CALCIUM TRANSFER ACROSS THE RAT PLACENTA

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Nicola R. Robinson
Department of Child Health
University of Manchester
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For Mum and Dad, with love and thanks.

'God grant me the **Serenity**
to accept the things I cannot change,
Courage to change the things I can,
And **Wisdom** to know the difference'.

ABSTRACT : Calcium Transfer Across The Rat Placenta

The mechanisms and control of calcium transfer across the near term rat placenta has been investigated using anaesthetized rats. To validate the model used, the permeability of the *in situ* perfused rat placenta was investigated by measuring the maternofetal transfer of a range of hydrophilic molecules and comparing this to the intact placenta. Unidirectional clearance (K_{mf}) of these molecules was directly proportional to their diffusion coefficients in water (D_w), except for sodium. Permeability of the rat placenta was almost identical in the intact and perfused placenta. The permeability was also comparable to that of other haemochorial placentas, so the extra layers of trophoblast in the rat seem to have no effect on permeability. A carrier-mediated pathway was suggested for sodium, as well as a route across the yolk sac.

Carrier-mediated maternofetal calcium transfer investigated using the *in situ* perfused rat placenta, increased dramatically between day 18 and 21 of gestation (term day 23). This maternofetal calcium transfer was sensitive to temperature and potassium cyanide, and, by comparison with the estimated net flux of calcium, was large compared to the fetomaternal calcium flux. A new method for the calculation of maternofetal calcium flux (J_{mf}) was suggested and also a route for calcium transfer across the yolk sac.

Alteration of maternal blood calcaemia, either acutely by infusion of calcium gluconate or EDTA, or chronically by maternal thyroparathyroidectomy and maintenance on a vitamin D

deficient diet, had no effect on J_{mf} or net calcium accretion by the fetus. This suggested that calcium transport is saturated even at low maternal plasma calcium concentrations and that maternal $1,25(OH)_2D_3$ has little effect on this transport.

Manipulation of the fetal parathyroid status by fetal decapitation, followed by replacement injections of parathyroid hormone (PTH) or $1,25(OH)_2D_3$, provided evidence that fetal PTH and $1,25(OH)_2D_3$ have a permissive role in controlling J_{mf} calcium. Addition of PTH or $1,25(OH)_2D_3$ to the fetal perfusate of a placenta from a decapitated fetus, further suggested that it is only fetal $1,25(OH)_2D_3$ which has a direct effect on placental calcium transfer in this respect. Finally, the addition of forskolin, a potent stimulator of adenylate cyclase, to the fetal perfusate markedly increased J_{mf} calcium, suggesting that a hormone (or hormones) acting via cyclic AMP, may also be involved in the control of calcium transfer across the rat placenta near term.

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CONTENTS

Page

List Of Illustrations

List of Tables

CHAPTER 1

PLACENTAL STRUCTURE AND TRANSPORT MECHANISMS

SECTION A : Placental Structure

Mossman (1937,1987) defines placentation as an approximation or combination of an embryo's tissues with those of its natural or surrogate parent for physiological interchange. The fetal membranes consist of the chorion, amnion, yolk sac and allantois. These membranes give rise to two major types of placenta: the choriovitelline placenta, which results from apposition of the yolk sac to the chorion; and the chorio-allantoic type in which the blood vessels of the allantois fuse with those of the chorion to form a vascular bridge. The former placenta is usually transitory in nature and forms a temporary means of support for the embryo whilst the chorioallantoic placenta is being formed, e.g. in man. However, in some species the yolk sac may form a placental link between the embryo and its mother, and may function side by side with the chorio-allantoic placenta, e.g. in the rat, rabbit and guinea-pig (Amoroso,1952).

In general, the definitive shape of the placenta is determined by the initial distribution of villi over the chorionic surface. In the diffuse placenta most of the outer surface of chorion is covered with small villi or folds, which lie in intimate contact with corresponding depressions in the uterine epithelium, e.g. in the pig. The cotyledonary or multiplex placenta is found in the majority of ruminants. Here, the chorionic villi are restricted to a number of well-defined circular or oval areas of the chorionic sac, which are separated by less specialized areas of relatively smooth chorion. The fetal cotyledons, which are the

rosettes of chorionic villi visible on the surface of detached fetal membranes, develop in those parts of the chorion which overlie specialized areas of the uterus known as caruncles. Fetal villi and uterine caruncles together form placental units known as placentomes. The zonary placenta is characteristic of the carnivores and here the chorionic villi are aggregated into a band of placental tissue which encircles the equatorial region of the chorionic sac. A discoid placenta is found in primates, rodents, lagomorphs and bats. The disc is single in man, but may be double, as in many monkeys (Martin and Ramsey,1970).

Cotyledonary placentas are all villous, whilst diffuse, discoid or zonary placentas are villous or labyrinthine (Ramsey, 1975). These two descriptions reflect the manner in which the fetal blood is conveyed to its point of contact with maternal blood. In villous, the fetal vessels travel in structures resembling the arborizing trunk, branches and twigs of a deciduous tree. In labyrinthine, fetal blood courses through intercommunicating anastomosing channels. This is a primitive form and may persist throughout gestation or later be superseded by villous configuration, e.g. in man (Amoroso,1952; Hamilton et al,1972).

Grosser (1909; as cited in Mossman,1987) classified chorio-allantoic placental stuctures, based on the number of tissue layers, which under the light microscope appeared to separate fetal from maternal blood streams. In the epitheliochorial placenta, which was considered to represent the simplest type, Grosser described six layers of tissue:

1) Endothelium of fetal capillaries

2) Fetal connective tissue or mesenchyme

3) Fetal chorionic epithelium (trophoblast)

4) Maternal uterine epithelium

5) Maternal connective tissue

6) Maternal endothelium

On the basis of gradual erosion of the layers, chorioallantoic placentas were divided into four groups:

a) Epitheliochorial- 3 maternal, 3 fetal layers, e.g. horse,pig.

b) Syndesmochorial- 2 maternal, 3 fetal layers (loss of maternal uterine epithelium), e.g. sheep.

c) Endotheliochorial- 1 maternal, 3 fetal layers (loss of maternal uterine epithelium and connective tissue), e.g. carnivores.

d) Haemochorial- maternal layers absent, 3 fetal layers (loss of all maternal tissue except free blood), e.g. man, rat.

An extension of Grosser's classification was proposed by Mossman (1926) who introduced a fifth group:

e) Haemoendothelial- maternal layers absent, loss of fetal epithelium and mesenchyme, leaving fetal endothelium in contact with free maternal blood, e.g. higher rodents, lagomorphs.

With the advent of the electron microscope it was revealed that placentas do not always conform to this classification. For example, some controversy surrounds the sheep placenta. Lawn et al (1969) suggested that the placental syncytium is formed from maternal uterine epithelium making it epitheliochorial in stucture, whilst Wooding et al (1981) support the syndesmochorial classification, having shown migration of fetal binucleate cells to form the placental syncytium. Also, the

fetal capillaries of certain haemoendothelial placentas seem to be covered by one or more layers of attenuated chorion (Wislocki and Dempsey,1955; Enders,1965). The latter observation has not only cast grave doubts on the existence of the haemoendothelial placenta in any species, but has given rise to a subdivision of the haemochorial category into: 1) haemomonochorial (e.g. man, guinea-pig), 2) haemodichorial (e.g. rabbit), 3) haemotrichorial (e.g. rat, mouse, hamster), relating to the number of layers of trophoblast.

Classification is also complicated by the fact that continual changes occur throughout gestation. For example, the chorio-allantoic placenta of the little brown bat undergoes a transition from endotheliochorial to haemochorial (Enders and Wimsatt,1968) and presumably this may be accompanied by changes in placental transfer throughout gestation (Cukierski,1987).

Enders' (1965) subdivision of the haemochorial group into mono-, di- and tri-, is in some ways more complicated than it first appears. For example, the human placenta (in which chorion consists of an outer syncytial and an inner cytotrophoblastic layer) is placed in the monochorial category. This is because the cytotrophoblast (or Langhans layer) is discontinuous and does not intervene between fetal capillaries and the syncytial trophoblast, at least in the latter stages of gestation. For the other placentas the situation is more straight forward, with the guinea-pig (labyrinthine) and the armadillo (villous) having a single layer of syncytium (Enders,1965). In the haemodichorial rabbit placenta the outer of the two layers is syncytial, the inner layer being relatively inconspicuous and partly cellular,

although regions of syncytium are probably present. In the haemotrichorial rat placenta, the three layers of trophoblast are found throughout the labyrinth. The outer layer (layer I) which encloses the maternal blood spaces, is cellular with cell boundaries present at all times. Distinct desmosomes (a type of cell junction) are present not only between this layer and the middle layer (layer II), but also occasionally between cells of the layer itself. In late pregnancy, the cells forming the outer layer are attenuated and irregular. Structures that are apparently pores are seen in these attenuated areas. Occasionally, vesiculated projections from the surface of this layer protrude into the maternal blood space. Many changes in fine structure occur throughout gestation and these probably affect placental transfer and have been detailed by Davies and Glasser (1968). The surface of the outer layer, which faces the maternal blood space, has irregular projections which only rarely have sufficient regularity to be designated microvilli. Such projections, over the thicker regions of the cells, e.g. the nuclear region, tend to be somewhat more regular and microvillous in appearance. Except where desmosomes are present, the outer layer is only loosely applied to the middle layer (Enders, 1965). The middle layer (II) also shows variation in thickness, being thicker in the area of the nucleus. In general it is the thickest of the 3 layers and is syncytial. It is closely opposed to the inner layer (III) and this junction is a series of undulations of rather large dimension, with secondary irregularities superimposed. The cytoplasm of the inner layer (III) can be traced for extensive distances and over several

fetal vessels without interuption, so it is basically syncytial.

The rat placenta is therefore quite different from the haemo-monochorial human placenta, which has a single syncytium consisting of a sheet of cytoplasm, elevated into minute microvilli on the free maternal side, the villus structure altering with increasing gestational age (Kaufmann,1985). The fetal plasma membrane of the human syncytiotrophoblast abuts onto the cells of the cytotrophoblast and, where these have disappeared, onto the collagen fibres and phagocytes of the mesoblast, which is relatively thin and contains the fetal capillaries.

The fetal capillary endothelium also appears to differ in structure in the human and rat placenta. In man, the fetal capillary endothelium is continuous, exhibiting exceedingly scarce micropinocytotic vesicles and tight intercellular junctions (Heinrich et al,1976), which seems to favour a predominant passage of low molecular weight substances. The fetal capillary endothelium in the rat exhibits patent intercellular clefts, micropinocytotic activity, many fenestrations with diaphragms and continuous transendothelial channels (Metz et al,1976). However, it is possible that these channels may be artefacts due to the fixation method used. The endothelium of the rat fetal capillary does not seem to be a significant barrier to low molecular weight substances, although a regulatory function is conceivable. The main barrier to placental transfer is thus thought to be the trophoblastic layers themselves, with the fetal endothelium playing a variable role.

As well as the chorioallantoic placenta, all mammals also have a yolk sac placenta, which is the site of formation of the first fetal vascular elements. In the rat, the yolk sac placenta persists throughout gestation, but by day 12 (term 23 days) a fully functional chorioallantoic placenta is also present (Jollie,1964), attaining its full size by about day 16. In man, the yolk sac placenta exists only early on in gestation, before the formation of the chorioallantoic placenta (Steven,1975).

SECTION B : Mechanisms of Placental Transfer

1) Simple Diffusion

This is the movement of a molecular species by random thermal motion from an area of high concentration to one of low concentration (Stein,1967). This mechanism can be used to explain the placental transfer of various substances, e.g. O_2, CO_2 (Meschia et al,1966; Gurtner et al,1982; see Chapter 1, Section D1); fatty acids (Thomas and Lowy,1983; Hendrickse et al,1985); electrolytes, e.g Na^+ (Štulc and Švihovec,1977; see Chapter 1, Section D2); and fat soluble vitamins, e.g. vitamin D (Koshy,1982).

When Fick's law is applied to placental diffusion, assuming that there is no metabolism in the placental membrane, the net transplacental flux is proportional to the difference between the concentration of permeant in the maternal blood ($[C_m]$) adjacent to the placental membrane, and the concentration in the fetal blood ($[C_f]$) adjacent to the placental membrane, multiplied by the permeability P (the ratio of the mobility of

molecules as expressed by the diffusion coefficient D_w, over the membrane thickness L) and the surface area of placental tissue lying between maternal and fetal blood streams available for exchange (S).

Hence, $J_{net} = PS ([C_m] - [C_f])$ (1)

The product PS is called the permeability surface area product and is the amount of blood cleared of a substance per minute per gram of tissue (Stein,1967).

Simple diffusion can be divided into two types : flow-limited and membrane-limited. Antipyrine has often been used as a standard for comparison of diffusion across placental membranes, as it is freely diffusible (Meschia et al,1967). Being lipid soluble it can dissolve in cell membranes, diffusing transcellularly through the tissue at rates that are orders of magnitude higher than the rates found for lipid insoluble molecules of similar molecular weight (Renkin,1952). The rate of diffusion of antipyrine is so high that it is no longer determined by the diffusion resistance of the tissue, but instead depends on the capacity of the vascular system to keep up the supply of the diffusing substance (Faber,1973); i.e. it is flow-limited. It is therefore a poor marker or standard in studies with hydrophilic molecules which are limited to the paracellular interstitial spaces. The diffusion of these hydrophilic molecules is usually diffusion or membrane-limited, i.e. their transfer is dependent on the surface area for diffusion rather than on blood flow. There is also a well established proportionality between tissue permeability and the coefficient of free diffusion (Renkin,1954; Landis and

Pappenheimer,1963). This relationship breaks down however at very large molecular weights where diffusion is restricted to some degree by steric factors.

Placental clearance (K) is often used as a measure of placental permeability and is empirically defined as the ratio of net flux over the maternofetal arterial concentration difference.

$$\text{Hence, } K = \frac{J_{net}}{[C_{ma}]} \quad \mu l min^{-1} g^{-1} \qquad (2)$$

It follows from above that if transfer of an uncharged solute is by membrane-limited diffusion only and the solute is not metabolized or restricted within the placenta then :

$$PS = K \qquad (3)$$

(Faber and Thornburg,1983; Sibley and Boyd,1988).

