during the permeability measurements, most of the exogenous cobalamin was present in unbound form and was available for diffusional transfer.

Mean placental permeability to exogenous cyanocobalamin was 14.3 ± 2.6 SEM μl(min gram placenta), see Table 3.

Discussion:

The purpose of the study is to determine the diffusion permeability of the human placenta for a molecule of a molecular weight above 1000. The method used is based on the assumption that fetal content of the tracer can be approximated as the product of fetal plasma concentration and a volume of

distribution and the assumption that the exogenous cyanocobalamin crosses the placental barrier by simple diffusion only.

The validity of the use of a volume of distribution of 0.28 ml per gram of fetal body weight is supported by the similarity of the permeabilities measured in guinea pigs with nonlabelled cyanocobalamin and with ^{57}Co labelled cyanocobalamin (Table 1) since the former did, while the latter did not, require the use of the distribution volume in the calculations.

We offer the following evidence that the exogenous cyanocobalamin behaved mostly as an inert tracer in spite of some additional nondiffusional transfer. The concentrations of exogenous tracer in human patients exceeded the concentrations of cobalamin bound by protein (10) by 20 to 40 times. Almost all of the exogenous cyanocobalamin was in free solution (Figure 3) and it is know that active transport of cyanocobalamin requires its binding to one of the transcobalamins (9). The unbound condition of the exogenous cyanocobalamin is in accord with a distribution volume in fetal guinea pigs that approximates extracellular water volume. Finally, the permeabilities (PS products) measured with either radiolabelled or nonlabelled cyanocobalamin in the guinea pig are in agreement with a diffusional mode of transfer and the known size discriminatory properties of this placenta and the molecular weight of cyanocobalamin (Figure 4).

Figure 4 highlights two other features of placental permeability. First, the range in the permeabilities of any given tracer in different animals is greater than 10 fold (13), far larger than any conceivable experimental error. There is, apparently, great individual variation. Second, the logarithmic scales in figure 4 illustrate that the effect of molecular weight on permeability is so great that small errors of measurement are of little consequence.

It can be calculated (6) from literature data (3,7) that the permeability of Na^+ in the human placenta of about 3.4 $ml \cdot min^{-1} \cdot g^{-1}$ is slightly greater than the Na^+ permeability in the guinea pig placenta (3.2 $ml \cdot min^{-1} \cdot g^{-1}$), when permeabilities are normalized per gram placental weight. The difference is in the same direction and somewhat greater for the permeabilities of the much larger cyanocobalamin (Tables 1 and 3). Unlike the epitheliochorial placenta, the hemochorial placenta does not discriminate the presence of electric charge on a diffusing molecule (5, 6). Thus, the permeability of the human placenta appears to parallel that of the guinea pig placenta at a slightly higher level, at least up to molecular weights like that of cyanocobalamin.

The average placenta of the guinea pig appears to be a quantitatively reliable analog of the average human placenta with regard to the permeability of hydrophilic materials of molecular weights below 1355, in accord with the ultrastructural similarity of the placentas of these two species.

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Table 1

Permeability of exogenous cyanocobalamin in the placenta of the guinea pig.

Cobalamin preparation	Wt^F (grams)	Wt^P (grams)	Amn.fl.vol. (ml)	PS ($\mu l \cdot min^{-1} \cdot g^{-1}$)	(N)
^{57}Co label	80 ± 7	5.3 ± 0.4	3.8 ± 0.6	6.6 ± 1.9	19
Nonlabelled	81 ± 4	5.0 ± 0.2	4.3 ± 0.4	6.3 ± 1.0	39

Means ± 1 SEM. Wt^F is fetal weight, Wt^P is placental weight. None of the differences between mesurements with radiolabelled and nonlabelled cyanocobalamin are statistically significant at $P<0.05$.

Table 2

Control cobalamin concentrations obtained on patients.

Maternal venous blood		Umbilical	Umbilical	Amniotic
pre-inf	post-inf	artery	vein	fluid
0.37 ± 0.06	0.32 ± 0.05	0.43 ± 0.08	0.41 ± 0.06	0.12 ± 0.02
(9)	(10)	(5)	(10)	(6)

Means ± SEM

Concentrations in ng/ml plasma

Difference between pre- and post-infusion values in maternal venous blood, $P < 0.005$.

Table 3

Permeability of exogenous cyanocobalamin in the human placenta.

	Fetal weight (grams)	Placental weight (grams)	Final concentration (ng/ml) Maternal plasma	Fetal plasma	Amniotic fluid	PS µl•/(min•g))
mean	3692	671	27.4	4.09	0.32	14.3
± SD	472	137	10.9	2.08	0.23	8.3
±SEM	149	43	3.4	0.66	0.09	2.6
(N)	(10)	(10)	(10)	(10)	(7)	(10)

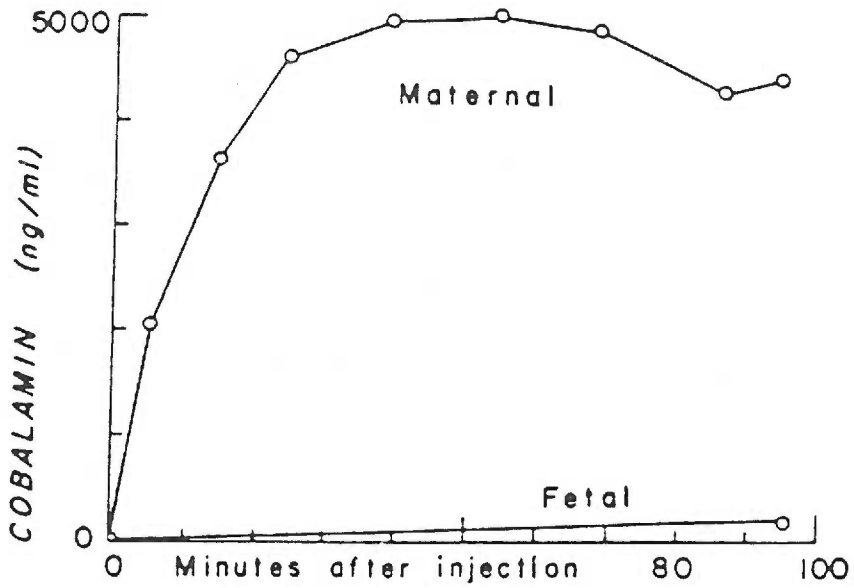

Figure 1. Cobalamin concentrations in maternal and fetal plasma in one of the guinea pigs after intramuscular injection of 1.4 mg/kg cyanocobalamin in the sow. Concentrations at the time of injection are not distinguishable from zero on the concentration scale used.

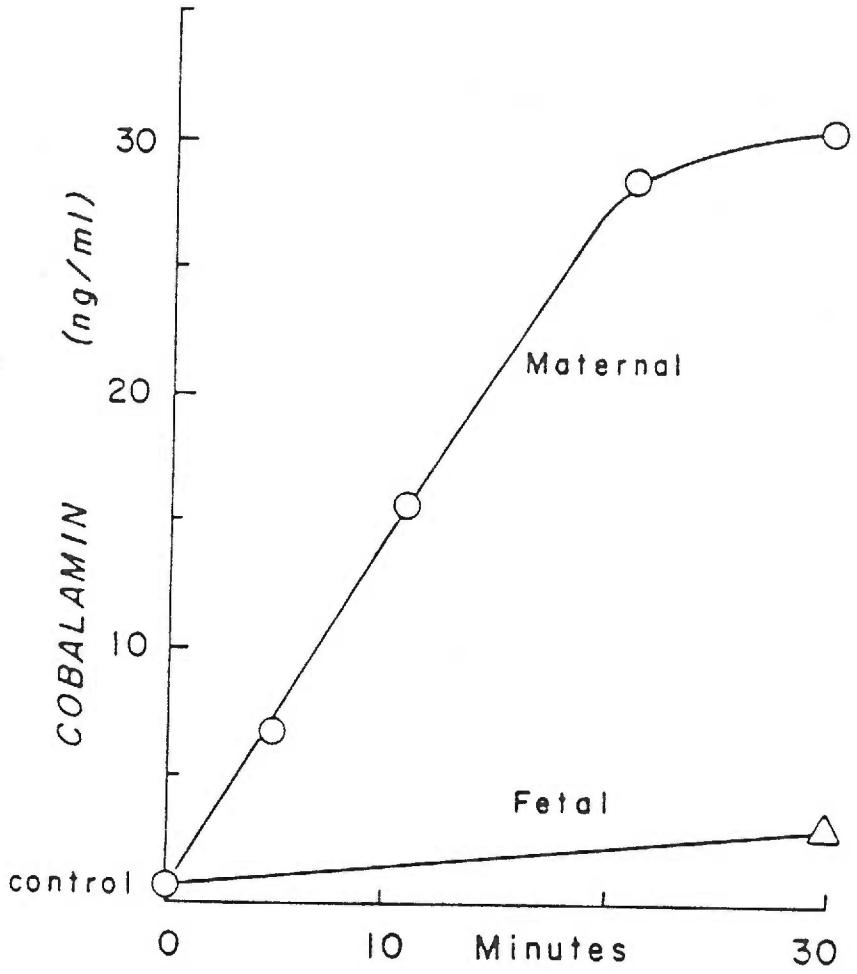

Figure 2. Cobalamin concentrations in maternal and fetal plasma after a patient received an intramuscular injection of 1 mg cyanocobalamin. Maternal control concentration, before injection, is used as starting point for both mother and fetus. The concentration time integral is the area between the maternal and the fetal concentration lines. The rise in fetal concentration is taken as the difference between the maternal control concentration before injection (time zero) and the fetal plasma concentration at the time of delivery of the baby (at 30 minutes after injection).