If transfer is by transcellular lipophilic diffusion, placental blood flow will be of major importance in determining placental transfer and an increase in placental surface area or a reduction in 'effective' placental thickness will have little effect. For simple paracellular diffusion of hydrophilic molecules an increase in the number of channels, a reduction in their length, or an increase in their radius, will alter their flux. These two controlling influences, namely the placental blood flow and the paracellular pathways of the placenta, will now be discussed in more detail.

a) Placental Blood Flow

Interfering with the growth of the placenta or with its blood supply leads to reduced fetal growth (Wigglesworth,1964; Bruce,

1977). In normal fetal development, growth rate and placental blood flow are closely related at a given gestational age (Bruce and Abdul-Karim,1973; Wootton et al,1977). However, this relationship changes with gestational age. In many species towards the end of gestation, the placenta stops growing while the fetus continues to gain weight and thus the fetal/placental weight ratio rises. Despite this, maternal placental blood flow continues to rise until term and the placental blood flow/fetal weight ratio falls or remains constant (Bruce and Abdul-Karim, 1973). Such observations have led to the concept that the placenta, except in late gestation, has a reserve of transport capacity above that required to sustain fetal growth (Harding et al,1985).

The application of radioisotope tracer techniques which have been used to investigate and quantify concepts of circulation, have been applied to the study of placental blood flow (Scheffs et al,1971). Isotopically tagged microspheres may be injected into the abdominal aorta via the femoral artery catheter. These microspheres are retained in the first capillary bed they encounter after intravascular injection. When properly mixed, they do not alter the circulation and will be distributed in the same manner as the inflowing blood. Thus, the localization of the radioactivity reflects relative blood flow (Rudolph and Heymann,1980). Steady state antipyrine infusion into the fetal circulation (using Fick's principle), has also been used to calculate uterine and placental blood flows (Meschia et al,1966; Cashner et al,1986).

Placental blood flow can obviously have a marked effect on the

transfer of flow-limited molecules. But the efficiency of exchange across the maternal and fetal placental blood flow systems will affect transfer of all molecules. Blood flow in the placenta has been measured in many species and the mean maternal placental blood flow on day 22 in the rat was 0.76 $ml min^{-1}$ or 121 $ml min^{-1} 100g^{-1}$ placenta (Bruce,1976 -measured using radioactive microspheres). However, there are marked species differences in maternal placental blood flow rates, e.g. 280 $ml min^{-1} 100g^{-1}$ placenta in sheep (Meschia and Battaglia,1973), 50-100 $ml min^{-1} 100g^{-1}$ placenta in man (Assali et al,1968), 50 $ml min^{-1} 100g^{-1}$ placenta in rabbit (Duncan,1969) and 50 $ml min^{-1} 100g^{-1}$ placenta in monkey (Lees et al,1971).

A crucial question is how the direction of flow in the fetal capillaries is orientated with respect to that of the maternal blood, as this should theoretically affect the efficiency of placental exchange. For example, Faber (1977) showed that transfer of oxygen is almost unlimited by the diffusional resistance of the placental barrier. The main determinants of oxygen exchange being the vascular geometry of the exchanger, the presence of shunts, perhaps maldistribution of flow and the oxygen consumption of the barrier itself. The presence of vascular shunts, deflecting blood away from exchange areas, can obviously greatly alter the efficiency of placental transfer, and these have been widely reported (Martin,1981; Faber and Thornburg,1983).

The countercurrent flow pattern, where the recipient blood entering the exchange capillary first comes into contact with the existing donor blood, is the most efficient system. In such

an exchanger, the concentration of an easily diffusible substance in the recipient vein can equilibrate with that in the donor artery, since a concentration gradient is maintained along the entire length of the exchange capillary pair. Crosscurrent exchangers occupy intermediate positions of efficiency according to whether the flows are unidirectional in both circulations or more or less random in one or both. The concurrent system is the least efficient, as the entering and recipient bloods rapidly come into equilibrium with one another and move together through the exchange area. So the concentration gradient is initially large, but falls rapidly along the length of the capillary. Even assuming no impediment to diffusion, the concentration in the recipient vein cannot exceed that in the donor vein (Martin, 1981).

Countercurrent flow has been suggested for the rabbit (Mossman,1926), the guinea-pig (Schröder and Leichtweiss,1977) and the rat (Lee and Dempsey,1976). The vascular arrangement which seems best able to describe circulatory anatomy in the haemochorial villous placenta of man and other primates is the multivillous flow pattern (Bartels et al,1962). A modification to this pattern was suggested by Gilbeau et al (1972), who pointed out that the villi are probably randomly orientated with respect to the direction of maternal flow. Thus, while some villous capillary loops will lie at right angles to the direction of maternal flows (crosscurrent), others will be orientated more or less parallel with it. In these latter villi, flow in two limbs of the capillary loop may be successively concurrent-countercurrent, or the reverse, with respect to the

maternal flow direction (Schröder et al,1985).

However, the evidence for these models is often only speculative or anatomical, so all placentas could show a variety of placental blood flow exchanger systems. Lee and Dempsey (1976) described the microcirculation of the rat placenta in a simplified form. Thin straight arteries at the fetal side traverse almost the entire thickness of the placenta before branching at the maternal side into a dense, tortuous mass of capillaries. This capillary network then forms anastomoses with a less tortuous, prevenous capillary network. The blood is then conducted into a series of wide-lumen venules which flow into large-size veins to be returned to the hilus and the umbilical cord. The maternal arterial blood of the rat placenta enters through a large central artery which then divides, forming numerous branches which spread out laterally to supply the organ. Then maternal blood flow is in the opposite direction to that of the fetal arterial blood, i.e. countercurrent flow. The evidence for this came from parallel examination of the microcirculation with the scanning and transmission electron microscopes. By examining casts of vessels injected with different coloured latex and prepared by corrosion in hypochlorite solutions, the fetal arteries, veins and capillaries of the placenta could be traced and the ultrastructural details of microcirculation and luminal surfaces could be studied. But one should remember that such fixation methods may cause artefacts and consequently, this is only a possible explanation of what happens in vivo.

b) Paracellular Pathways of the Placenta

As already explained, placental clearance (K) of a substance can be readily measured experimentally and for inert hydrophilic molecules crossing by unrestricted simple diffusion, K will equal PS if there are no electrical effects or flow limitation (Meschia et al,1967). Such measurements allow interspecies comparison of placental diffusional permeability to a range of molecules (Faber and Thornburg,1983). The ratio of K to the diffusion coefficient (D_w) of each molecule in a group of such hydrophilic molecules of increasing molecular size will be a constant, provided they are crossing by diffusion alone. So a K/D_w ratio could suggest whether the mode of placental transfer is simple diffusion or not (Sibley and Boyd,1988). K is usually further defined as K_{mf} or K_{fm} depending on whether maternofetal or fetomaternal clearance respectively is being measured. However, for molecules crossing by simple diffusion only, then $K = K_{mf} = K_{fm}$. Thus if $K_{mf} = K_{fm}$ there can be no active transplacental transport for that molecule.

Clearance measurements have clearly shown that the permeabilities of haemochorial placentas in rabbit (Faber et al, 1971; Faber,1973), guinea-pig (Thornburg and Faber,1977; Hedley and Bradbury,1980) and man (Willis et al,1986; Bain et al,1988) are as much as fifty-fold that of the epitheliochorial sheep placenta (Boyd et al,1976). One simple way to explain this is to suggest that the water-filled channels or pores constituting the paracellular route through which diffusion is occurring have, in the sheep, a radius only slightly greater than the permeant molecules themselves. This leads to restricted diffusion, with

increasing restriction as molecular size increases. Estimation of a pore size (Renkin,1954) of 0.4nm for the sheep (Boyd et al, 1976) and 10nm for the guinea-pig (Hedley and Bradbury,1980), reflect these differences in permeability. However, it is often hard to actually visualize these pores within the placental syncytiotrophoblast and often difficult to fit an isoporous model (Štulc et al,1969; Štulc,1985). In the pig, the chorionic epithelium is made up of distinct cells, so the paracellular route is probably between these cells. However, in the haemochorial placentas of man, guinea-pig and rat, a true syncytium is found and the nature of transtrophoblastic channels here is a matter of some debate (Kaufmann et al,1982; Berhe et al,1987; Kaufmann et al,1987).

The actual resistance barrier to diffusion has been investigated in many species. Faber and co-workers (1968; Faber and Stearns,1969) looked at the haemodichorial placenta of the rabbit, investigating the total diffusion resistance made by each of the placental layers. They found that the fetal endothelium contributed only 10% of the total resistance to diffusion of a range of lipid insoluble solutes, as did the syncytiotrophoblast. This led to the hypothesis that most of the diffusion resistance is located somewhere in the middle layer. But whether this resistance lies in the junctions between the cells of the cytotrophoblast or in the long pathways between the intercellular spaces of the various cell layers, is at present unresolved.

A similar study was carried out in the rat haemotrichorial placenta, but using ultrastructural techniques (Aoki et al,

1978; Metz et al,1978). The three trophoblastic layers (I-III) were identified, numbered from maternal to fetal side. Layer I showed fenestrations and patent intercellular clefts (Robertson et al,1971) and macromolecules were not restricted by this layer. Layer II is syncytial and macromolecules appear to cross by vesicular uptake (Metz et al,1976). Extensive gap junctions located at the interface between layer II and III may control transport of subtances between these two syncytial layers. So layer II may be the main barrier to placental transfer, but Aoki et al (1978) proposed that layer III was the real barrier, since they observed no passage of horseradish peroxidase (HRP) across this layer. The fetal capillary endothelium is probably a significant barrier to high molecular weight substances (Sibley et al,1982 - at least it is in the guinea-pig), leaving layers II and III to control the passage of low molecular weight substances. Molecular charge does not seem to be important with respect to the placental transfer of small molecules like sodium and chloride (Faber and Hart,1967; unless influenced by a trans-placental potential difference; see Chapter 1, Section D2), but does seem to affect the permeability of macromolecules. Indeed, in the perfused guinea-pig placenta (Sibley et al,1983; Berhe et al,1987), anionic HRP did not penetrate the endothelial cell layer of the fetal capillaries, whereas cationic HRP penetrated into subendothelial spaces. Thus, in guinea-pig placentas the lateral intercellular spaces seem to be an important site, both of macromolecule penetration and of its restriction. The fact that both size and charge of a particular protein affect its permeation of placental (as well as other) capillaries, raises

the question of the relative importance of these two macromolecule characteristics, especially as most endogenous plasma proteins are anionic. The transfer of HRP across the human placental cotyledon perfused in vitro, indicated that the pathway of the transfer from mother to fetus was different and more selective than from fetus to mother (Sideri et al,1987), thus suggesting that transfer was not by simple diffusion alone.

It must also be noted that many of these measurements have been made using perfused placentas and perfusion itself seems to increase placental permeability, at least in the guinea-pig (Hedley and Bradbury,1980). It has also been suggested that the fixation methods involved in the study of permeability may produce artefacts themselves and spurious localization of tracers (Orgnero de Gaisán and Aoki,1985;1987; Orgnero de Gaisán et al,1985).

The permeability of the near term rat placenta in comparison to other species will be discussed in more detail in Chapter 2. Also, the role of a transplacental potential difference in controlling placental transfer via paracellular pathways will be discussed in Chapter 1, Section D2.

2) Facilitated Diffusion

This is defined as the chemical combination of a given substance with a 'carrier' in the membrane, the carrier-substrate crossing the membrane at a rate faster than that of the substrate alone (Stein,1967). Dextro sugars seem to cross the placenta in this way (Johnson and Smith,1980; Bissonnette et al,1981; see Chapter 1, Section D3).

Since diffusional processes follow Fick's law, the following conditions indicate the presence of a carrier in transfer processes :

a) the appearance of a transfer process saturable at high substrate concentration;

b) a faster rate of transfer than would be predicted on the basis of the physicochemical properties of the molecule alone;

c) reduced transfer in the presence of similar molecules, i.e. competitive inhibition;

d) specificity of the transport system (Stein,1967).

Carrier molecules exhibit Michaelis Menton type kinetics :

$$J = \frac{S.V_{max}}{K_m + S} \qquad (4)$$

where J= unidirectional flux
S= substrate concentration
K_m= Michaelis constant or substrate concentration for half the maximal unidirectional flux
V_{max}= maximal transport rate for unidirectional flux

Hence, an increase in the V_{max} might be induced by carrier synthesis or recruitment or by an increase in placental surface area, provided the number of carriers per unit area remains constant.

3) Active Transport

This is the transport of molecules 'uphill' against an electrochemical gradient, probably by a carrier-substrate mechanism, but in this instance linked to an energy source (Stein,1967). In the placenta several substances seem to cross by active transport, e.g. amino acids (Yudilevich and Sweiry, 1985; see Chapter 1, Section D4), calcium (Twardock and Austin,

1970; Štulc and Štulcová,1986; see Chapter 1, Section D5) and water soluble vitamins, i.e. vitamin C (Ingermann et al,1986) and B_2 (Dancis et al,1985).

4) Endocytosis

This can be divided into two types, i.e. fluid-phase or receptor-mediated endocytosis. The first is the engulfment of tiny droplets of solute and water by invaginated cell membranes, which then cross the cell and discharge their contents on the other side. The second is more specific, with substances binding to receptors in the membrane before invagination (Steinman et al,1983). Immunoglobulins seem to cross the placenta in this way (Brambell,1966; Wild,1981; see Chapter 1, Section D6).

5) Bulk Flow or Ultrafiltration

In this mechanism, hydrostatic or osmotic pressure of gradients may cause the transfer of water molecules carrying dissolved particles, e.g. electrolytes. But this is not thought to be important in placental transfer (Faber and Thornburg, 1983). Similarly, breaks in the placental villi may account for placental transfer of some substances, for example, as in the passage of fetal erythrocytes into the maternal circulation. However, there is no hard evidence to confirm this at present.