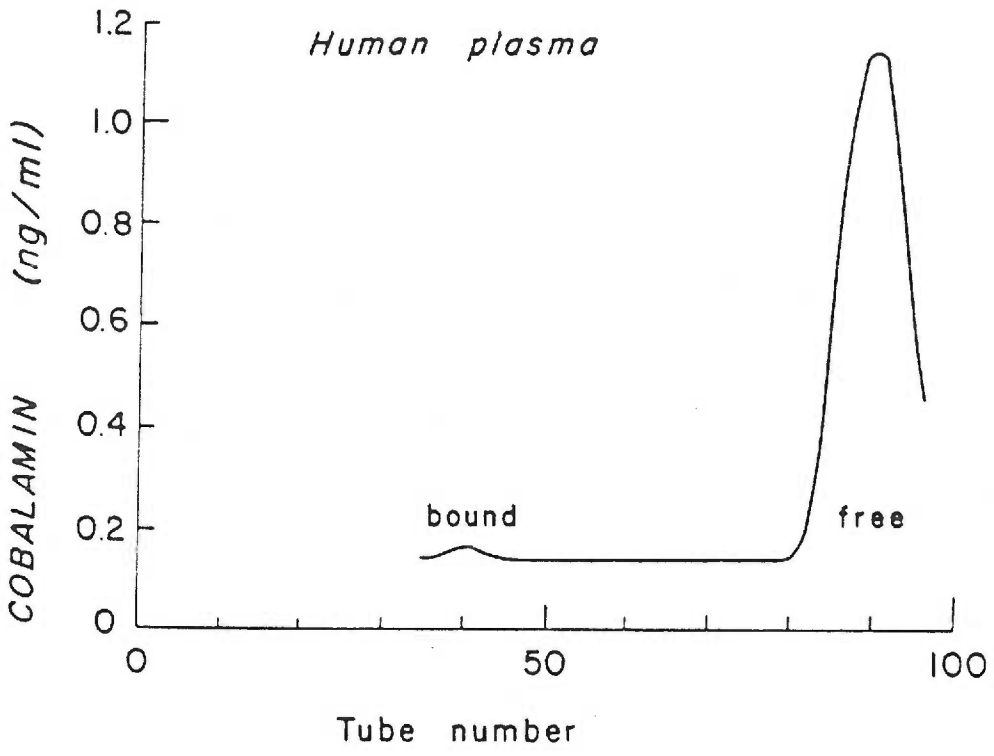

Figure 3. Separation of free and protein bound cobalamin in the last plasma sample from a pregnant patient given 1 mg cyanocobalamin by intramuscular injection.

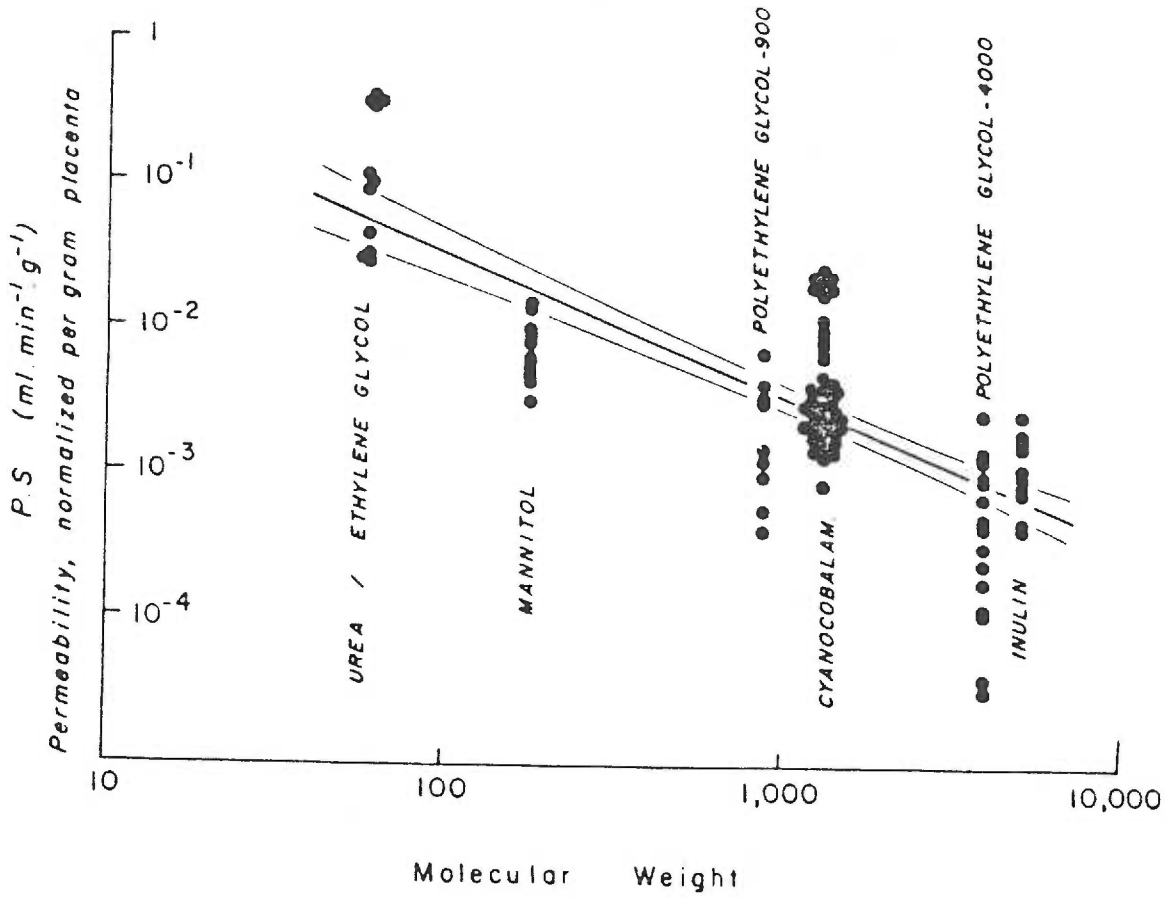

Figure 4. Placental permeabilities of cyanocobalamin in guinea pig placenta and permeabilities measured (13) with other test molecules as functions of molecular weight. Note logarithmic scales. Lines are least squares linear regression with 95% confidence limits of the means.

Paper 2

The Effect of Vitelline Vessel Ligation on the Ratio of Fetomaternal Flux and Concentration Gradients of Unbound Cyanocobalamin

Abstract

Cyanocobalamin has been shown to cross the guinea pig placenta by diffusion when injected in quantities sufficient to cause a large excess of unbound cyanocobalamin. Endogenous cyanocobalamin, however, circulates in maternal blood mostly bound to carrier proteins. To establish that the apparent permeability of cyanocobalamin measured previously in guinea pig placenta was not significantly altered by transfer of free or bound cyanocobalamin by the yolk sac, the transfer of ^{57}[Co]-cyanocobalamin was assayed in the presence of excess nonlabelled cyanocobalamin in four pregnant guinea pigs in which the vitelline vessels of two fetuses in each pregnancy were ligated. There was no difference in the clearance of cyanocobalamin per gram placental weight between fetuses with ligated vitelline vasculature and those with intact vitelline vessels. An increase in permeability per gram placental weight occurred as fetal weights increased. We conclude that the previous measurements of placental permeability in the guinea pig were not significantly affected by yolk sac transfer of cyanocobalamin.