SECTION C : Methods Used To Study Placental Transfer

Placental transport has been studied using various methods and due to the fragility of the feto-placental unit many problems

have been encountered. Any invasive technique in the uterine cavity can cause uterine contraction due to muscle spasm and loss of amniotic fluid, both of which may cause death, hypoxia or asphyxia. Manipulation of the fetus may alter placental blood flow and stimulate the autonomic nervous system or increase hormonal activity. The practicality of catheterizing fetal blood vessels is determined not only by their size, but also by their fragility and reactivity. Blood volume of the fetus is important in relation to the amount of blood withdrawn during blood sampling and to the quantity of any fluid administered. Maternal anaesthesia is also crucial, since it may affect blood pressure and thus indirectly affect the fetus and placenta, especially as it is believed that the uteroplacental unit cannot auto-regulate (Wallenburg,1981). Hence validation of all placental transport studies must be rigourous.

1) 'In Vivo' Studies

Techniques for intravascular catheterization of the ewe and her fetus were introduced many years ago (Meschia et al,1965). This method is useful since it can be maintained over long periods of gestation in conscious animals. However, it must be remembered that there are no criteria to ensure that recovery is ever complete, with a return to perfectly normal intrauterine conditions. The simplest type of investigation requires a source of fetal and maternal blood and a peripheral venous infusion catheter. More information can be gained if umbilical and uterine venous blood can also be obtained at the same time, thus enabling the arterio-venous (A-V) differences to be measured.

Potential differences can also be measured between fetal and maternal catheters, or between fetal blood and allantoic or amniotic fluid (Mellor,1980; see Chapter 1, Section D2). A combination of A-V or venous-arterial (V-A) differences and flow measurements enable estimates of fetal and uteroplacental uptakes and outputs to be made. This can be done by direct methods, e.g. flow probes, or indirectly by Fick principle methods or microspheres (Faber and Thornburg,1983; Myers et al, 1986). Ruminants seem to be the best species for chronic catheterization (Silver,1981), this method being more difficult to establish in non-ruminants for several reasons. The fetus may be too small to catheterize (rat, rabbit, guinea-pig), the placenta may be diffuse and therefore susceptible to damage at surgery (horse, pig), the uterus may be too reactive or the animal too difficult to handle (monkey).

Perfusion of the placenta <u>in situ</u> refers to perfusion of the fetal placental vascular bed(s) without severing the normal anatomical connections to the mother and without removing the placenta from the maternal organism. Only the umbilical vascular bed is perfused, the fetus having been removed (Dancis and Money,1960; Fenton,1977; Robinson et al,1988; see Chapter 2). Advantages of this type of study include : 1) Under steady state conditions the rate of transfer can be simply calculated as the product of the pump flow rate and the difference of the inflow and outflow concentrations. 2) The composition and circulation of the fluid on the fetal side of the placenta can be easily manipulated. 3) Toxic or even lethal doses of drugs can be introduced into the fetal perfusion medium. 4) Effects of fetal

metabolism and fetal homeostatic mechanisms are eliminated. On the other hand, transport is studied under unphysiological conditions, especially with respect to blood flows and this may be affected by maternal anaesthesia, operative trauma and the perfusion procedure itself.

Another *in vivo* technique involves injecting radioisotopes into the maternal circulation, then sacrificing fetuses at timed intervals and determining their radioactivity content by carcass analysis (Flexner and Pohl,1941; Hedley and Bradbury,1980). *In vivo* techniques in the human subject are difficult from an ethical point of view, but several indirect studies of placental transfer have been made (Willis et al,1986; Bain et al,1988).

2) *'In Vitro' Techniques*

The complete isolation and dual perfusion of the guinea-pig placenta (Leichtweiss and Schröder,1981) enables the fundamental mechanisms of transfer to be investigated without the influence of the mother or the fetus. However, it is a very unphysiological model and perfusion itself may alter placental permeability and transport (Berhe et al,1987). Yudilevich et al (1979) used a rapid single-circulation paired-tracer dilution technique to examine sugar transport, at either maternal or fetal surfaces of the syncytiotrophoblast, in the isolated dually-perfused guinea-pig placenta. This method involves the simultaneous dilution of a test substrate and an extracellular tracer of similar size and diffusibility.

Single human cotyledons have been isolated and perfused *in vitro* (Schneider et al,1972; Bloxam and Bullen,1986), but less

precise control over the maternal circulation is possible. Several cannulae are placed in maternal blood-filled lacunae, as there are no distinct blood vessels to catheterize. The usual approach involves open-circuit perfusion of both maternal and fetal circulations through a branch of the umbilical cord with a modified Earles' solution. Antipyrine is usually used as a diffusional marker when looking at maternal to fetal transfer. Recently, an alternative method has been described where the placental lobule is perfused through both circulations with diluted autologous fetal blood (Contractor and Stannard,1983). This closed-circuit preparation has an additional benefit in that large, slowly transported molecules such as immunoglobulins, can be examined over a period of several hours (Contractor et al,1983). However, antipyrine equilibrates too quickly, as it is flow-limited and is unsuitable for use in such experiments. Creatinine has been used instead, since its rate of diffusion provides an index of the overlap of the two circulations and the available exchange area (Eaton et al,1985). Another in vitro technique is the mounting of tissue in an Ussing chamber, which has been used for the specialized pig placental membrane (Sibley et al,1986) and for yolk sac membranes (Chan and Wong,1978; Gibson and Ellory,1984). Here potential difference measurements can be made across the tissue, being generated by the differential passage of ions from one side of the membrane to the other, and thus can reflect a net flux of ions, e.g. Na^+ (Mellor,1969).

Dissection, slicing and homogenizing are essential when studying the component parts of the biochemical mechanisms

underlying the physiological function of organs. Three such placental tissue preparations, are the incubation of slices of placental tissue (Dancis et al,1968) or uniform fragments of placental villous tissue (Smith et al,1973) and the incubation of isolated microvillous membrane vesicles (Smith et al,1974). When incubating isolated placental villous tissue, portions are taken from maternal and fetal surfaces to avoid decidua and stem villi (Smith,1981). Uptake of radioactive substances is usually taken as a rough equivalent of uptake by microvillous membrane from maternal blood. While this is probably a valid approximation, it involves a number of assumptions and limitations : 1) The microvillous membrane is only one of a number of plasma membranes to which medium solutes are exposed. Its large surface area and free exposure to the medium suggest that it will be responsible for most of the uptake but there is no quantitative data to demonstrate that this is so. 2) The syncytiotrophoblast is known to be suceptible to damage in vitro (Panigel,1972) and may be poorly preserved during prolonged incubations. Incubation of microvillous membrane vesicles allows measurement of transport into vesicles formed from the maternal plasma membrane of the human (Smith et al,1974) and more recently the rat (Glazier et al,1988) syncytiotrophoblast. Vesicles from basolateral (fetal-facing) membranes of the human syncytiotrophoblast (Boyd et al,1979; Smith,1981) have also been isolated. Uptake of solute can be determined by incubating vesicles with substrate and isolating them by filtration (Bissonnette,1982). The microvillous membrane vesicle preparation can be used more flexibly and handled more rapidly

in short-term incubations and is of course more homogenous; however, the isolated villous tissue preparation contains the membrane in a more natural cell environment. Neither preparation takes into account that transport normally occurs across both maternal and fetal plasma membranes, as well as fetal endothelium, in series.

Biochemical and histochemical analysis of placental tissue can show up the location in fetal and maternal placental membranes, of various enzymes which are used in transport processes. This is especially important for substances transported actively or via receptor-mediated methods. Ultracytochemical localization of adenylate cyclases involved in producing energy for 'active' processes, has been widely reported in the placenta (Firth et al,1979; Mukherjea et al,1986; Matsubara et al,1987). Various hormone receptors have also been isolated from placental tissue (Truman and Ford,1984), which give insight into some of the controlling influences governing placental transport. Similarly, qualitative determination of transport properties can be obtained by using ultracytochemistry, electron microscopy and electron probe localization of solute distribution within tissue (Sibley et al,1982;1983; Kaufmann,1981). Morphometry investigates the quantitative aspects of morphology. The size of the total placental surface area being a useful parameter to measure (Baur,1981), with respect to transfer studies.

Finally, it is possible to culture embryos <u>in vitro</u> with their attached extra-embryonic membranes (Payne and Deuchar,1972). Transfer of small metabolites to the embryo seems to depend on the number of embryonic layers present. By modifying this method

(Dunton et al,1986), it has been possible to grow rat visceral yolk sac separated from the embryo. The yolk sac vesicle obtained after the extended culture is called a 'giant yolk sac' and can be used to study the passage of substances from maternal to embryonic tissue in early development. Various placental cell lines have also been developed which are capable of expressing characteristics of extra-embryonic membranes in vitro and after in vivo transplantation (Soares et al,1987). Rat placental cells have now been cultured in vitro (McArdle et al,1985) and cultured human placental cytotrophoblast has been shown to differentiate into syncytiotrophoblast (Kliman et al,1987). Special microinjection techniques have also been developed, which can be readily applied to cultured cells or fresh placental tissue (Gaunt et al,1986). By this method it should be possible to define directly the internal structure of the tissue using detectable probes and explore possible organelle and molecular transplacental transport routes (Gaunt and Ockleford, 1986).

SECTION D : Examples Of Types Of Placental Transport

1) Oxygen

The placenta has often been described as the fetal 'lung', it is however, far less efficient as an organ for gas exchange (Longo,1981). The characteristics and requirements for respiratory gas exchange have been analysed in some detail (Longo et al,1972). It seems generally agreed that the transfer of oxygen (O_2) is almost unlimited by the diffusional resistance

of the placental barrier (Meschia et al,1967), thus crossing by simple flow-limited diffusion. The important factors determining placental gas exchange are the blood gas tension gradients across the exchange area, which will obviously depend on the rate and distribution of blood flow on either side, the vascular architecture and O_2 usage by the placental tissue itself. There are other features which are specifically involved in placental O_2 transfer, for example, the affinity and capacity of maternal and fetal haemoglobin for O_2. Analysis of the relative importance of these various factors is hampered by technical problems of obtaining reliable data representative of the *in utero* condition, i.e. ideally from unanaesthetized *in vivo* preparations.

The mean pressure gradient for O_2 across the sheep placenta has been reported to be 19-24mmHg (Barron,1951), however, it is only 2-4mmHg in the mare (Comline and Silver,1970). Thus a relatively large O_2 partial pressure (pO_2) gradient would indicate a low diffusion capacity, suggesting that O_2 delivery to the fetus is partially limited by diffusion resistance of the membrane. However, the presence of 'shunting' in maternal and fetal circulations could introduce sizable errors. A second complicating factor being the O_2 consumption by the placenta, which appears to be as much as 10-30% of the O_2 delivered to it (Campbell et al,1966). Power and co-workers (1967) studied the diffusion characteristics of carbon monoxide in sheep and dog placentas. With this information they estimated the diffusion capacity for O_2 and concluded that diffusion resistance of the membrane was not limiting for O_2 transfer. The diffusing

capacity was four times that previously estimated from partial pressure gradients in uterine and umbilical veins. All these results have been obtained using indwelling catheters, therefore similar results are not available for smaller animals like the rat. However, blood gas tensions have been measured in the fetal guinea-pig with maternal anaesthesia, using a catheter in the right atrium (Carter and Grønlund,1982; Girard et al,1983).

The rates of O_2 transfer across the placenta will be further influenced by the amount and type of haemoglobin present, the shape of the O_2 dissociation curves and the influence of pH changes at exchange areas. In fetal blood, O_2 uptake is favoured by a higher haemoglobin concentration (c.10g% maternal, 12g% fetal) and a high O_2 affinity, i.e. saturation with O_2 at a relatively low pO_2. The transfer of O_2 to the fetus is also favoured by the transfer of carbon dioxide in the opposite direction (Bohr effect). Both an increase in temperature or a decrease in pH, will cause the O_2 dissociation curve to move to the right and in this position a higher pO_2 is required for haemoglobin to bind to a given amount of O_2 (and vice versa). 2,3-diphosphoglyceric acid (2,3-DPG) found in red blood cells, combines with reduced β-haemoglobin chains and reduces the affinity of haemoglobin for O_2, i.e. more O_2 is liberated. The cause of the greater affinity of fetal haemoglobin for O_2, is the poor binding of 2,3-DPG by the ɣ-polypeptides that replace the β-chains in the fetal haemoglobin. Thus, the fetal O_2 dissociation curve is to the left of the maternal O_2 dissociation curve (Green,1976). Consequently, despite its low pO_2, fetal blood is capable of transporting large amounts of O_2

from the placenta to fetal organs, due to its high affinity for O_2 and because the rate of perfusion of fetal organs is high in comparison to their O_2 requirements (Meschia,1984).

In the placenta the double Bohr effect is noted. The maternal blood releases O_2 to fetal blood and accepts fetal metabolites, which causes a fall in pH of maternal blood. This results in a shift of the maternal O_2 dissociation curve to the right, which automatically increases the transfer of O_2 to the fetus by releasing O_2 from oxy-haemoglobin. At the same time, fetal blood in shedding its metabolites, undergoes a rise in pH which shifts the fetal O_2 dissociation curve to the left; thus the double Bohr effect (Bartels et al,1962).

Recently, there has been some evidence for a carrier-mediated diffusion mechanism for O_2, via cytochrome P_{450} (Gurtner et al, 1982). They measured the diffusing capacities of O_2 and argon in the presence of carbon monoxide and showed a fall in O_2 diffusing capacity with carbon monoxide, but not for argon. They interpreted these results as being consistent with the hypothesis that placental O_2 transport is partially carrier-mediated and that O_2 and carbon monoxide compete for the same carrier. However, technical objections to the interpretation of the data have been raised and therefore the evidence is not compelling (Faber and Thornburg,1983).

The stability of blood gas tensions in the mother and fetus seem to be maintained throughout gestation (Comline and Silver, 1972). Also, neither maternal hyoxia or hyperoxia seem to affect fetal blood pO_2 to any great extent in ruminants (Comline et al, 1965), man (Meschia,1979) or rhesus monkey (Jackson et al,1987).

However, the fetal pO_2 in the horse can be altered by changes in maternal pO_2 (Comline and Silver,1970). These interspecies variations may be explained by differences in placental structure, especially with respect to the blood micro-circulation.

Girard et al (1983) have tried to look at placental O_2 transport in the anaesthetized guinea-pig. They found that the carotid arterial O_2 saturation in the fetal guinea-pig was 59%, well within the range quoted in other species, e.g. 60% for sheep (Dawes et al,1954). The O_2 supply to fetal tissue was 1.1 μmolmin^{-1}g^{-1}, whilst uteroplacental O_2 uptake was 0.35 μmol min^{-1}g^{-1} fetal body mass. It can be seen that 3 times more O_2 is supplied than is actually consumed, hence O_2 deprivation should not be a problem. In theory, there must be a minimum O_2 supply at which fetal O_2 consumption and acid-base balance are within normal limits. There are numerous factors which can produce a decrease in the supply of O_2 to the fetus. At any time, the pO_2 and therefore the O_2 content of fetal blood can decrease, because of changes either in the maternal organism or the placenta, over which the fetus has no control. Primarily these changes are :1) a decrease in the area of placental exchange (e.g. placental infarction), 2) a decrease in maternal placental blood flow (e.g. maternal hypotension, constriction of placental arterioles, uterine contraction), and 3) a decrease in maternal arterial O_2 saturation (e.g. hypoventilation), (Meschia,1979).

In the ordinary usage of the term, fetal hypoxia means any decrease below normal in the level of fetal oxygenation. Such a decrease may come about in several ways, most commonly as a

reduction in :1) the pO_2 of umbilical venous blood (caused by the above placental/maternal changes), 2) in the O_2 capacity of fetal blood (e.g. due to elevated levels of carbon monoxide), or 3) in the perfusion rate of the umbilical circulation and the fetal body. As the pO_2 falls, there is found to be a progressive increase in blood flow to the central nervous system and the heart, although cardiac output and placental blood flow tend to remain constant (Meschia,1984).

Fetal O_2 extraction is the ratio between fetal O_2 consumption and O_2 delivery. In the fetal lamb, O_2 delivery is high relative to consumption and hence extraction is low, normally about 30%. When O_2 delivery falls, O_2 consumption can be maintained via an increase in O_2 extraction to about 60%. In man, umbilical blood flow is reported to be lower than in sheep (Erskine and Ritchie, 1985), but O_2 consumption appears to be similar in the two species (Bonds et al,1986). Estimation of fetal O_2 extraction in the human, gave a much larger value than that in the sheep (Rurak et al,1987). Although the conditions under which the measurements were made differed, they suggest significant quantitative differences in the systems for fetal O_2 delivery in man and sheep, with the human system being more efficient for placental O_2 transfer.

de Grauw et al (1986) have looked at the effect of maternal hypoxia on fetal growth retardation in rats. They showed a correlation between the severity of maternal hypoxia and the magnitude of fetal growth retardation. Liver weights were more and brain weights less markedly reduced than body weight. This same pattern is also observed in studies in which either the

uterine (Wigglesworth,1964) or the umbilical (Myers et al,1971) blood flows are reduced. The hypoxic animals also all show enlargement of the placenta in comparison to fetal size and through this mechanism, possibly increased O_2 transfer to the fetus. Intra-uterine growth retardation (IUGR) in sheep following restriction of placental growth, is associated with a reduced supply of O_2 to both the pregnant uterus and fetus, and a redistribution of O_2 to the fetus. Furthermore, the greater uterine and fetal extraction of O_2 found, suggests that a smaller margin of safety may exist between supply and demand in IUGR (Owens et al,1987A).

Fetal lamb O_2 consumption falls with long-term reduction of umbilical blood flow (Anderson et al,1986), which is in harmony with a reduced rate of fetal growth in the lamb when umbilical flow is reduced for longer periods of time (Anderson and Faber, 1984) and with the known effect on newborn human weight of maternal smoking (Longo,1977), or such a comparatively mild provocation as living at altitude (Moore et al,1982). Acute uterine blood flow occlusion also reduces O_2 delivery to the fetus in sheep, but has no effect on fetal O_2 consumption. This suggests that under normal physiological conditions the supply of O_2 to the pregnant uterus of sheep exceeds the minimum necessary to sustain fetal oxidative metabolism (Wilkening and Meschia,1983). However, as suggested above, a long term reduction in uterine blood flow would probably restrict fetal growth.

It is not known whether O_2 per se has any regulatory influence on placental growth and maternal placental perfusion. There

being no clear experimental evidence for or against the hypothesis that maternal and/or fetal chronic hypoxia induces compensatory changes in placental perfusion and the area of transplacental exchange (Meschia,1979).

2) Sodium

Some of the earliest placental studies investigated unidirectional sodium transport from mother to fetus in vivo in several species (Flexner and Gelhorn,1942), including man (Flexner et al,1948). They showed that in all species studied, unidirectional maternofetal sodium flux (J_{mf}) increased throughout gestation, falling off just before term. Comparison of net sodium flux with J_{mf} revealed the varying size of the fetomaternal sodium flux (J_{fm}) in the different species. Another interesting observation correlated placental morphology with the magnitude of the J_{mf} sodium; the pig epitheliochorial placenta having a much smaller J_{mf} sodium than the human haemochorial placenta (Flexner et al,1948). The permeabilities of the different placental types to hydrophilic molecules (Boyd et al, 1976; Hedley and Bradbury,1980; Robinson et al,1988; see Chapter 2), as well as the similar sodium concentrations in maternal and fetal plasma, suggest that passive, paracellular diffusion is important for sodium and it is widely assumed that this route predominates. However, some evidence now points to an important transcellular component for sodium transport, either in co-transport with other solutes or alone. A transplacental potential difference (p.d.) would also exert an important influence on the transfer of sodium and other ions, but there is

controversy concerning the existence of such a p.d.

A wide range of maternofetal p.d.'s have been measured between maternal and fetal vascular catheters, ranging from -70mV (fetal side negative) in goats (Mellor,1970), to +15mV in rats (Mellor, 1969). However, it is not clear whether the maternofetal p.d. as measured in chronically catheterized animals, is identical to any transplacental p.d. acting at the placental exchange area and therefore important in governing ion transfer. Mellor (1970) stated that the p.d. appears to originate in the placenta itself, as in sheep it can be reduced to 0mV (from -50mV) during temporary occlusion of the umbilical vessels. In the guinea-pig, fetal membranes other than the placenta, e.g. amniotic membrane, have no ability to generate a p.d. (Mellor, 1969). It is clear that the epitheliochorial pig placenta mounted in an Ussing chamber, generates a p.d. (Crawford and McCance,1960; Bazer et al,1981; Sibley et al,1986), but of a different magnitude to the maternofetal p.d. (Boyd et al,1986). A p.d. across a membrane is usually generated by active transport of one ion or by active exchange of two ionic species, other ionic species being distributed across the membrane in conformance with the Nernst equation and, the placenta is presumably no exception to this. However, whether the Nernst equation is really applicable is uncertain (Sibley and Boyd, 1988), as it is really only applicable when there is no net flux of ions. The transplacental p.d. calculated using the Nernst equation, from the plasma concentrations of Na^+, K^+, Mg^{2+} and Cl^-, is actually found to be zero. A zero p.d. was also calculated from the steady state concentration ratios found when guinea-pigs and sheep were

injected with ions not usually present, e.g. Li^+, Rb^+, Br^-, SO_4^{2-}, etc. (Binder et al,1978; Thornburg et al,1979). It follows from this, that if the maternofetal p.d. between mother and fetus really is generated at the placental exchange barrier, then this equal distribution of electrolytes in maternal and fetal plasma would have to be maintained by the expenditure of energy. Thornburg's group (1979) thought it unlikely that specific ionic pumps would exist for each of these 'exogenous' ions. However, since then, there has been increasing evidence for ionic pumps for these species in other tissues (Hoffman, 1982). For example, SO_4^{2-} and Cl^- fluxes are probably mediated by a single carrier protein in the syncytiotrophoblast human brush border membrane (Boyd and Shennan,1986A;1986B). Also, existing pumps may have the appropriate affinity for these 'exogenous' ions and consequently you may get Li^+/Na^+, Rb^+/K^+ exchange, etc. Faber et al (1987) still favour their view of a zero transplacental p.d., but highlight the continuing lack of experimental information concerning the resistance of elements in the circuit during maternofetal p.d. measurement and the paucity of evidence for other electrogenic sites; without which the controversy still remains.

Sibley et al (1986) have been investigating this problem directly, using the membranous pig placenta mounted in an Ussing chamber <u>in vitro</u>. A p.d. of +5.9mV (fetal side positive) was measured, which was different to that measured by Crawford and McCance (1960; -50mV) and Bazer et al (1981; -6mV). There is unfortunately no obvious explanation for these differences. However, in the study of Sibley et al (1986), addition of

adrenaline to the fetal side stimulated the p.d. (fetal side becoming more positive) and produced a net sodium flux towards the fetus; both the p.d. and the sodium flux being inhibitable by ouabain. This has been followed up by maternofetal p.d. measurements *in vivo* using chronic fetal catheterization (Boyd et al,1986). The maternofetal p.d. measured was fetal side negative and was much larger in magnitude than the *in vitro* transplacental p.d., but adrenaline added to the fetal circulation stimulated this maternofetal p.d., in the same way as it did *in vitro*. The difference in the actual p.d. measured, may reflect deterioration of the tissue in the *in vitro* preparation or may be due to the fact that the tissue mounted in the Ussing chamber is not precisely the exchange barrier found *in vivo* (which includes extra connective tissue layers). However, both preparations demonstrate the involvement of fetally facing β-adrenergic receptors in the stimulation of electrical activiy and presumably, ion flux across the pig placenta (Boyd et al, 1987). Clearly the nature of the transplacental p.d. requires further investigation.

Besides this direct evidence of active sodium transport across the pig placenta, other evidence is now mounting to suggest a carrier-mediated transcellular component to transplacental sodium movement in other species. Indeed, the clearance measurements for sodium in the haemochorial placentas, show that sodium is cleared faster than would be expected if crossing by simple diffusion alone (Flexner et al,1948; Hedley and Bradbury, 1980; Robinson et al,1988). This may simply reflect the influence of a transplacental p.d., fetal side negative or the

existence of heterogenous sized pores (Štulc et al,1969), but it may be the result of transcellular sodium transport.

Simultaneous measurements of sodium and potassium fluxes in the in situ perfused guinea-pig placenta (Bailey et al,1979), suggested the presence of two ouabain-dependent Na^+-K^+ pumps, one orientated with its active K^+ component directed towards the fetus and the other towards the mother. In support of this argument, histochemical studies in the same species revealed large concentrations of a K^+-dependent ouabain-sensitive enzyme located in the maternal-facing membrane of the trophoblast (Firth et al,1979) and the demonstration of a dense Na^+-K^+-ATPase in relation to the maternal microvilli (Firth and Farr, 1977). Firth and Farr (1977) also found Na^+-K^+-ATPase on the fetal-facing plasma membrane and in the pig, it is localized in the fetal-facing chorionic epithelium of the areolar regions of the placental surface (Firth et al,1986). In the human placenta Na^+-K^+-ATPase activity and ^3H-ouabain binding sites were also present on the fetal-facing plasma membrane of the syncytiotrophoblast (Whitsett and Wallick,1980). It is also interesting to note that in the rat, Zamora and Arola (1987) found an increase in rat placental Na^+-K^+-ATPase activity between day 12 and 21; perhaps in parallel with an increasing placental transfer of sodium.

Sodium transport across human placenta microvillous plasma membrane vesicles, was inhibited by amiloride and stimulated by a proton gradient, demonstrating the presence of Na^+/H^+ exchange (Balkovetz et al,1986; Chipperfield et al,1986). There is also evidence for several more Na^+-coupled transport systems in this

preparation. For example, a Na^+-coupled amino acid uptake system has been demonstrated (Ruzycki et al,1978; Boyd and Lund,1981; see Chapter 1, Section D4), a Na^+-Cl^- co-transporter (Boyd et al,1980) and a Na^+-dependent PO_4^{2-} transport system (Brunette and Allard,1985).

Thus, it would appear that the transplacental movement of sodium is via two routes :1) By simple diffusion via a paracellular pathway through wide aqueous channels, dependent on concentration and electrical gradients (Štulc and Švihovec, 1977), 2) A transcellular pathway which may have several components (Sibley et al,1985; Sibley and Boyd,1988). The relative importance of these two routes and the ratios of J_{mf} to J_{fm} for sodium, probably vary from species to species. The existence of such a transcellular route may allow control of active sodium transport, e.g. by adrenaline (Sibley et al,1986; Boyd et al,1987). Hormonal control of passive paracellular sodium permeability may also be important, sodium transfer being a rate-limiting factor in fetal growth (Conrad and Faber,1977), but this requires further investigation.

Finally, the yolk sac has also been investigated as a possible route for sodium transfer (see Chapter 2). When mounted in an Ussing chamber, the rat yolk sac placenta gave a p.d. of +3.85mV (fetal side positive), which varied throughout gestation (Chan and Wong,1978; Gibson and Ellory,1984). This p.d. could be reduced by cooling, metabolic inhibtors and ouabain, suggesting an active transport of sodium across the rat yolk sac placenta, dependent on the presence of a p.d.

3) Glucose

Glucose is the main substrate for oxidative metabolism in the fetus and is an important precursor for the synthesis of fetal glycogen and lipids in man (Gabbe and Quilligan,1977). The major source of glucose for the fetus, is that transferred across the placenta from maternal blood (Kalhan et al,1979). Fructose levels are very low and it is not used as a fetal fuel or as a building material in significant amounts in man (Gabbe and Quilligan,1977). However, in ungulates, fructose is the primary sugar in fetal blood and glycogen content of the placenta is minimal (Longo,1972). The concentration of D-glucose is higher in maternal than in fetal plasma (Tsoulos et al,1971) in both man and sheep, suggesting a purely passive process for D-glucose transport across the placenta.

In 1952, Widdas analysed the data obtained by Huggett et al (1951) in sheep, showing that glucose transfer was much more rapid than fructose, which is of similar size. He thus postulated that glucose was transferred by facilitated diffusion, that is, a mechanism that shows stereospecificity, competition between isomers and analogues, saturation phenomena and chemical inhibition (Leichtweiss,1981). Since then, these criteria have been satisfied for placental glucose transfer in many different species, using various experimental models.