Hydrophilic molecules cross the placenta by diffusion or by means of active transport mechanisms. Cyanocobalamin has previously been shown to cross the guinea pig placenta by diffusion when injected in quantities sufficient to cause a large excess of free cyanocobalamin (1). At a molecular weight of 1355 Daltons, it is within the weight range of molecules previously shown to traverse the guinea pig placenta by diffusion (2).

Endogenous cyanocobalamin is mostly protein bound. To confirm that the apparent permeability measured in a previous study (1) was not significantly invalidated by transfer of free or protein bound cyanocobalamin by the yolk sac, the the vitelline vessels of the guinea pig fetuses were ligated and the transfer of ^{57}[Co]-cyanocobalamin was assayed in the presence of excess nonlabelled cyanocobalamin.

Methods:

Four pregnant guinea pigs at approximately 40 to 50 days gestation were obtained from the Department of Animal Care at Oregon Health Sciences University. Pregnancy was determined by palpation and the gestational age estimated by fetal weight at autopsy according to the data of Draper (3). Term gestation is about 65 days in the guinea pig.

The sows were anesthetized with a gas mixture of one volume percent halothane, 80 percent nitrous oxide, and 20 percent oxygen. Using sterile technique, an indwelling catheter was placed in a maternal carotid artery. An abdominal incision was made to expose the gravid uterus. In each uterine horn the vitelline vessels were identified. A small incision was made in the uterus near the entrance of the vitelline vessels into the fetus in one or two fetuses in each sow. The vessels were ligated. This causes deterioration of the yolk sac (2). A uterine incision was made over one fetus in each pregnancy, but the

vitelline vessels were not ligated. This provided a sham operated control in each sow. The remaining fetuses in each sow were nonoperated controls. The uterus and abdomen were closed and the externalized carotid artery catheter taped to the sow's neck for protection. The sow was allowed to recover from anesthesia and surgery.

Twenty-four hours later the sow was given an intramuscular injection containing 1.4 mg/kg maternal weight of cyanocobalamin (Cyanocobalamin injection, U.S.P., 1mg/ml, Elkin-Sinn, Inc., Cherry Hill, N.J. 08002) and an additional injection of 2 μCi of radiolabelled cyanocobalamin. Cyanocobalamin labelled with ^{57}Co (10 μCi/mg) was obtained from the medical products division of Amersham Corporation, Arlington Heights, IL 60005. The injection of 1.4 mg/kg of unlabelled cyanocobalamin into the pregnant guinea pig has previously been shown to result in a cyanocobalamin content in maternal plasma that was 99% unbound (1).

Seven maternal blood samples were collected over the next 90 minutes at 10 to 15 minute intervals after which the guinea pig was reanesthetized, the abdomen opened and the uterine vessels clamped. The time of clamping was noted and a final maternal blood sample was drawn. The fetuses were examined to ensure they were alive, as noted by the presence of movement, respiration or a pulsating heart . Samples of fetal blood and amniotic fluid were taken and the fetuses and sow killed. Fetuses and placentas were collected and weighed. The fetuses were ashed and total fetal radioactivity was determined.

Levels of ^{57}Co were determined in maternal and fetal blood samples, amniotic fluid samples, and ashed fetuses in a Nuclear Data ND600 system with a Packard Auto-gamma detector and sample changer.

Results:

Transfer of radiolabelled cyanocobalamin was studied in seventeen fetuses carried by four sows. Of these fetuses, five had ligated vitelline vasculature, three were sham operated littermates, and nine were nonoperated littermates.

The placental clearance was calculated from the maternal and fetal plasma concentrations and fetal content by the ratio,

$$K_{Wt}=N / (Wt^P \times \int (C^M-C^F)\, dt$$

where K_{Wt} is the clearance, normalized for placental weight, in µl/min/g placental weight, N is the total fetal content of labelled cyanocobalamin in the fetus, amniotic fluid, and fetal blood sample, and Wt^P is the placental weight in grams. $\int (C^M-C^F)\, dt$ is the integral of maternal and fetal concentration difference over the time from injection of the radiolabelled cyanocobalamin until the clamping of the uterine vasculature.

Fetal plasma concentrations of radiolabelled cyanocobalamin at the end of the experiment were only a small percentage of maternal plasma levels and could be ignored in the calculation of the concentration-time integral, as shown in a typical graph of the maternal-fetal concentration gradient (figure 1).

The mean duration of the experiment was 101 ± 2 (SD) minutes. The mean value of K_{Wt} was 1.02 ± 0.38 (SD) µl/min/g placental weight. There was no statistical difference ($p > 0.05$) between the three groups. The values for each group are presented in table 1.

To determine if the weight normalized placental clearance for cyanocobalamin changes with fetal weight as previously reported (4), the logarithms of K_{Wt}/D, where D is the coefficient of free diffusion in water for cyanocobalamin, are plotted against the logarithm of fetal weights for the

previously reported data (4) and the present study, there is a highly significant relationship between these parameters (figure 2).

Discussion:

The yolk sac placenta in the guinea pig has been shown to be important in the transfer of macromolecules from the sow to the fetus (2). Endogenous concentrations of cyanocobalamin are bound to carrier proteins, and these complexes are of a size which could be transported by the yolk sac placenta. This data shows that the rate of transfer of cyanocobalamin per unit concentration to the guinea pig fetus does not change if the yolk sac is eliminated. We can, therefore, conclude that the parameter, K_{Wt}, is the diffusion permeability of the chorionic placenta normalized for placental weight and that the permeabilities measured in the previous report (1) were not significantly effected by transfer across the yolk sac placenta.

Although the value for placental permeability normalized for placental weight for cyanocobalamin in this study differs from that previously published (1), the fetal weights are also significantly lower in the present experiment (table 2). If the effect of fetal weight on placental permeability is considered, the ratios of permeability per gram placental weight divided by the coefficient of free diffusion in water of cyanocobalamin (4.8×10^{-6} cm/sec) are within the 95% confidence intervals predicted by extrapolation of the data of Adams et al., (4).

The permeability per gram placental weight of cyanocobalamin increases with the gestational age of the fetus. This is in agreement with previous reports of the permeability of inulin, cyanocobalamin, and morphine glucuronide, which demonstrated an increase in permeability with fetal weight for fetuses in the last third of gestation, when placental weight in the guinea pig

does not significantly increase (4,5,6). Flexner and Pohl reported an increase in placental flux of sodium ion with increases in the fetal weight of the guinea pig from 28 days of gestation to term (7). In the present study the fetal weights are compatible with an estimated gestational age of 40-50 days. At this gestational age, fetal weights (7.24 to 26.29g) and placental weights (1.7 to 3.56g) increase with gestational age. The increase in permeability per gram placental weight with increases in fetal weight, suggests that the membrane area or diffusion distance does not change as a constant function of placental mass alone, but that restructuring of membrane surface area or diffusion distance occurs throughout gestation. This would be consistent with the thinning of the placental interhemal barrier during gestation described by Firth and Farr (8), the increase in placental vascular volume with gestational age reported by Kaufmann and Davidoff (9), and the increases in the carbon monoxide diffusion capacity with fetal weight in the guinea pig (10). This increase in permeability of the placenta to cyanocobalamin per gram placenta weight occurs during a period when the ratio of placental to fetal weight is decreasing. These observations indicate that the placenta is a dynamically changing structure and not simply a fixed membrane serving only as a diffusional barrier between the mother and fetus, even near the end of gestation.

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Table 1

	Yolk sac tied	Sham operated	Nonoperated	Combined
K_{Wt} (µl/min/g)	0.994	1.261	0.962	1.024
SD	0.269	0.640	0.326	0.368
N	5	3	9	17

No statistical difference exists between these clearances normalized for placental weight, $p>0.5$ (Bonferroni modified t-test for multiple comparisons).

Table 2

	fetal weight (g)	PS/Wt^P†(μl/(min·g)	N
Willis, et al. (1)	80.5 ± 24 (SD)	6.4 ± 6.8 (SD)	58
Present values	17.5 ± 6.9 (SD)*	1.02 ± 0.36* (SD)	17

†Wt^P is placental weight in grams.

* Significantly different ($p \leq 0.002$) than values reported by Willis, et al. (1) .

Table 3

	present study	Willis, et al.(1)
Range of fetal weights (grams)	7.24 - 26.29†	31.7 - 119.9
estimated range of gestation (days)*	40 - 50	50 - 65

*Based on the data of Draper (3).

† Significantly different ($p < 0.001$) than weights reported by Willis, et al. (1).

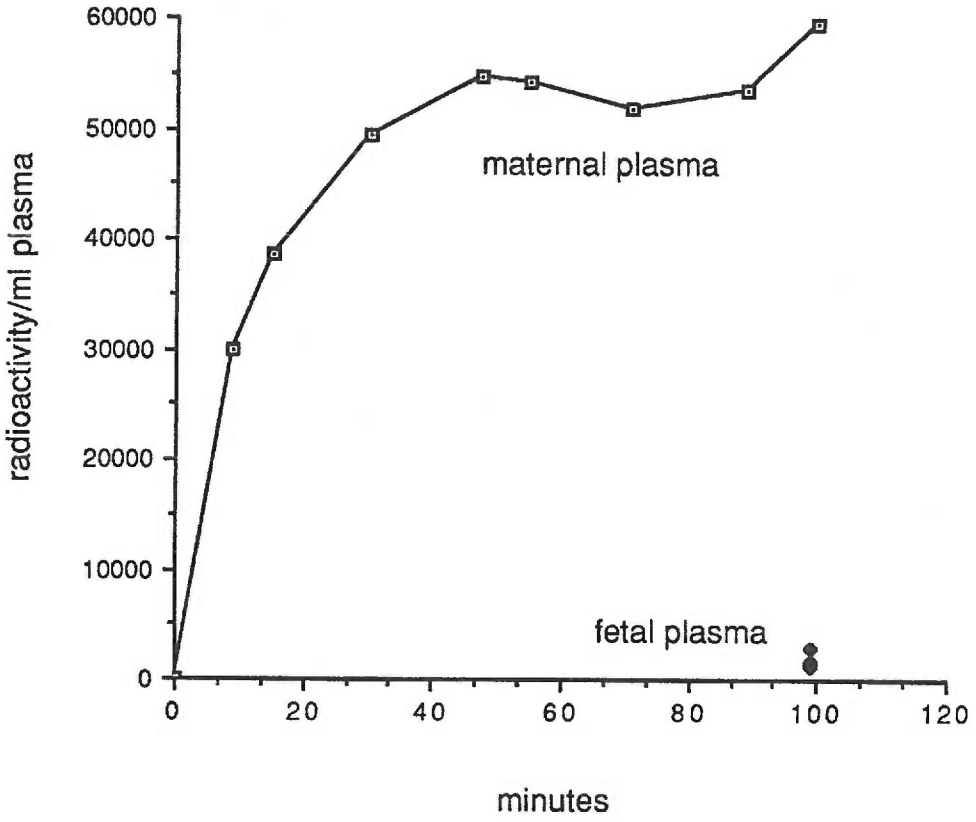

Figure 1. Radioactivity per ml plasma detected in a typical sow and her six fetuses as a function of time after maternal injection of 1.4 mg/kg of nonlabelled cyanocobalamin and 0.2 mg of ^{57}Co labelled cyanocobalamin.

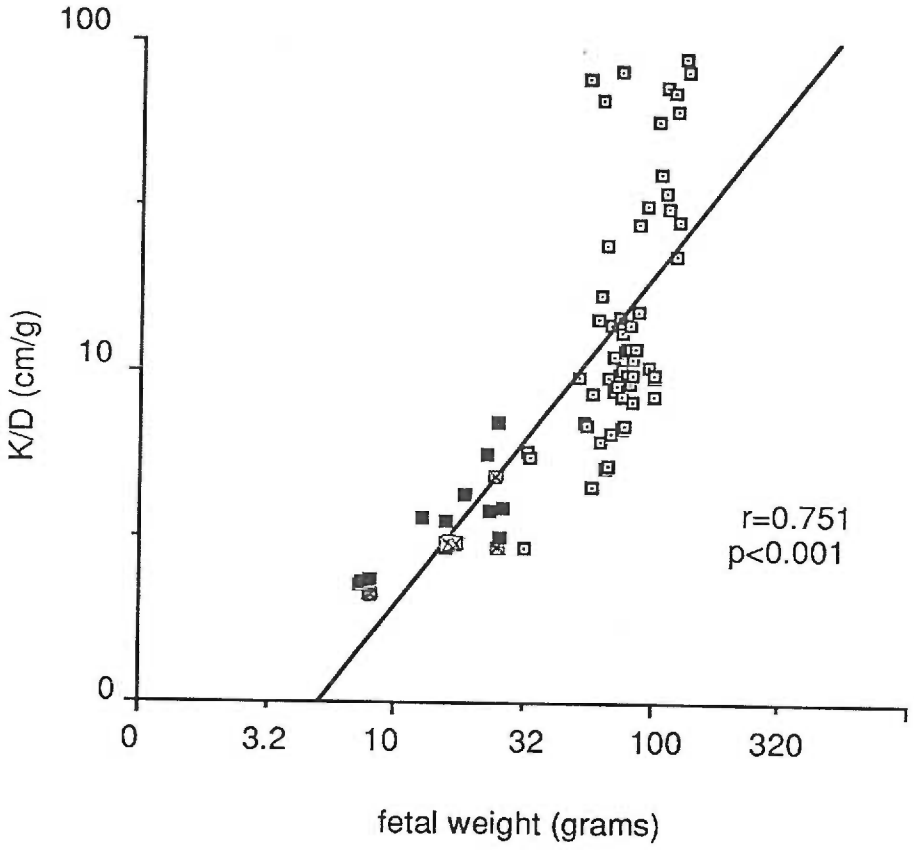

Figure 2. The ratio of K/D (cm per gram placental weight) as a function of fetal weight. Filled squares are sham and control fetuses from this study. X are from fetuses with ligated vitelline vessels. Open squares are the individual measurements from Adams, et al. (4). Note logarithmic scales.

Paper 3

The Transfer of Protein Bound Cyanocobalamin Across the Guinea Pig Placenta

Abstract

We have previously shown that when injected in quantities sufficient to cause a large free concentration, cyanocobalamin crosses the guinea pig placenta at a rate expected for inert hydrophilic substances of a similar molecular weight. To demonstrate that the maternofetal transfer of protein bound cyanocobalamin was a negligible fraction of the flux of cyanocobalamin which occurs when a large excess of free cyanocobalamin is present in maternal plasma, the rate of transfer of protein bound cyanocobalamin to the fetus was measured *in vivo*. An intramuscular injection of a small amount of ^{57}[Co]-cyanocobalamin was given to nine pregnant guinea pigs at approximately 50 to 65 days gestation and fetal and placental content of ^{57}Co measured at either 2, 6, or 24 hours after injection. The majority of ^{57}Co was bound in the maternal plasma. A gradual transfer of radiolabelled cyanocobalamin to the fetus occurred over the 24 hours after injection. The rate of transfer to the fetus of bound cyanocobalamin exceeded the rate of transfer of an equal amount of unbound cyanocobalamin. The total amount of cyanocobalamin transferred to the fetus from the fraction bound in maternal plasma was only a small fraction of the transfer measured previously when a large excess of unbound cyanocobalamin was present.

Cyanocobalamin (vitamin B_{12}) is essential for growth and development of the fetus as well as for the daily metabolism of the adult mammal. Since cyanocobalamin is synthesized exclusively by microorganisms, adult animals acquire this essential vitamin from dietary sources or by bacterial production in the rumen or intestine. The fetus cannot obtain vitamin B_{12} by these means and must, therefore, obtain vitamin B_{12} from the maternal blood by either active or passive transport.

The presence of cyanocobalamin in fetal plasma has been demonstrated in mice, rats, guinea pigs, and humans (1-5). Though yolk sac or chorioallantoic placental binding of cyanocobalamin has been demonstrated *in vitro* in mice, rats, guinea pigs, rabbits and humans, the method and rate of transfer has not been established (1,2,6,7).

The distribution of radiolabelled cyanocobalamin in pregnant mice after intravenous injection was studied by Ullberg et al., (1). They found that the uptake of radioactivity was greater in the chorioallantoic placenta than in maternal organs. Over hours the radiolabel shifted from the maternal and placental tissues into fetal tissues. The fetal concentration of radioactive cyanocobalamin, expressed as the fraction of injected radioactivity per gram tissue, exceeded maternal concentration at 2 hours and peak fetal concentration was reached at 24 hours. The amount of cyanocobalamin that circulated as a protein bound complex in maternal plasma after injection into the pregnant mouse was not measured. The percentage of injected cyanocobalamin excreted in the urine of the pregnant mouse decreased when the amount of cyanocobalamin injected decreased. This suggests the presence of unbound cyanocobalamin in maternal plasma which is excreted in the urine. Unbound cyanocobalamin has been shown to cross the placenta by diffusion (5).