Dancis et al (1958) reported that dextro-sugars are transported across the intact guinea-pig placenta quicker than laevo-sugars, implying stereospecificity for the carrier system. Ely (1966) using the fetal side perfused guinea-pig placenta, provided evidence for competition between glucose, fructose and

galactose, with a saturable glucose transfer. Carstensen et al (1977) looked at the effect of phloretin, which has been shown to inhibit carrier-mediated transport of monosaccharides across the membranes of erythrocytes (LeFevre,1961), in the perfused human placenta. Phloretin caused a 60% inhibition of human placental D-glucose transport, with no effect on L-glucose and a similar effect was seen in the dually-perfused guinea-pig placenta (Schröder et al,1975). Use of the non-metabolizable glucose analogue, 3-O-methyl-D-glucopyranose (3MEG) in sheep (Stacey et al,1978) and in man (Rice et al,1976A;1976B), has suggested that 3MEG is a good marker for glucose, both sharing the same carrier. The transfer of 3MEG being as rapid as that for glucose and competitively inhibited in the presence of D-glucose. Yudilevich et al (1979) using the indicator dilution method in the in situ perfused guinea-pig placenta, demonstrated stereospecific, saturable transport for D-glucose, at both maternal and fetal surfaces. Tracer backflux and transplacental flux were equal from both sides, as would be expected for a facilitated diffusional system. They concluded that similar hexose carriers which resemble the human erythrocyte carrier (LeFevre,1961), exist at the membrane on both sides of the trophoblast. Rice et al (1979) demonstrated in the perfused human placenta that unlike transport across, e.g. gut mucosa (Murer and Hopfer,1974) or renal tubule (Kinne et al,1975), placental D-glucose transfer was independent of sodium and did not require energy, in that it was not inhibited by dinitrophenol (DNP). In this respect, the placental glucose system resembles that of adipose tissue (Carter et al,1972).

The kinetics of facilitated transport of glucose have been investigated in placental microvillous membrane vesicles. This system has a high K_m (31mM) and is independent of sodium gradient coupling (Johnson and Smith,1980; Bissonnette et al, 1981). Transport is not altered by insulin, but several steroids inhibited transport, including oestriol and progesterone which are abundant in utero. The K_m and V_{max} (20 nmolmg^{-1}protein) suggest a large capacity in relation to calculated fetal needs (some 16 times greater). In consequence of this capacity, intrasyncytial concentrations of glucose are probably maintained near those of maternal blood (Johnson and Smith,1980). D-glucose transport systems which are Na^+-dependent are markedly inhibited by phlorizin. Thus, 50% inhibition is seen at 5-10μM in renal brush border vesicles (Kinne et al,1975) and 50μM in intestinal luminal vesicles (Hopfer et al,1973). The fact that this level of inhibition is only appproached with a phlorizin concentration of 1.0mM in placental vesicles (Johnson and Smith,1980) is consistent with the lack of sodium enhancement of the placental glucose transport mechanism (Rice et al,1979). The finding that phloretin inhibits D-glucose uptake at a concentration which is less than that of phlorizin, implies that the placental vesicle preparation qualitatively resembles the human erythrocyte (LeFevre,1961).

Johnson and Smith (1985) have more recently, studied uptake into vesicles prepared from the human basal (fetal-facing) syncytiotrophoblast plasma membrane. The glucose carrier here is very similar in terms of sodium independence, stereospecificity and kinetics (K_m=23mM). With a high K_m for the carrier on both

sides of the syncytiotrophoblast it is likely that transport is proportional to the maternofetal arterial concentration gradient, at least in the physiological range. Hauguel et al (1986) showed a comparable K_m value for the carrier on the maternal side of the perfused human cotyledon, whilst similar conclusions were drawn for the carriers on both sides of the sheep placenta (Stacey et al,1978; Simmons et al, 1979). Isolation of the glucose carrier from human microvillous membrane vesicles has been accomplished by photo-affinity labelling (Johnson and Smith,1982; Ingermann et al,1983). They found a protein of between 52-60,000 molecular weight and the isolated carrier retained its function when reconstituted into lipid vesicles (Bissonnette et al,1982).

It is interesting to note that in the perfused human placental cotyledon, D-glucose transfer was 4 times greater than L-glucose (Challier et al,1985), whilst in the intact sheep placenta there is a 600-fold difference between 3MEG and L-glucose permeability (Boyd et al,1976; Stacey et al,1978) and in the intact guinea-pig placenta, a 50-fold difference (Bissonnette et al,1979). A similar stereospecificity is thus shown by each of the different species, with L-glucose simply showing a higher passive permeability in the haemochorial placentas, as is found for other inert hydrophilic substances (see Chapter 2).

A major problem with the study of glucose transport is that it is metabolized by the placenta itself. Simmons et al (1979) measured the A-V glucose concentration differences and blood flow on maternal and fetal sides of the sheep placenta. Their assessment of placental glucose consumption ($44.9 mg min^{-1}$),

showed that only a third of the glucose entering the uteroplacental mass actually crossed the placenta into the fetal tissues. Similarly, in the perfused human cotyledon, Hauguel et al (1986) showed that placental utilization always represented about 50% of the total glucose uptake by the placenta.

The placental glucose transfer system is thus able to provide a more than adequate supply of glucose to the fetus, using a system of unenergised, symmetrical, low affinity, high capacity, stereospecific carriers, at both maternal and fetal faces of the trophoblast. These enable the rate of transfer of D-glucose to be much higher than that for other molecules of the same size and lipid solubility. Recently, Owens et al (1987B) showed that restriction of placental growth in the sheep, caused a compensatory increase in the clearance of 3MEG across the placenta. This suggests control of placental glucose transfer, which will be discussed later in this chapter (Section E1).

4) Amino Acids

Amino acids taken up from the maternal circulation will (like glucose) either serve the metabolic functions of the placenta itself or be rapidly transferred to the fetus (Battaglia and Meschia,1978). The placenta utilizes the amino acids for protein synthesis (Young et al,1982) and for its energy requirements. However, most of the amino acids do seem to be transferred directly to the fetus, at least in the sheep (Munro et al,1983). Carroll and Young (1983) have suggested a model linking placental protein synthesis to the generation of a transfer pool of amino acids, arising from experiments in the guinea-pig where

inhibition of protein synthesis reduced the amount of amino acids available for transfer. However, there are several other control mechanisms which may play important roles in placental amino acid transfer and these will be discussed later in this chapter (Section E1).

It has been known for many years, that the total fetal plasma amino-nitrogen concentration exceeds that in maternal plasma in man, guinea-pigs and rabbits. This led to the assumption that diffusion alone could not explain placental transfer (Christensen and Streicher,1948). Fetal protein is thus synthesised in situ from free amino acids transported across the placental membrane from the maternal circulation. These amino acids are probably 'essential' for the fetus, until its full complement of enzymes has developed. Net transfer of amino-nitrogen is quantitatively small and occurs mostly during the last third of gestation. The rate of protein accumulation however, differs between and within species due to the interaction of the genetic capacity of the fetus for growth and the opportunity for placental exchange of nutrients between fetus and mother (Young,1976).

It is now clear that the fetal plasma concentrations of most, but not all, individual amino acids are also higher than their maternal counterpart (Young and Prenton,1969; Hill and Young, 1973; Young and McFayden,1973). Furthermore, the placental intracellular free amino acid concentrations are far in excess, even of fetal plasma levels (Hill and Young,1973; Yudilevich and Sweiry,1985). This has suggested that active accumulation of amino acids occurs at the maternal surface, producing the high

placental tissue concentrations, followed by passive movement down the concentration gradient into the fetal circulation (Reynolds and Young,1971). In sheep and rats, the placental transfer of amino acids seems to be independent of the maternal plasma level (Young and McFayden,1973; Domenech et al,1986). However, transfer may be slightly but significantly, influenced by fetal plasma levels, being inversely proportional to the concentration of amino acids in the fetal perfusate of the perfused guinea-pig placenta (Hill and Young,1973). Levels of amino acids measured in amniotic fluid seem to lie between those found in fetal and maternal plasmas in the rhesus monkey. It has been suggested that as well as the active transport of amino acids to the fetus, there are facilitated diffusion systems existing for amino acid transfer into other compartments, but this is purely speculative (Pueschel et al,1986).

Active transport of amino acids across the placenta has to satisfy four main criteria : stereospecificity, competitive inhibition, uphill transfer against concentration and electrochemical gradients, and an energy requirement. Selective transfer of L-isomers (rather than D-isomers) of amino acids has been observed in sheep and guinea-pig placentas (Reynolds and Young,1971; Young and McFayden,1973). However, competition exists between the mechanisms for glycine and histidine, and methionine and proline, and there is a saturation of the mechanism for histidine transport. 'Uphill' transport has been shown in the <u>in situ</u> perfused guinea-pig placenta (Hill and Young,1973). With the closed circuit perfusion, amino acids accumulated in the perfusion fluid until an equilibrium

concentration was reached, equivalent to twice that of the fetal plasma, indicating the reserve capacity of the placenta for amino acid transfer. An energy requirement for placental intracellular amino acid accumulation has been clearly shown using cyanide and DNP, in human and guinea-pig placental slices (Dancis et al,1968).

The transport of amino acids by animal cells is generally mediated by specific carrier systems that have overlapping substrate reactivities (Christensen,1968). Competitive interactions between amino acids have demonstrated at least six distinct systems for neutral amino acids. It is not yet clear whether there are separate carriers for acidic and basic amino acids, but uptake and placental transfer is certainly present. Only three of the neutral carriers, the 'A', 'ASC' and 'L' systems, have been clearly demonstrated in the placenta (Yudilevich and Sweiry,1985). System 'A' is most reactive with amino acids having short polar or linear side-chains, such as alanine, glycine and amino-isobutyric acid (AIB). This system is sodium dependent and is slowed reversibly by lowered extracellular pH. System 'L' is most reactive with large apolar branched-chain and aromatic amino acids, such as leucine, isoleucine, tyrosine, tryptophan, valine, methionine and phenylalanine. This system is sodium independent and is sometimes stimulated by lowered extracellular pH. The 'ASC' system is sodium dependent, being more or less restricted in reactivity to alanine, serine and cysteine; although at low pH it may transport aspartate and its analogues (Makowske and Christensen,1982). This system is distinguished from system 'A'

on the basis of its relative pH-insensitivity, higher stereo-specificity and intolerance to N-methylated substrates. These three neutral amino acid transport systems were identified in human microvillous membrane vesicles (Ganapathy et al,1986; Kudo et al,1987) and human villous tissue fragments (Enders et al, 1976). However, in the perfused guinea-pig placenta, the 'A' system seems to be absent (Eaton et al,1982), with the 'L' and 'ASC' systems being present on both maternal and fetal sides. It is presumed that in the intact placenta, energy requiring Na^+-K^+-ATPase maintains an inwardly directed sodium gradient thus energising concentrative uptake by system 'A' and 'ASC' carriers, co-transporting amino acids with sodium (Ruzycki et al,1978). In the absence of sodium co-transport, it is unclear what energises the concentrative movement of amino acids using the 'L' carrier (Ganapathy et al,1986). The maternal and fetal sides of the guinea-pig placenta have carriers for neutral and basic amino acids (Eaton and Yudilevich,1981). Using the dually-perfused guinea-pig placenta and the indicator dilution technique, uptake of neutral and basic amino acids was similar when measured from either the maternal or fetal circulation, whereas backflux and transplacental transfer were asymmetric. Backflux being less pronounced at the maternal side, with transplacental transfer being in a maternofetal direction (Eaton and Yudilevich,1981).

There is good evidence that basic amino acids are also readily transferred across the placenta. In fact, the transfer of basic amino acids closely resembles that for neutral amino acids in many placental models, e.g. in human and guinea-pig perfused

placentas (Schneider et al,1979; Eaton and Yudilevich,1981). Although some interaction between lysine and alanine was noted in the guinea-pig (Eaton et al,1982; Yudilevich and Sweiry, 1985), in which lysine uptake may be sodium dependent (Eaton et al,1982), there is little direct information available about the basic amino acid carriers.

Placental handling of acidic amino acids seems to be quite different and they are not transferred readily. Holzman et al (1979) noted a negative A-V concentration difference for glutamate in the sheep umbilical circulation (ie: placental uptake), but no significant A-V difference in the uterine circulation. A similar situation was reported in man at several stages of gestation (Hayashi et al,1978). In the guinea-pig, uptake of glutamate and aspartate by either maternal or fetal faces was low or absent, according to indicator dilution studies (Eaton and Yudilevich,1981), although some unidirectional flux of glutamate into the placenta from the fetal circulation was seen during steady state perfusion (Bloxam et al,1981). Here, a net flux from placenta to fetus was evidenced by a positive umbilical A-V difference (Hill and Young,1973; Bloxam et al, 1981). Consequently, there seems to be species differences in the placental handling of acidic amino acids and their carriers have not been characterized.

Placental uptake of dipeptides might provide another source of amino-nitrogen for the fetus (Crandell et al,1981). Ganapathy et al (1985) demonstrated transport of the dipeptide glycyl sarcosine in human microvillous membrane vesicles. The dipeptide was transported intact and independent of sodium, transfer

probably occurring down a concentration gradient. The transfer showed competition also, being inhibited by other dipeptides. It is quite possible that small, hydrolysis-resistant peptides are generated during intracellular protein catabolism (as well as free amino acids) and hence enter the maternal circulation. Thus the concentration of small peptides in maternal plasma may increase during the last trimester of pregnancy and become an important source of amino acids for the fetus. Furthermore, placental tissue contains peptidases and may augment the supply of amino acids available to the fetus. The activity of one such peptidase, γ-glutamyltransferase, has been measured in the rat placenta (Baumrucker and Stover,1987) throughout gestation. Its activity seems to show two peaks, one at the period of greatest placental growth and the other at greatest fetal growth. The occurrence of these changes in enzyme activity supports the concept of a developmental role for γ-glutamyltransferase, in providing amino acids from glutathione to the placenta and fetus. However overall, the activities of enzymes related to amino acid metabolism are not well-developed during fetal rat life (Remesar et al,1987).

Carrier mechanisms for active amino acid transport into and across the placenta are thus present, but how their activity is controlled is still unclear. Their activity can be modulated in a variety of *in vitro* systems, e.g. by calcium (Karl et al, 1988), but how much of this can be extrapolated to the intact tissue *in vivo* is under question (see Chapter 1, Section E1).

5) Calcium

a) Cellular Calcium Homeostasis

Before looking at placental calcium transport in detail, it is interesting to review how calcium homeostasis is maintained in other tissues (see Godfraind-De Becker,1980) and especially in epithelial cells (Gmaj and Murer,1988). The intracellular ionized ('free') calcium concentration in most mammalian cells is extremely low (≈ 10^{-7}M), in contrast to a much higher extracellular concentration (≈ 10^{-3}M). It is this distribution of ionized calcium that is believed to be important in the regulatory role of the cation (England,1986). The large transmembrane electrochemical gradient ensures that calcium is an ideal, fast 'second messenger' in stimulus-secretion coupling (Rubin,1982). Total plasma calcium in man is between 9-10mg $100ml^{-1}$ (2.3-2.5mM) and exists in various forms : 1) ionized calcium, 5-6mg$100ml^{-1}$ (1.3mM); 2) protein-bound calcium, 3-4 mg$100ml^{-1}$ (1.0mM); 3) unidentified complexes, 0.5-1.0mg$100ml^{-1}$ (0.2mM) (Moore,1970). The large protein-bound fraction serves as an ion buffer system, keeping the ionized fraction relatively constant. It seems that the ionized calcium is of physiological importance, since the protein-bound calcium is only poorly diffusible through plasma membranes (Racker,1980).

Cellular calcium homeostasis is thus maintained by a low natural permeability of the plasma membrane to calcium and by at least 3 systems by which calcium is pumped out of the cell against an electrochemical gradient. The first is a specific $Ca^{2+}/2H^{+}$-ATPase or calcium pump (Schatzman,1985), whilst the second is a Na^{+}/Ca^{2+} exchanger driven by the sodium gradient

across the membrane, which is in turn maintained by Na^+-K^+-ATPase or the sodium pump (Baker,1986). There are also calcium channels, which are either voltage (Stanfield,1986) or agonist-dependent (Berridge,1982). Two other membrane systems, besides the plasma membrane, are also involved in this homeostasis : the inner mitochondrial membrane and the endoplasmic reticulum (Nicholls,1986). Each membrane possesses distinctive transport mechanisms which act in concert to regulate and modulate intracellular calcium (Carafoli,1987).

The major sources of an increase in intracellular free calcium are the endoplasmic reticulum (Somylo and Somylo,1986), the extracellular fluid (via the plasma membrane) and possibly the nucleus of the cell (Brown et al,1985). Release of calcium from intracellular stores can be brought about by activation of adenylate cyclase, with consequential cyclic adenosine monophosphate (cAMP) production (Morel,1981; Tomlinson et al,1985) or by hydrolysis of membrane phosphoinositides, releasing inositol trisphosphate (IP_3) and diacylglycerol (DG) (Berridge and Irvine,1984). IP_3 can act directly on the endoplasmic reticulum, releasing calcium by interacting with a specific receptor. It can also act via the intracellular binding protein, calmodulin (Cox,1986), usually producing only brief, rapid responses or the initial phase of a sustained response. Calmodulin, in the presence of calcium, can modulate the activity of a number of enzymes, including adenylate cyclase and Ca^{2+}-ATPase (Klee et al,1980). On the other hand, DG can produce more sustained phases of cellular reponses, acting via protein kinase C (Berridge,1986; Putney,1987). So there are two branches

of information flow in the cell, the calmodulin branch and the C-kinase branch, and they interact along with cAMP via calcium (Rasmussen,1986A;1986B).

All these mechanisms bring about a calcium-linked response to a stimulus. This can be terminated by feedback mechanisms, again involving calcium pumps in the membrane, translocation of calcium from the cytosol to cellular organelles or calcium binding to specific binding proteins within the cell (see Carafoli,1987). It follows that the capacity of different cell types to use internal calcium as an initiator of cell response, depends on the size of the calcium pool contained in their smooth endoplasmic reticulum (Rasmussen and Barrett,1984).

b) Mechanisms of Placental Calcium Transfer

It has been known for many years that total plasma calcium concentration of the fetus at term is much higher than that of the mother (Bogert and Plass,1923). Later, Delivoria-Papadopoulos et al (1967), showed that the maternofetal calcium gradient is not the result of increased calcium binding capacity of fetal serum proteins, since the ionized calcium levels were also higher in the fetus. Table 1.1 indicates that this seems to be the case in all the species which have been studied. Maternal plasma total calcium concentration falls with increasing gestation, however this is only due to a change in bound calcium, whilst ionized calcium concentration is maintained within a narrow physiological range (Pitkin and Gebhardt,1977). With analogy to other tissues, it is also assumed that it is the ionized calcium which is important in placental transfer. This

in is direct contrast to placental iron transfer which occurs in a protein-bound form, bound to transferrin (Baker and Morgan, 1969).

Table 1.1 : Total and ionized calcium concentrations (mM) in maternal and fetal blood (mean values)

Species	Maternal blood		Fetal blood	
	Total calcium	Ionized calcium	Total calcium	Ionized calcium
Rat[a b]	2.23[a] 1.97[b]	1.10[b]	2.75[a]	1.42[b]
Sheep[c]	2.12	0.99	3.30	1.59
Guinea-pig[d]	1.70	1.23	2.30	1.53
Monkey[e]	2.05	1.05	2.10	1.33
Human[f]	2.13	1.12	2.65	1.41

a Chalon and Garel,1985B (19.5 g.a.)
b Our data (21 g.a., see Chapter 3)
c Care et al,1981 (near term)
d Twardock et al,1971 (ultrafilterable calcium, not ionized)
e Northrop et al,1977 (150 ± 5 g.a.)
f Schauberger and Pitkin,1979 (36-42 weeks g.a.)
g.a. Gestational age
(adapted from Sibley and Boyd,1988).

Throughout gestation and especially in the last trimester, placental calcium transfer increases rapidly, rising exponentially until near term (Chef,1969A). This places a demand on the mother for calcium, which is met by increased intestinal absorption of calcium (Chef,1969B) and an increased reliance upon maternal skeletal calcium stores (Symonds et al,1978).

There are certainly many species differences in fetal calcium accretion during pregnancy (see Chapter 3, Table 3.4). In the rat, estimates of net calcium flux between day 20 and 21 (term

is day 23) were similar to measurements of J_{mf} calcium in the *in situ* perfused placenta, suggesting that J_{fm} for calcium is small. Thus, calcium transfer to the rat fetus is highly asymmetrical, maternofetal transport prevailing, with only a small bidirectional diffusional component (Štulc and Štulcová, 1986). Similar observations were made in sheep, where the rate of transport of calcium from ewe to fetus was 215 $mgday^{-1}kg^{-1}$ fetal weight, whereas the rate of transport in the opposite direction was only 12 $mgday^{-1}kg^{-1}$ fetal weight (Symonds et al, 1972; Ramberg et al,1973). Braithwaite et al (1972) showed that the rate of net bone resorption in the fetus was negligable and that the rate of bone accretion approximated to the rate of net transport of calcium from ewe to fetus. That is, in the ovine fetus, almost all the calcium that passes to it from the mother is used by the developing skeleton. This is in sharp contrast to the monkey, in which the bidirectional rates of calcium transfer across the placenta are comparatively similar to one another (MacDonald et al,1965; Ramberg et al,1973). It also shows a higher absolute rate of calcium transfer across the placenta in either direction, than in the sheep (J_{mf}= 391$mgday^{-1}kg^{-1}$; J_{fm}= 326$mgday^{-1}kg^{-1}$). The differences in net calcium flux between sheep and monkey is reflected in their relative growth rates (Battaglia and Hay,1984), with primates producing a much smaller fetal mass in proportion to maternal size, over a longer gestation period.

Several pieces of evidence have suggested that placental calcium transfer is an active process, presumably whilst still maintaining low intracellular calcium levels. For example, the

transplacental calcium gradient can be maintained in an in situ perfused placental preparation and does not seem to be dependent on the presence of the fetus (Twardock and Austin,1970). Also, in the rat the measured maternofetal p.d. is fetal side positive, another observation incompatible with the idea that the higher fetal plasma ionized calcium concentration is maintained by maternofetal electrical gradients, rather than active transport (Van Dijk,1981; see Chapter 1, Section D2). Twardock and Austin (1970) found a significant rise in the total calcium concentration of fetal perfusate after a single pass through the in situ perfused guinea-pig placenta, despite the fact that the perfusate calcium concentration started higher than that of the maternal plasma. Similarly, closed-circuit perfusion of the fetal circulation of the guinea-pig (Sweiry and Yudilevich,1984) and human placenta (Abramovich et al,1987A) resulted in accumulation of calcium despite the adverse maternofetal ionized calcium concentration gradient. Metabolic inhibitors such as DNP and cyanide, reduced calcium transport in the perfused human (Abramovich et al,1987B) and rat (Štulc and Štulcová,1986) placentas. However, in the in situ perfused guinea-pig placenta, the diuretics ethacrynic acid and furosemide which are known to inhibit Ca^{2+}-ATPase activity, had no effect on maternofetal calcium transfer (McKercher et al, 1983;1984). This may simply be a reflection of the increased permeability which is seen with perfusion of the guinea-pig placenta (Hedley and Bradbury,1980), especially as McKercher et al (1983) did not actually show that their model could support active calcium transport. The K_{mf}/D_w ratio for unidirectional

maternofetal calcium flux can be calculated from the data of Twardock and Austin (1970) and Štulc and Štulcová (1986), both values being considerably higher than the ratios for extracellular tracers (see Chapter 2 and 3), providing further support for the importance of transcellular transfer of calcium across the placenta.

There is also evidence for calcium carriers located at both surfaces of the placental trophoblast. Sweiry and Yudilevich (1984) demonstrated that unidirectional calcium influx across both surfaces of the trophoblast in the perfused guinea-pig placenta, conforms to Michaelis-Menton saturation kinetics, being saturated even at low maternal plasma calcium concentrations. On the maternal side, kinetic analysis indicated affinity constants (ranging from 0.18-1.12mM) lower than those estimated on the fetal side (1.8mM) and the transport capacity at the fetal interface (V_{max}= 1.66 μmolmin^{-1}g^{-1}) far exceeded that measured on the maternal side (0.12-0.59 μmolmin^{-1}g^{-1}). Asymmetries in unidirectional influx into and efflux out of the trophoblast, indicate a mechanism which results in net transfer of calcium from the maternal to the fetal circulation and maintenance of the maternofetal calcium gradient. This has now also been observed in the perfused human placenta, but here asymmetry was only seen with efflux (Sweiry et al,1986). In the <u>in situ</u> perfused rat placenta, doubling of the maternal ionized calcium concentration caused a 60% reduction in $K_{mf}{}^{45}Ca$, but only a 30% increase in J_{mf} calcium (Štulc and Štulcová,1986). This suggests that the affinity of the maternofetal transport system for calcium is high.

In the monkey (MacDonald et al,1965) and pig (Ross et al, 1980A; Care et al,1982), maternal hypercalcaemia caused a rise in fetal blood ionized calcium concentration, whilst in the sheep (Bawden and Wolkoff, 1967), rabbit (Graham and Porter, 1971) and guinea-pig (Greeson et al,1968) fetal blood ionized calcium levels remained constant. This may suggest that the active transport mechanisms are the same in both groups, but that the diffusional components differ. However, Derewlany and Radde (1985) found that maternal hyper- and hypocalcaemia in the perfused guinea-pig placenta caused a significant rise and fall respectively, in maternofetal calcium transfer. These results may again be due to the susceptibility of the perfused guinea-pig placenta to perfusion artefacts (Hedley and Bradbury,1980). Chalon and Garel (1985C) showed that acute maternal hypercalcaemia in rats, caused a small but significant rise in fetal plasma calcium concentration. This rise was independent of the degree of maternal hypercalcaemia, suggesting a saturation of placental calcium transport. The lack of correlation between maternal and fetal calcaemia makes it likely that the placental transfer of calcium to the fetus is an active process, which normally works at maximal or submaximal levels in different species. Maternal hypocalcaemia in rats had no effect on fetal calcium levels (Payne and Sansom,1963). Similarly, alterations of fetal perfusate calcium concentration in perfused placental systems, had no effect on maternofetal calcium transport in the guinea-pig (Twardock and Austin,1970) or rat (Štulc and Štulcová,1986).

All this data points to there being active placental transfer

of calcium to the fetus, at least near term. Intestinal calcium transport has been investigated in detail and changes have been noted during pregnancy. Analogies have been drawn between the mechanism of intestinal calcium transfer and placental calcium transport, therefore it is worth reviewing the former (see Van Os,1987).

Schacter and Rosen (1959) demonstrated active transfer of ionized calcium across the small intestine of the rabbit, rat and guinea-pig, using everted gut sacs in vitro. There is also a large passive diffusion of calcium from the intestinal lumen into the bloodstream, down the electrochemical gradient via a paracellular pathway. However, it is the active component of calcium transfer that is increased in growing animals and during pregnancy (Schacter et al,1960). The active transport mechanism is also vitamin D dependent (Dowdle et al,1960), so regulation of intestinal calcium transfer could occur via changes in $1,25(OH)_2D_3$, the active metabolite of vitamin D, and possibly indirectly, by changes in parathyroid hormone (PTH) and calcitonin metabolism. $1,25(OH)_2D_3$ seems to act directly, having a 'lipnomic' effect, causing changes in the phospholipid composition of the brush-border membranes (Rasmussen et al, 1982). It also acts indirectly, stimulating messenger ribonucleic acid (mRNA) synthesis and the production of calcium binding proteins (Wasserman et al,1978). The active transcellular pathway for calcium transfer, involves an initial passive influx of calcium from the intestinal lumen across the brush-border membrane into the cell, followed by intracellular diffusion (which is probably the rate-limiting step) and an

active extrusion of calcium into the circulation. This is brought about by Na^+/Ca^{2+} exchange (Blaustein and Nelson,1982) and ATP-dependent calcium transport, mechanisms located exclusively in the basolateral membrane of enterocytes (Gmaj et al,1979). It is interesting to note that Ca^{2+}-ATPase activity is reduced in vitamin D deficient chick enterocytes, suggesting a dependence on vitamin D for active calcium transfer (Davis et al,1987).