At normal physiologic concentrations cyanocobalamin circulates bound to carrier proteins in the plasma. One major carrier protein, transcobalamin II, is involved in the transfer of cyanocobalamin into cells, which is thought to occur by endocytosis (8,9,10). Transcobalamin II may be involved in the transfer of cyanocobalamin from the maternal blood to the fetus as well. A placental receptor has been identified for the transcobalamin II- cyanocobalamin complex (7,11,12). Binding of the transcobalamin II-cyanocobalamin complex to human placental slices has been demonstrated *in vitro* from early in gestation (13,14). The mechanism and the rate of transfer to the fetus of the protein bound cyanocobalamin in the maternal plasma is not known.

To demonstrate that the maternofetal transfer of the protein bound cyanocobalamin in maternal plasma was a negligible fraction of the flux measured previously by us when a large excess of free cyanocobalamin was present in maternal plasma (5), the rate of transfer of protein bound cyanocobalamin to the fetus was measured *in vivo* . An intramuscular injection of a small amount of ^{57}[Co]-cyanocobalamin was given to pregnant guinea pigs and the fetal and placental content of ^{57}Co was measured at 2, 6, and 24 hours after injection.

Methods:

Nine pregnant guinea pigs at approximately 50 to 65 days gestation were obtained from the Department of Animal Care at Oregon Health Sciences University. Pregnancy was determined by palpation and the gestational age estimated by fetal weight according to the data of Draper (15). Term gestation is about 65 days in the guinea pig.

The sows were anesthetized with a gas mixture of one volume percent halothane, 80 percent nitrous oxide, and 20 percent oxygen. Using sterile

technique an indwelling catheter was placed in a maternal carotid artery. After two hours for recovery from anesthesia, the sow was given an intramuscular injection of approximately 10 ng radiolabelled cyanocobalamin. ^{57}[Co]-cyanocobalamin (10.5 μCi/50ng) was obtained from the medical products division of Amersham Corporation, Arlington Heights, IL 60005. The amount of radiolabelled cyanocobalamin solution injected, approximately 0.2ml, was determined by weighing the syringe before and after injection. A measured aliquot was counted with the samples collected from each experiment.

Three sows had five blood samples drawn during the two hours after injection (0, 15, 30, 60, and 120 minutes). The sows were reanesthetized, their abdomens opened, the uterine vessels clamped, and the final maternal blood sample was drawn. All fetuses were alive at the time the uterine vessels were clamped as demonstrated by fetal movement, respirations, or pulsating hearts. The sow and fetuses were killed. Placentas, fetuses, fetal blood, amniotic fluid, and amniotic and yolk sac membranes, and uterus were collected and weighed. Urine voided by the sow after injection and urine in the maternal bladder were collected. The sows, fetuses and placentas were ashed.

Three sows had blood samples drawn over the next 6 hours (0, 30, 120, 210, 300, and 360 minutes) and fetal and maternal samples were collected as above. Three sows had blood samples drawn over the next 24 hours (0, 360, 1080, and 1440 minutes) and fetal and maternal samples were collected.

The fraction of free and bound cobalamin in the maternal plasma was determined by separation on a Sephadex G-50 gel filtration column. The column was calibrated with blue dextran, ovalbumin, IgG, and cyanocobalamin. Half a milliliter of plasma in 1g/ml sucrose was placed on the column. Typically 60 tubes of 2 milliliters each were collected. The protein bound cobalamin fractions were separated from the free cyanocobalamin peak

by twenty tubes. All fractionations were performed at 4° C. Relative cobalamin concentrations in each fraction were determined by radioisotope counting.

The activity of ^{57}Co was determined for maternal and fetal blood samples, amniotic fluid samples, amnion and yolk sacs, maternal urine, uterus, ashed fetuses and placentas, and gel separation fractions in a Nuclear Data ND600 system with a Packard Auto-gamma detector and sample changer.

Significance of differences was analyzed by t-tests and linear regressions. Means are arithmetic means plus or minus one standard deviation unless otherwise stated. Differences were considered significant at probabilities less than or equal to 0.05.

Results:

Transfer of cyanocobalamin was studied in nine pregnant sows containing thirty-two fetuses. The mean fetal weight was 67 ± 22 (SD) grams and mean placental weight was 4.8 ± 1.2 (SD) grams. The highest concentration of ^{57}Co in maternal plasma was 0.2% of the injected radioactivity per milliliter. The majority of ^{57}Co circulated with the protein fraction of maternal plasma (table 1). An average of 1.5% of the injected ^{57}Co was cleared in the maternal urine (range 0 to 2.8%), primarily in the first two hours after injection.

The ^{57}Co radioactivity in counts per minute per milliliter maternal or fetal plasma is plotted as a function of time (figure 1). After normalizing these concentrations by dividing by the amount of ^{57}Co injected into each sow, it is evident that the fraction of injected ^{57}Co per milliliter in the fetal plasma is significantly higher than the fraction per milliliter in maternal plasma at 24 hours (table 2). The mean fetal plasma concentration of ^{57}Co was eleven times the maternal plasma concentration 24 hours after injection (range 5 to 17).

The apparent volume of distribution of ^{57}Co in the fetus was determined by dividing the fetal concentration of ^{57}Co in counts per minute per gram fetal weight divided by the concentration of fetal plasma in counts per minute per millilter plasma. The apparent volume of distribution increased with time (figure 2, table 3).

The fraction of injected ^{57}Co transferred into the fetus increased over the duration of the experiment (figure 3, table 4). By 24 hours 34 ± 6 (SD) % of the injected ^{57}Co was transferred from the sow to the fetuses. The fraction of ^{57}Co in the placenta decreased with time (figure 4). Less than 0.5% of the injected ^{57}Co was found in the combined yolk sac and amnion of each fetus. Although the yolk sac and amnion content of ^{57}Co increased with time, there was no statisical difference between the radioactivity per milliliter of fetal plasma and the radioactivity per gram of the yolk sac and amnion (table 5; $p>0.05$, paired t-test).

Only 0.6 ± 0.2 (SD) % of the injected ^{57}Co was present in the empty uterus. This amount did not change with time. Only six fetuses with samples collected 6 hours or earlier after injection had measurable radioactivity in their amniotic fluid. The maximum amount present was 0.035% of the radioactivity injected. Although all fetuses at 24 hours had detectable radioactivity measured in their amniotic fluid, this amount was still a small fraction (0.4 ± 0.4 (SD) %) of the total radioactivity injected.

Discussion:

When run through a Sephadex G-50 gel filtration column, radioactivity measured in maternal blood, eluted into fractions that corresponded to the elution volumes of protein bound and free cyanocobalamin fractions as determined by us previously using nonlabelled cyanocobalamin and a

cobalamin specific radioassay (5). Previous studies have demonstrated both direct and indirect evidence that cyanocobalamin retains its cobalt label after injection into an animal. In tissue extraction studies, Reizenstein showed that when small amounts of ^{60}[Co]-cyanocobalamin were injected into humans the radioactivity was present as microbiologically active B_{12} even 11 days after injection (16). By similar means, Glass and Mersheimer demonstrated in dogs that radioactivity stored in the liver after injection of ^{60}[Co]- B_{12} was microbiologically active ^{60}[Co]-B_{12} and in dogs who delivered 233 days after injection, radioactive B_{12} was present in the pups' livers (17). Further indirect evidence also suggests that radiolabelled cyanocobalamin remains in body stores as a cobalamin (18). First, vitamin B_{12} stores influence the amount of radiolabel retained after injection of radiolabelled cyanocobalamin. Second, the turnover rate of the injected radioactivity corresponds to a biological half-life of months rather than the half-life of radio-cobalt of 8 days. Third, excretion of radioactivity in the urine after injection of radiolabelled cyanocobalamin corresponds to the excretion of vitamin B_{12} seen by bioassay. Fourth, the pattern of absorption and distribution of radiolabelled cyanocobalamin is radically different than the pattern seen after injection of Co-Cl. Fifth, the distribution of radioactivity after injection of radiolabelled cyanocobalamin into animals correlates with the distribution of microbiologically active cobalamins. From this evidence we conclude that the ^{57}Co radioactivity measured in this experiment was in the form of ^{57}[Co]-cobalamin.

A much smaller fraction of the injected radiolabelled cyanocobalamin was transferred from the maternal plasma to the fetus as unbound cyanocobalamin than was transferred as protein bound cyanocobalamin. By 24 hours 34% of injected cyanocobalamin entered the fetus. This is much higher than would be predicted by the free diffusional equilibrium of unbound

cyanocobalamin. At its highest measured concentration in maternal plasma, unbound cyanocobalamin was only 1.5 pg/ml and our studies demonstrated that this was partially excreted in the maternal urine. The remainder was available for distribution between maternal and fetal tissues. The contribution transfer of unbound cyanocobalamin made to the measured transfer of cyanocobalamin was small and the concentration profiles in figure 1 are incompatible with diffusional equilibrium. The majority of transfer to the fetus occurred after 2 hours when over 99% of cyanocobalamin was bound, again confirming the insignificant contribution the unbound cyanocobalamin made in these transfer measurements.