So there seems to be two transport processes, a saturable transcellular one, that is regulated by $1,25(OH)_2D_3$ and predominates in the proximal intestine where nuclear receptors for $1,25(OH)_2D_3$ have been found (Lawson and Emtage,1974), and a non-saturable paracellular pathway similar in intensity throughout the intestine (Bronner,1984; Bronner et al,1986).

Active intestinal calcium transport varies directly and proportionately with the concentration of calcium binding protein (CaBP), a specific molecular expression of the action of $1,25(OH)_2D_3$ (Corradino and Wasserman,1971) and a quantitative index of vitamin D deficiency (Bronner and Freund,1975). Since CaBP is a cytosolic protein, it is suggested that it may act to facilitate calcium diffusion (Kretsinger et al,1982). However, $1,25(OH)_2D_3$-stimulated CaBP does not appear before the increased calcium transport and outlasts it, after the removal of $1,25(OH)_2D_3$ (Spencer et al,1976). Also, blockage of protein synthesis prevents CaBP synthesis after $1,25(OH)_2D_3$, but does not abolish the stimulation of calcium transport (Bikle et al, 1978; Nemere and Norman,1987). Therefore, there is no explanation for how CaBP and active calcium transport are

correlated and it may simply act as a buffering protein, keeping free intracellular calcium levels low (Morrissey et al,1980).

With intestinal calcium transport mechanisms in mind, it seems logical to study the placenta in a similar way, consequently, attempts have been made to localize CaBP's, Na^{+}/Ca^{2+} exchange, Ca^{2+}-ATPase and methods for ensuring that free intracellular calcium is kept low. However, the actual mechanisms involved in placental calcium transfer are still not clear. Use of pyroantimonate to complex calcium into a granular form, visible under the electron microscope, suggests that calcium is bound to endoplasmic reticulum immediately after entry into the trophoblastic syncytium and subsequently is taken up by mitochondria (Croley, 1973), so maintaining low intracellular calcium levels. A sequestrating role may also be played by non-membranous ligands such as inorganic phosphate, citric acid, adenosine nuleotides and by CaBP's. However, it is not clear if these ligands can change the free calcium levels in response to physiological needs. Croley (1973) showed calcium leaving the trophoblast cells associated with single membrane vesicles, suggesting that this is how calcium traverses the intracellular space and perhaps, also the basement membrane of the trophoblast (but vesicles would have to be very small to do this).

An attractive hypothesis has been put forward by Terepka et al (1976), however it is not based on any direct evidence. They suggest that coated vesicles known to take up materials by micropinocytosis, are involved in transcellular transfer of calcium. Invaginations of the plasma membrane would lead to the formation of endocytotic vesicles, into which the now inwardly

directed calcium pump would accumulate and transport the calcium that has diffused into the cytosol along its electro-chemical gradient. After fusion of the vesicles with the opposite membranes, calcium would be discharged by means of exocytosis. It may be that the calcium efflux across the plasma membrane (or the calcium influx after formation of the endocytotic vesicle) is driven by the electrochemical gradient for sodium. In this case, a carrier for sodium and calcium must be present. On the other hand, the calcium pumps may be driven by membrane-bound Ca^{2+}-ATPase. The relevance of the Terepka model remains to be established, but a similar model has been suggested for sodium transport (Bradbury,1981) and recently, for intestinal calcium transport (Nemere et al,1986).

The presence of a CaBP has been demonstrated in the placenta of rat and man (Bruns et al,1978; Fowler et al,1978), in the rat uterus (Warembourg et al,1987), as well as in rat and mouse fetal membranes, especially the yolk sac (Delorme et al,1979; 1983; Bruns et al,1985;1986). In fact, CaBP of rat intestine and placenta appear to be immunologically identical (Bruns et al, 1978; MacManus et al,1986), although the concentration of CaBP RNA and CaBP itself, is much less in the placenta than in the intestine (Warembourg et al,1986). Both CaBP's show increasing levels during pregnancy, in parallel with the increase in intestinal calcium transfer (Schacter et al,1960), fetal growth (Bruns et al,1978) and net flux of calcium to the fetus (Chef, 1969A).

Tuan (1985) showed that microsomal membrane vesicles isolated from human term placentas exhibited active calcium transport in

vitro. After incubation with specific anti-CaBP antibodies calcium uptake was inhibited, suggesting that human CaBP is involved in placental calcium uptake. Localization of CaBP in placental tissue has also suggested that it has a role in placental transfer. In the rat and mouse yolk sac it was found in the endodermal cells, which are known to be absorptive cells (Delorme et al,1983; Bruns et al,1985). In the rat chorio-allantoic placenta, CaBP's have been predominantly located in the syncytiotrophoblast (Delorme et al,1979) and in the human placenta (Tuan,1982) they were localized in the chorionic villi. Three calcium-dependent binding proteins (molecular weights = 68,36,34kDa) have been found associated with the human placental syncytiotrophoblast microvillous cytoskeleton and they were immunologically related to human lymphocyte cytoskeletal CaBP's (Webb and Mahadevan,1987). These are thought to play a role in the maintenance of microvillous structure, especially during endocytosis and exocytosis. Therefore a range of CaBP's may be found in the placenta, each possibly having different actions depending on their localization.

Maternal levels of $1,25(OH)_2D_3$ increase during late gestation in the rat (Pike et al,1979), which correlates well with the rise in CaBP and suggests that placental CaBP synthesis, like that in the intestine, is dependent on $1,25(OH)_2D_3$. Consistent with this hypothesis is the observation that $1,25(OH)_2D_3$ is accumulated in the rat placenta, though it may be produced by the placenta itself (Weisman et al,1978B). Indeed, nephrectomized pregnant rats can still synthesisze $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ (both of which are usually made in the kidney).

Consequently, the fetoplacental unit has been suggested as a possible site of production (Weisman et al,1978B) and the placenta certainly possesses $1,25(OH)_2D_3$ receptors (Pike et al, 1980). It has also been shown that human placenta can synthesize $24,25(OH)_2D_3$ *in vitro* (Weisman et al,1979) and both the human (Whitsett et al, 1981) and the rat (Tanaka et al,1979) placenta can convert $25(OH)D_3$ to $1,25(OH)_2D_3$ *in vitro*. However in mice, $1,25(OH)_2D_3$ had no effect on placental CaBP levels when given to the mother. So perhaps the CaBP is dependent only on placental or fetally derived $1,25(OH)_2D_3$ (Bruns et al,1982). Thus, $1,25(OH)_2D_3$ certainly seems to be available for a role in placental calcium transfer, possibly via its action on CaBP, but again as in the intestine, CaBP may simply act as a buffering protein.

The presence of a Na^+/Ca^{2+} exchanger in the placenta has not been investigated in detail. However, removal of sodium from fetal perfusate of the *in situ* perfused rat placenta, had no effect on J_{mf} calcium, suggesting that a Na^+/Ca^{2+} exchange mechanism is not involved in maternofetal calcium transfer in the rat (Štulc and Štulcová,1986).

A Ca^{2+}-ATPase has been shown to be present in the plasma membranes isolated from a guinea-pig placental homogenate (Shami and Radde,1971), in human placental homogenate (Miller and Berndt,1973) and in human placental microvillous membrane vesicles (Whitsett and Tsang,1980). In the rat, a sudden increase in Ca^{2+}-ATPase activity was noted after day 17 of gestation (Legrand et al,1978). Again, this correlates well with the onset of fetal ossification, appearance of fetal hyper-

calcaemia and increasing placental calcium transfer. The Ca^{2+}-ATPase located primarily in the brush border membranes of human placenta (Whitsett and Tsang,1980), is ideally placed for the regulation of intracellular calcium levels, but it is difficult to see how this Ca^{2+}-ATPase can play a role in maternofetal calcium flux. However, this Ca^{2+}-ATPase was probably a low affinity non-specific (Mg^{2+} or Ca^{2+}) ATPase as is found in most tissues (Penniston,1983), since it was studied using millimolar concentrations of calcium. Treinen and Kulkarni (1986;1987) have since identified a high affinity Ca^{2+}-ATPase in human placenta brush border membrane vesicles. Whether the high and low affinity Ca^{2+}-ATPases are 2 separate proteins or further activation of a single ATPase is not known. It is of interest however, that the properties described for this high affinity Ca^{2+}-ATPase are similar to those of other Ca^{2+}-ATPases known to be involved in the active transport of calcium, particularly that of the liver (Carafoli,1984). However, Treinen and Kulkarni (1986) suggest that the main role of this enzyme is probably the regulation of intracellular calcium concentration by pumping calcium out of the placental cells. It would certainly seem to be in the wrong location if involved in placental transfer.

In their investigation of calcium binding to plasma membranes isolated from a guinea-pig placental homogenate using a flow dialysis system, Shami et al (1974) could identify two types of Ca^{2+}-binding sites, a low and a high affinity site. These sites had similar capacity, specificity and affinity to those found in other membranes involved in active calcium transport. They may be linked to Ca^{2+}-ATPase (Shami et al,1975), but it is not clear

how they are involved in placental calcium transport.

Tuan and Kushner (1987) found that the inhibitors of Ca^{2+}-ATPase activity, phenothiazin and erythrosin B, effectively inhibited the active uptake of calcium by placental microsomal membranes in vitro. However, since the actions of these agents are by no means limited to the Ca^{2+}-ATPase, this correlative finding is only consistent with, not confirmatory of, the hypothesis that Ca^{2+}-ATPase is involved in active placental calcium transport.

Recently, Fisher et al (1987) have observed ATP-dependent calcium transport across basal (fetal-facing) plasma membranes of human placental trophoblast. This was saturated at submicromolar concentrations (K_m =0.119μM, V_{max} =2.0 nmolmin^{-1} mg^{-1}protein), was not sensitive to a sodium gradient, required magnesium and was stimulated by calmodulin. The greater magnitude of calmodulin stimulation at lower concentrations, suggests that the primary effect is to increase the apparent affinity rather than the V_{max} of the transport system for calcium. This transport mechanism is ideally situated for active extrusion of calcium into the fetal compartment, as well as maintenance of low intracellular calcium concentrations.

The detailed analysis of systems for intracellular and extracellular calcium regulation, has only recently started to be investigated in placental tissue. Moore et al (1986) found that calcium in conjunction with phospholipid, enhances the phosphorylation of a distinct group of proteins in human placental cytosol. This raises the possibility that hormones which modulate the metabolism of cell membrane phospholipids,

with the intracellular release of phospholipid components, may exert physiological effects by the activation of placental kinase C and subsequent phosphorylation of specific proteins. An extensive list of hormones has been postulated to act via protein kinase C in this manner, e.g. insulin (Kuo et al,1980). Enhancement of calcium-activated calmodulin-dependent phosphorylation may also be important in the placenta, possibly playing a role in placental calcium transport (Moore et al, 1986). Okuyama et al (1986) have detected protein kinase C in the membrane fraction of the rat placenta, where it specifically phosphorylates several endogenous membrane proteins. The membrane-bound protein kinase C was shown to increase markedly during pregnancy and seems to have properties similar to those reported for protein kinase C in other tissues (Kuo et al,1980). Several components of the inositol phosphate pathway have also been isolated from the placenta (Urumow and Wieland,1986; Ross and Majerus,1986). Evidence is therefore accumulating which suggests that the placenta possesses all the usual calcium-regulating mechanisms as are found in the intestine and other tissues. However, it is still unclear how a background of low intracellular calcium is maintained, during rapid, active transplacental calcium transport and what role, if any, CaBP's and Ca^{2+}-ATPase play in this transfer.

Finally, it is interesting to note that active calcium transport has been measured across the guinea-pig yolk sac and amnion <u>in vitro</u> (Derewlany et al,1983), suggesting another route for calcium transfer. Similarly, active calcium transport across the chick embryonic chorioallantoic membrane has been

investigated in detail, suggesting involvement of CaBP and Ca^{2+}-ATPase (Tuan et al,1986A;1986B; Tuan,1987).

In conclusion therefore, placental calcium transport certainly seems to occur by active mechanisms, but the details of these are unclear. The control of placental calcium transfer is discussed later in this chapter (Section E2).

6) Immunoglobulin G

The transport of proteins across the placenta shows a high degree of specificity and the route of immunoglobulin transfer, is also very species specific. Thus in man, immunoglobulin G (IgG) is transferred *in utero* across the placenta (Dancis et al, 1961), rabbits and guinea-pigs absorb IgG through the yolk sac endoderm from the uterine cavity (Brambell et al,1949; Kulangara and Schechtman,1962), whilst rats and mice acquire some IgG *in utero* by way of the yolk sac endoderm (Brambell and Halliday, 1956), but also receive some via the intestinal epithelium *in utero* by swallowing amniotic fluid and postnatally by ingesting colostrum (Halliday,1955). The colostrum of ruminants contains very high concentrations of IgG, which is absorbed by the newborn during the first day after birth (Mason et al,1930). In man, there is no appreciable postnatal transmission of antibodies through the gut wall, although IgA present in breast milk controls local intestinal bacterial flora.

Transmission of immunoglobulins is selective, both as between albumin and globulin, and between heterologous and homologous globulins (Brambell,1958). It would seem that both the structure of the immunoglobulin molecule (Hemmings and Jones,1962; Gitlin

et al,1963) and the cell surface membrane components present (Hemmings,1976), are important in determining transfer. Morris (1956) found that the F_c portion of IgG can interfere with the transfer of the other gamma globulin residues, however, the F_{ab} portion cannot. The F_c portion is responsible for most of the antigenic activity of the molecule and is important in transfer (Brambell et al,1960). Specific F_c receptors have been identified in the rabbit yolk sac endoderm (Wild and Dawson, 1977) and on human placental membranes (Johnson and Brown,1981), suggesting that IgG tranfer could be by saturable receptor-mediated endocytosis.