The concentration of radiolabelled cyanocobalamin at the placenta and the gradual transfer of radiolabelled cyanocobalamin to the fetus measured over the next 24 hours is consistent with findings in the pregnant mouse and with studies in rats (1,2,19,20). Other reports in rabbits show a rapid uptake of cyanocobalamin by the combined placental and fetal tissues within one hour of injection of transcobalamin II-cyanocobalamin complexes, but no uptake after one hour when transcobalamin I or transcobalamin III-cyanocobalamin complexes were injected (21). The radiolabelled cyanocobalamin in the present study would be predicted to be largely bound to transcobalamin II since transcobalamin II comprises the largest fraction of unsaturated binding sites for cyanocobalamin in plasma (3,22,23).

The increase in the apparent volume of distribution of radiolabelled cyanocobalamin with time is consistent with the uptake of fetal plasma cyanocobalamin into fetal tissues. The small amount of radiolabelled cyanocobalamin present in the combined yolk sac and amnion suggests that these tissues do not contribute significantly to the transfer of cyanocobalamin to the fetus. This finding is consistent with previous experiments in the guinea pig,

which showed binding of cyanocobalamin to the yolk sac per gram tissue was less than the concentration of cyanocobalamin in other fetal tissues (6). This is in distinct contrast to the amount of binding that occurred in rabbit and rat yolk sacs (6,24). The small amount of radioactivity in the uterus suggests that uterine uptake does not play a large role in the transfer of cyanocobalamin into the fetus.

The transfer of cyanocobalamin into cells is thought to occur by binding of the transcobalamin II-cyanocobalamin complex to a membrane receptor and slow transfer into the cell by endocytosis (8,9,10). Measurable diffusion of previously bound cyanocobalamin back into the plasma as free cyanocobalamin has been reported, as well as release by cells of cyanocobalamin bound transcobalamin I (8,25). The small amount of radioactivity in the amniotic fluid after 24 hours (0.2 pg) may reflect either urinary excretion of free cyanocobalamin by the fetus or transfer into the amniotic fluid across fetal membranes. The proportion of the total injected radiolabelled cyanocobalamin that was present in amniotic fluid was minute (0.002%).

The ratio of the concentrations of radioactivity in maternal and fetal plasma at 24 hours is close to the ratio of endogenous cyanocobalamin concentrations measured previously by us in the guinea pig. Endogenous concentrations were 1.53 ng/ml in maternal plasma and 11.3 ng/ml in fetal plasma (5). This suggests that the transfer of cyanocobalamin measured here provides a reasonable estimate of the normal redistribution of cyanocobalamin from the mother to the fetus.

From the known amount of radiolabelled cyanocobalamin injected, the quantity of radiolabelled cyanocobalamin transferred in 24 hours can be calculated. The quantity of radiolabelled cyanocobalamin transferred to the

fetus in 24 hours is determined by calculation of the product of the amount of radiolabelled cyanocobalamin injected (10 ng) and the fraction of injected radioactivity in the fetus at 24 hours (0.126), yeilding a value of 1.26 ng per 24 hours.

The maximum fraction of the injected radiolabelled cyanocobalamin per milliliter maternal plasma sampled during the experiment was 0.002. This represents about 1.3% of the endogenous maternal plasma level of cyanocobalamin of 1.53 ng/ml. If it is assumed that injected radiolabelled cyanocobalamin is distributed between transcobalamin II and other circulating binding compounds in a manner similar to the endogenous vitamin B_{12}, an absolute rate of transfer of vitamin B_{12} can be estimated at 97 ng per 24 hours. This is probably an overestimate of the transfer rate of cyanocobalamin into the fetus. Most unbound cyanocobalamin entering the circulation becomes bound to transcobalamin II and a higher proportion of the cyanocobalamin bound to transcobalamin II may be transferred to the fetus than vitamin B_{12} bound to other carrier proteins (22).

Although it is not certain how this would compare to transfer of endogenous vitamin B_{12} to the fetus, an estimate can be made. This can be based on the rate of fetal growth, if a known volume of distribution in the fetus and a constant concentration of vitamin B_{12} in fetal plasma is assumed. From the data of Draper (15), the increase in weight of a guinea pig fetus during the last 2 weeks of gestation is about 2.5 g per day. If the fetal cyanocobalamin concentration remains constant at 11.3 ng/ml and is distributed in fetal tissues with the same apparent volume of distribution as the radiolabelled cyanocobalamin 24 hours after injection (0.98 ml / gram fetal weight), the calculated rate of transfer of cyanocobalamin to the fetus is 28 ng per 24 hours (11.3 ng/ml X 0.98 ml/g fetal weight X 2.5 g fetal weight/day). The validity of

these assumptions have not been confirmed. The estimated rate of fetal acquisition of cyanocobalamin agrees well with the rate of transfer measured in this study.

The rate of transfer of protein bound radiolabelled cyanocobalamin exceeds the rate of diffusional transfer for an equal amount of unbound cyanocobalamin. If for purposes of comparison, it is assumed that all the radiolabelled cyanocobalamin was free in maternal plasma and the fetal concentration is ignored in calculating a maternal-fetal concentration difference, a permeability surface area product would be 50 µl/min/g placental weight for the fetuses 24 hours after injection. The mean diffusional permeability of unbound cyanocobalamin normalized for placental weight is 14.3 µl/min/g placenta weight (5).

These calculations indicate that cyanocobalamin bound in the plasma is transferred into the fetus at a rate exceeding the diffusional flux. Further, it is likely that the majority of transfer occurs after concentration of the bound cyanocobalamin in the placenta, and that the slow transfer observed from the maternal plasma to the fetus is sufficient to account for the increase in fetal cyanocobalamin content during the last part of gestation.

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Table 1

Sephadex separation of bound and free cyanocobalamin in maternal plasma

sample time	≤ 2 hours	>2 hours	all samples
Fraction protein bound	0.936*	0.992*	0.970
standard deviation	0.05	0.012	0.039
n	7	7	14

*The fraction of radiolabelled cyanocobalamin protein bound in maternal plasma at 2 hours or before statistically differs (unpaired t-test, p=0.017) from the fraction of radiolabelled cyanocobalamin protein bound more than 2 hours after injection.

Table 2

Fraction of injected ^{57}Co per milliliter in maternal and fetal plasma at 24 hours

	maternal plasma	fetal plasma
Fraction of injected ^{57}Co per milliliter	0.2×10^{-3}	2.1×10^{-3}
standard deviation	0.06×10^{-3}	0.7×10^{-3}
n	3	8

The fraction of injected ^{57}Co is significantly higher in fetal plasma than in maternal plasma 24 hours after injection ($p=0.001$).

Table 3

Apparent volume of distribution of ^{57}Co in the fetus

Hours after injection	2	6	24
Milliliters/gram fetal wt	0.34	0.56*	0.98*
Standard deviation	0.06	0.12	0.16
N	11	13	8

*significantly different from preceeding mean point ($p<0.002$) by modified t-test using the Bonferroni correction for multiple comparisons.

Table 4

Fraction of injected ^{57}Co in the fetus after 2, 6, and 24 hours

Hours	2 hours	6 hours	24 hours
Fraction of injected ^{57}Co per fetus	0.014	0.044*	0.126*
Standard deviation	0.004	0.021	0.018
N	11	13	8

* significantly different from preceeding point ($p<0.002$) by modified t-test using the Bonferroni correction for multiple comparisons.

Table 5

Increase in combined yolk sac and amnion radioactivity with time

Hours	2	6	24
Fraction of injected ^{57}Co in yolk sac and amnion ($x10^{-3}$)	0.59	1.9*	4.1*
Standard deviation ($x10^{-3}$)	0.16	0.96	0.54
N	11	13	8

*significantly different than preceeding point ($p<0.002$) by modified t-test using the Bonferroni correction for multiple comparisons.

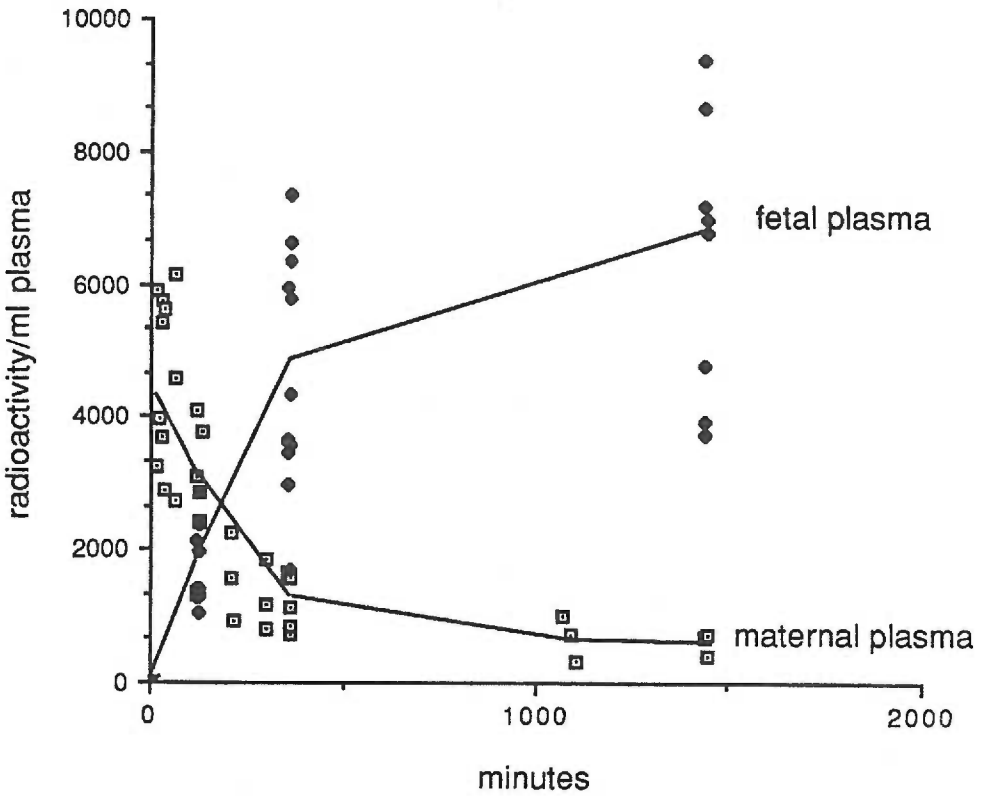

Figure 1. Concentration of ^{57}Co radioactivity per milliliter maternal or fetal plasma as a function of time from injection.

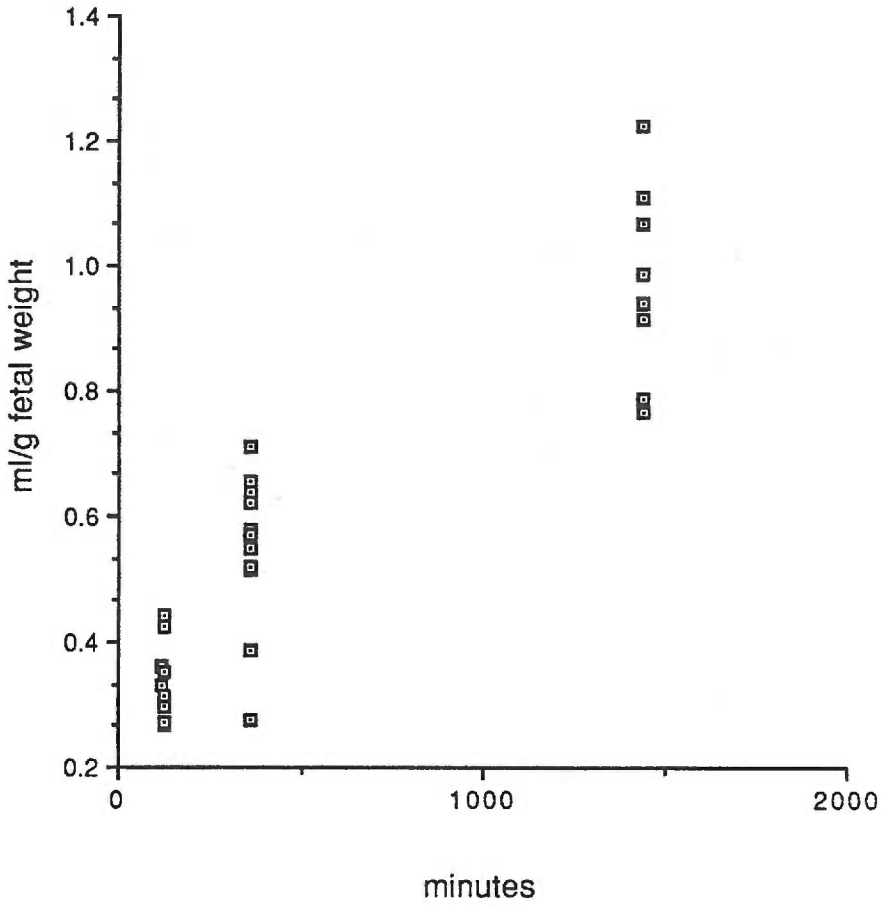

Figure 2. Increase in the apparent fetal volume of distribution of ^{57}Co as a function of time from maternal injection.

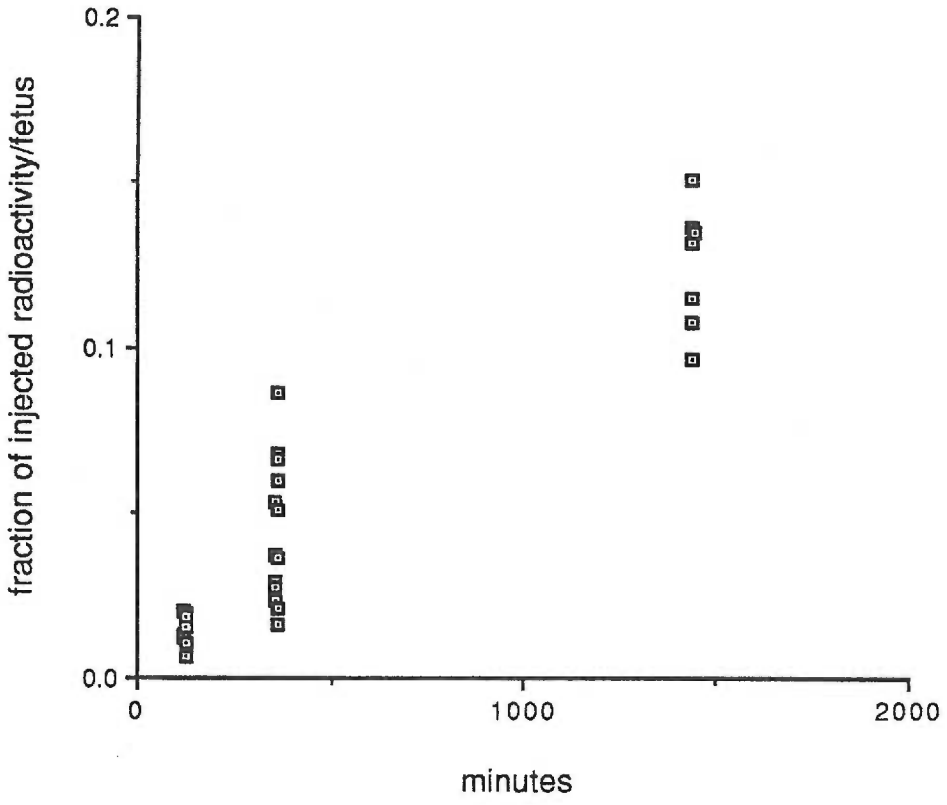

Figure 3. Fraction of ^{57}Co transferred to each fetus as a function of time from maternal injection.

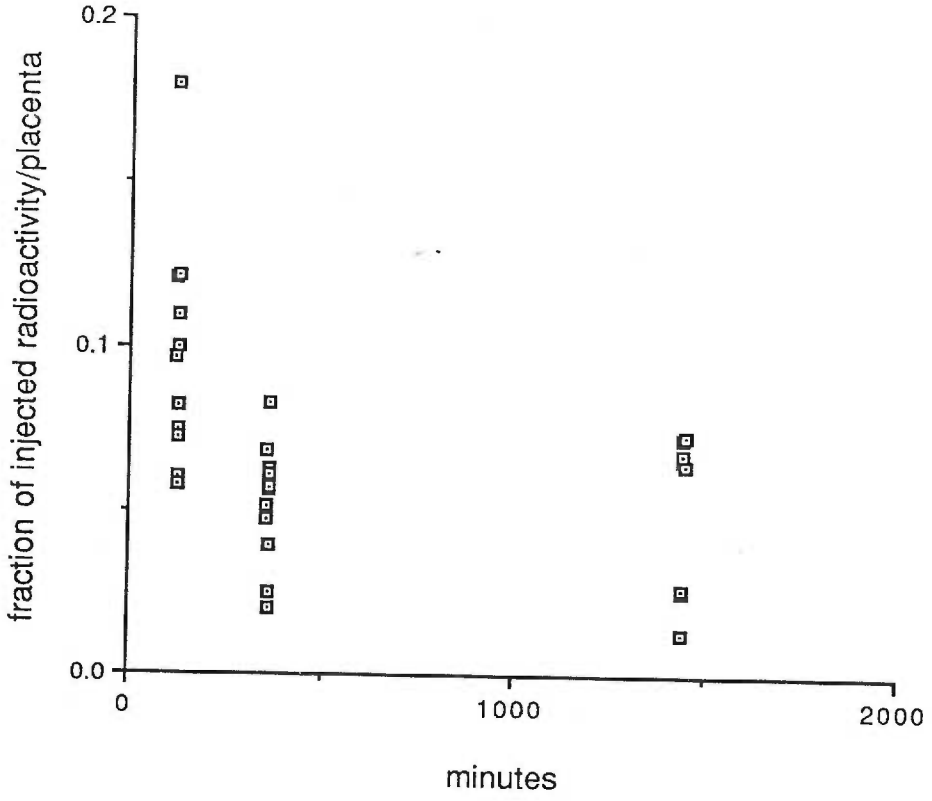

Figure 4. Fraction of ^{57}Co in the placenta as a function of time from maternal injection. The fraction of ^{57}Co in the placenta at 2 hours is significantly higher ($p \leq 0.003$) than the fraction at 6 or 24 hours, but the fraction of radioactivity in the placenta at 6 hours does not differ from the values for 24 hours ($p > 0.8$) by modified t-test using the Bonferroni correction for multiple comparisons.

Discussion:

Potential routes for transfer of cyanocobalamin (vitamin B_{12}) are passive or facilitated diffusion, endocytosis or active transport. In paper 1, the transport of cyanocobalamin across the guinea pig and human placenta by passive diffusion was studied. Evidence was supplied to support the validity of the methods used to measure permeability. Further evidence supports the interpretation that the ratio of flux and maternofetal concentration difference represent a measurement of diffusional permeability. Paper 2, demonstrates that ligation of the vitelline vessels of the guinea pig did not significantly effect permeability measurements when a similar amount of cyanocobalamin was injected into pregnant guinea pigs as in paper 1. We conclude that the yolk sac placenta does not contribute significantly to the flux of unbound cyanocobalamin in the guinea pig.

In the experiments of papers 1and 2, almost all of the cyanocobalamin present in maternal plasma was present as unbound cyanocobalamin (99%). The remaining cyanocobalamin would have been transferred at the same rate as the protein bound cyanocobalamin in paper 3. If one calculates an apparent permeability for the protein bound fraction of cyanocobalamin in guinea pig plasma at 2 hours (PS= 26.8 µl//min/g placental weight), the active transport of bound cyanocobalamin would account for only about 1% of the total cyanocobalamin transferred to the fetus. This amount is smaller than the intraspecies variability seen in this paper or in measurements of placental permeability by other investigators (1,30,34,42,43,45).

There are two subsequent reports of diffusion permeability of the human placenta. Bain et al., measured the clearance of inulin and mannitol across the human placenta (42). Bolus and continuous intravenous infusion of inulin and

mannitol were given to six pregnant women 1 to 2 hours before delivery by cesarean section. The time integral of the flux of inulin and mannitol to the fetus was taken to be the excretion of these substances in the infant's postnatally voided urine. Recalculating their measured clearances to PS products gives permeabilities of 1.35±0.19 (SD) µl/min/g placental weight for inulin and 11.9±3.7 (SD) µl/min/g placental weight for mannitol. These are lower values than would be predicted by our measurement of the cyanocobalamin permeability of 14.3 µl/min/g placental weight.

Thornburg et al., also measured the permeability of the human placenta to inulin. Their value for inulin permeability of 9.2±3.7 (SD) µl/min/g placental weight is also higher than the mean value reported by Bain et al., (42,43). However, by a t-test on the individually reported permeabilities in both papers, the means do not differ (unpaired t-test, p=0.118). Thornburg et al., calculated the net flux of inulin to the fetus by multiplying the fetal plasma concentration by the volume of distribution in the fetus. This is similar to the method used to calculate the net flux to the fetus in this study of cyanocobalamin permeability and has the advantage that it does not require an accurate collection of the newborns' urine. In paper 1, Thornburg et al., and Bain et al., values for human placental permeability are very similar to the permeabilities obtained for guinea pig placenta (1,41,45) and much larger than permeabilities for the epitheliochorial placenta of the sheep (34).

The increase in the placental permeability to cyanocobalamin normalized for placental weight with increases in fetal weight observed in paper 2 agrees with previous reports (45). Our study extends these previous observations to an earlier gestational age. We agree with the previous studies in concluding that the change in permeability with fetal weight represents a

change in diffusing distance or an increase in the surface area for diffusion, i.e., a change in pore area.

Previous reports on the endogenous transfer of cyanocobalamin agree with our finding that cyanocobalamin is initially bound to the placenta and transfers slowly to the fetus (52,53). Dencker showed that the binding to the placenta in mice was localized between the first and second trophoblastic layer (54). The larger dose and intravenous injection of the radiolabelled cyanocobalamin given to smaller animals (pregnant mice) probably resulted in a larger unbound concentration of cyanocobalamin than measured in our study.

The intramuscular injection in our study resulted in a slower absorption of the 10 ng of cyanocobalamin into the maternal circulation. The free concentration of cyanocobalamin in maternal plasma was at most 1.3 pg/ml, less than 0.1% of the endogenous concentration of cobalamins. The remaining exogenous cyanocobalamin was protein bound. According to previous reports, free cyanocobalamin entering the circulation is preferentially bound to transcobalamin II (56,57). Transcobalamin II is the carrier thought to be responsible for transport of cyanocobalamin into cells (55,58). Transcobalamin II may also be important for transfer of cyanocobalamin into the fetus, as a transcobalamin II receptor has been identified on the placenta (61,62,63).

The uptake of cyanocobalamin at the placenta by means of a transcobalamin II-cyanocobalamin complex is supported by the work of Fernandes-Costa and Metz, who reported rapid uptake in the combined fetal and placental tissue of rabbits when ^{57}[Co]-labelled transcobalamin II-cyanocobalamin complex was injected (67). Transcobalamin I- and III-cyanocobalamin complexes showed little uptake by the combined fetal and placental tissues.

Ng et al., have shown uptake of transcobalamin II-cyanocobalamin complex by placental tissue slices (65). When the tissue cytosol fraction was isolated after two hours of incubation a transcobalamin II peak and a transcobalamin I-like peak were found bound to the cyanocobalamin. No free cyanocobalamin was present. When only free cyanocobalamin was added to the media (modified Krebs Ringer phosphate buffer) similar uptake and binding of the cyanocobalamin was found in the cytosol fraction at two hours. The origin of the transcobalamins was not determined. The shift of cyanocobalamin from a transcobalamin II complex to a transcobalamin I complex has been reported in the uptake of cyanocobalamin by other tissues (55,58).

These studies suggest that the bound cyanocobalamin in our study was largely bound to transcobalamin II because of the large proportion transcobalamin II contributes to the unsaturated binding capacity of plasma. The specificity of placental uptake to the transcobalamin II-cyanocobalamin complex as reported by Ferdanabdes-Costa and Metz also suggests that the bound cyanocobalamin in his study was bound by transcobalamin II (67). Uptake of the transcobalamin II- cyanocobalamin complex by the placenta may occur by endocytosis as proposed in other tissues. The means by which the cyanocobalamin is transferred from the placenta to the fetus has not yet been determined. Porck et al., have shown that in humans the fetal transcobalamins are not derived from the maternal circulation (64).

In our studies we have demonstrated that the diffusional permeability of the human and guinea pig placenta to cyanocobalamin (1355 Daltons) is similar, and both differ greatly from the less permeable sheep placenta. The yolk sac placenta of the guinea pig does not contribute significantly to the diffusion of cyanocobalamin to the fetus. Diffusional permeability to

cyanocobalamin increases as a function of fetal weight as previously reported for later gestational ages. The transfer of bound cyanocobalamin occurs at a rate much greater than the transfer by diffusion of free cyanocobalamin and this rate appears sufficient to account for the normal acquisition of cobalamins by the fetus.

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