Endocytosis of immunoglobulins could be by fluid-phase or adsorptive endocytosis. Fluid-phase endocytosis is the non-selective uptake of substances by cells from a fluid suspension, without binding to the cell surface. Adsorptive endocytosis involves the binding of extracellular molecules, either to non-specific binding sites or to specific and saturable binding sites (i.e. receptor-mediated) on the plasma membrane and subsequent internalization by pinocytosis. There are then two cellular pathways for the ultimate disposition of the materials that are taken up, one for digestion and the other for transport, both involving lysosomes. Furthermore, two types of microvesicle are found, one that is smooth-surfaced, associated with fluid-phase endocytosis and the other which is clathrin-coated, associated with receptor-mediated endocytosis (Steinman et al,1983).

Brambell (1966) hypothesized preferential binding of homologous proteins by receptors on the surface and in the

vacuoles of cells and suggested that steric hindrance due to this receptor binding, protected these proteins from attack by the cathepsins of the lysosomes. Unprotected (i.e. unbound) heterologous proteins would be degraded and discharged from the cell. An analogous transport mechanism has been shown across the small intestine of suckling rats (Jones and Waldmann,1972). A slightly different mechanism was postulated by Wild (1973), who suggested that the larger smooth-coated vesicles found in human placenta were engaged in non-selective adsorptive endocytosis, whilst the smaller micropinocytotic vesicles which form on the lumenal surface of the cell as 'coated pits', have many specific binding sites (receptors) present and carry out receptor-mediated adsorptive endocytosis, with the 'coated vesicles' being protected from lysosomal attack. This mechanism has also been proposed for transport across the neonatal rat gut (Rodewald,1973). However, not all immunoglobulin transfer seems to be so specific. The rabbit yolk sac endoderm showed no receptor-bound material, suggesting simple non-selective endocytosis (Hemmings and Williams,1976). King (1982) revised Wild's hypothesis, presenting ultrastructural evidence that clathrin-coated vesicles (containing bound IgG) form at the apical surface of endodermal cells, migrate to the periphery and fuse with the basolateral membrane. He also found that small coated vesicles appear to bud from larger vesicles, the larger vesicles losing their clathrin coats and their contents being digested.

In the yolk sac endodermal epithelium of the rat, visualization of uptake of materials, both by fluid-phase and

receptor-mediated adsorptive endocytosis, has been facilitated by cytochemical localization of the protein HRP (Jollie,1985; 1986). Receptor-mediated endocytosis was seen to be most active at day 12 of pregnancy, intermediate in activity by day 17 and minimal the day before term (day 22; Jollie,1985). Fluid-phase endocytosis was also seen at day 12 and 17, but not at day 22. These results suggest that antibody transfer via this route is important early in gestation. Jollie (1985) also suggests that both fluid-phase and receptor-mediated endocytosis are involved in antibody uptake throughout pregnancy by the rat yolk sac placenta. However, only the latter process seems to account for transcytosis of material, with the former being involved in the digestion of proteins by lysosmal action and several other groups have come to similar conclusions (Masters et al,1969; McArdle and Priscott,1984). It has recently been shown that such digestion of protein by the visceral yolk sac membrane, in the early rat embryo at least, can provide all of the amino acids necessary for growth and development (Freeman et al,1981;1982; Freeman and Lloyd,1983). It may well be that the primary function of the membrane is to provide amino acids for embryonic development, through digestion of such maternal proteins as are internalized by fluid-phase endocytosis. It is by a circumvention of this protein digesting system, through receptor-mediated endocytosis near term, that the fetus acquires passive immunity.

LaLiberté et al (1984) have looked at the kinetics of antibody transfer across the rat yolk sac endoderm. They showed very rapid binding of IgG to the cell membrane (15 seconds), with

internalization occurring by 5 minutes and appearance of antibody in intracellular spaces within 15 minutes of incubation. Specific F_c receptor binding has been identified in this tissue (LaLiberté et al,1981), the receptor-bound IgG entering the cell through coated pits. Once the protein was bound to the cell membrane (30 seconds), the transfer time was 15 minutes, which is well within the range for receptor-mediated endocytosis as reported in other tissues (Goldstein et al,1979).

Similarly, in the human chorioallantoic placenta, IgG uptake has been visualized (Ockleford and Clint,1980) and associated with coated vesicles (Pearse,1982). However, it is not clear whether Wild's hypothesis (1973) can account for the selectivity of the human placenta for IgG. Lin (1980) localized endogenous IgG in the term placenta using electron microscopy. IgG was detected on the apical (maternal-facing) surface, in endocytotic vesicles and in vesicles fused to the basal (fetal-facing) cell membrane of the syncytiotrophoblast. However, neither Lin (1980) nor King (1982) detected large numbers of coated vesicles. Consequently, Lin (1980) proposed a mechanism of transfer involving fusion of endocytotic vesicles with lysosomes, where some IgG escapes proteolytic digestion (i.e. similar to Brambell's hypothesis, 1966). He suggested that escape from digestion may be possible if there is a paucity of proteolytic enzymes in the lysosomes or alternatively, that protease inhibitors may be present. However, how IgG escapes digestion, whilst other bound proteins, e.g. transferrin, ferritin, etc. are metabolized (Pearse,1982), is unclear.

Most transfer of IgG seems to occur late in human gestation,

after 32 weeks (Sidiropoulos et al,1986). Contractor et al (1983) showed transfer of IgG across the perfused human term placenta, with 70-80% of the IgG being broken down intracellularly and the smaller fragments transported to the fetal circulation, perhaps by an active process. A similar breakdown of IgG occurs during its transfer across the rabbit yolk sac placenta (Wild,1981) and across the gut of suckling rodents (Rodewald,1976). Quinlivan and Fox (1964) actually showed fetomaternal transfer of IgG across the rat chorio-allantoic placenta, but whether this occurs in vivo is not clear.

In many cases, it is reported that cord serum IgG concentrations are higher than maternal serum at birth (Kohler and Farr,1966). This suggests that in late pregnancy, transfer may be taking place against a concentration gradient and consequently may involve an active transport mechanism (Pitcher-Wilmott et al,1980), but this has not been verified. It could be due to the additive effect of fetal production of IgG, but this is unlikely as fetal synthesis of IgG is slow (Williams and Gershowitz,1979).

Therefore, immunoglobulin transfer seems to involve various specific endocytotic mechanisms and is most apparent near term. How the small amount of IgG that reaches the fetus avoids digestion, remains unclear, but the evidence to date does suggest the involvement of coated pits and vesicles.

SECTION E : Control Of Placental Transfer

1) Types of Control Mechanisms

As indicated in previous chapters, the control of placental transfer is probably very complex and is largely not understood. The control mechanisms probably vary between species and change throughout gestation, involving a variety of maternal, fetal and placental modulators (Štulc,1988). The following controlling mechanisms may be involved :

1) In the case of flow-limited transport (e.g. O_2), changes in rates of flow in maternal and fetal circulations will greatly influence transfer (Meschia et al,1966; Lasunción et al,1987). Such changes will be occurring throughout placental growth and development, accompanying structural changes. These structural changes will probably cause changes in the permeability to hydrophilic diffusion-limited molecules. Any increased blood flow which leads to the recruitment of previously unperfused placental exchange areas, might also increase the surface area available for membrane-limited transfer. Howard (1987) has considered the control of human placental blood flow and suggests that maternoplacental vascular resistance is controlled humorally by release of hormones into maternal blood from the fetoplacental unit. Similarly, fetoplacental vascular resistance may be controlled locally by a reversible hypoxic fetoplacental vasoconstriction, in response to reduced local maternoplacental oxygen delivery. It is also possible that prostaglandins are involved locally in controlling placental blood flow (Rankin,1976). All such mechanisms would of course indirectly

affect the efficiency of placental transfer. However, Anderson and Faber (1984) contend that no such control of blood flow exists.

2) One of the simplest means of control is found for non-active transport mechanisms, e.g. for glucose. Here the net transplacental flux depends on maternal and fetal plasma glucose concentrations and hence will reflect the balance of glucose supply, metabolism and excretion within the mother, fetus and placenta (Lasunción et al,1987; see Chapter 1, Section D2). The administration of insulin to the mother and fetus will directly influence plasma levels of glucose and hence will probably alter glucose flux (Paxson et al,1978; Crandell et al,1982).

3) Carrier-mediated transfer will be influenced by changes in carrier synthesis or recruitment (Kudo et al,1988) or by an increase in placental surface area, if the number of carriers per unit area remains constant. This is true for facilitated diffusion (e.g. glucose), carrier-mediated active transport (e.g. amino acids) and receptor-mediated endocytosis (e.g. IgG). Changes in the affinity of the carrier and competition by other molecules is obviously also important. There is however little direct evidence for such changes, except in the case of amino acid transport. Here, the 'A' transport system seems to be subject to two modes of regulation, as studied in human placental tissue. Firstly, increased amino acid uptake by placental tissue slices was seen following pre-incubation of the tissue (Enders et al,1976), an effect which was abolished by inhibition of protein synthesis during pre-incubation (Gusseck et al,1975). It was suggested that synthesis of new transport

carrier was involved, but this is still uncertain (Smith,1981). Secondly, there was reduced uptake in the presence of high intracellular concentrations of amino acids (Smith and Depper, 1974). Steel et al (1982) found that this suppression resulted from a direct action of intracellular amino acids on carrier activity, as the K_m of AIB uptake into vesicles was reduced. This process is called transinhibition (Guidotti et al,1978) and it may have the potential for in vivo regulation of placental amino acid transport. The generally accepted mechanism of this interaction between carrier and substrate, is that binding occurs at the substrate site usually involved in transport and that the mobility of the occupied carrier across the membrane, is much slower than that of the empty carrier (Christensen, 1972). An alternative mechanism, is that binding occurs at an allosteric site, causing a change in carrier activity.

4) The transfer of individual solutes might be influenced by specific endocrine control. The placenta is well-endowed with a variety of receptors for various endocrine factors (Sibley and Boyd,1988), although many of these may be primarily controlling the endocrine and metabolic functions of the placenta itself. However, there could also be effects on transport and these have been investigated in some detail. For example, the placental transfer of water is mainly by flow-limited simple diffusion and ultrafiltration, but it can also be influenced by arginine vasotocin and arginine vasopressin in the same way that they control water flux in other tissues (Leake et al,1983). Insulin receptors have been located on the maternal surface of the placental trophoblast in several species (Posner,1974; Whitsett

and Lessard,1978), but there is no evidence of a direct effect on the placental glucose carrier. The elevation of insulin levels in the fetal lamb (Hay et al,1985) and dam (Rankin et al, 1986), or in the perfused human placenta (Challier et al,1986), whilst keeping glucose levels constant, had no effect on placental glucose transfer. The effect of lowering insulin concentrations below normal in vivo has not been investigated and therefore a permissive effect on the glucose carrier cannot be ruled out (Sibley and Boyd,1988). Recently, a new role for insulin has been suggested by Leturque et al (1987A;1987B), who showed a striking effect of insulin on rat placental glucose utilization, with lowered insulin levels causing reduced placental glucose metabolism and vice versa, but not at term. Insulin injections into rabbit fetuses caused a significant increase in placental weight and RNA content (Fletcher and Bassett,1986), in keeping with its mitogenic activity in other tissues (Hill and Milner,1985). Maternal hyperinsulinemia in rats caused fetal growth retardation (Ogata et al,1987). Insulin may therefore play a role in the control of growth, rather than in the control of placental glucose transfer (Ingermann,1987). Several of the steroids which are abundant in utero, e.g. oestrogen and progesterone, have been shown to inhibit glucose transport into human placental microvillous membrane vesicles (Johnson and Smith,1980;1985). Such down-regulation by steroids may serve a regulatory function (Bissonnette et al,1981), but whether this occurs in vivo is unknown. In human placental slices, neither progesterone and oestradiol (Litonjua et al, 1967; Sybulski and Tremblay,1967), nor insulin (Steel et al,

1979), all of which have been shown to stimulate protein synthesis in various tissues (Guidotti et al,1978), had any consistent effect on placental amino acid uptake.

Although the placenta has no nervous system as such (Reilly and Russell,1977), it does contain large amounts of acetylcholine and choline acetyltransferase (Sastry and Sadavongrivad,1979). Studies with isolated villi suggest that acetylcholine is released from the trophoblast into maternal blood. Nicotine increases this release and the effect is blocked by atropine, suggesting a muscarinic type receptor. The role for this cholinergic system is unclear, but it may be involved in the modulation of placental amino acid transfer (Yudilevich and Sweiry,1985). Rowell and Sastry (1978) showed that high levels of acetylcholine could block AIB uptake by placental villous tissue, whilst basal acetylcholine levels could facilitate amino acid transfer by a muscarinic effect.

Finally, the fetus may play a bigger role than was originally believed, in the control of placental transfer. Experiments involving fetal decapitation, have shown that nutrient metabolism may be altered by modifying the fetal endocrine status (Jost and Picon,1970). Fetal decapitation in the pig produced changes in placental lipid and amino acid metabolism, but no effect on fetal or placental growth (Ramsay et al,1985). Obviously numerous factors may be involved in this response, but it is well known that fetal endocrine glands are active before birth (e.g. parathyroid glands; Thomas et al,1981) and therefore have the potential to be involved in the control of materno-fetal and fetomaternal placental transfer. Similarly, genetic

factors may possibly play a role in the control of placental transfer. The possible endocrine control of placental calcium transfer has probably received most attention and this is considered in the next section (E2).

2) Control of Calcium Transfer

Calcium homeostasis in the mammalian body is complex, involving many factors, including vitamin D metabolites, parathyroid hormone (PTH), calcitonin and the prevailing serum calcium concentration. During pregnancy, the need to maintain adequate maternal plasma calcium levels whilst providing for the increasing demands of the fetus, obviously adds to the degree of complexity. Much research has been carried out using maternal and/or fetal surgical or dietary techniques, to produce acute or chronic changes in calcium metabolism. Before looking at these in detail, it is important to understand the actions of the following calcium-regulating hormones : the vitamin D metabolites, PTH and calcitonin. Other hormones such as glucocorticoids, oestrogens, androgens, growth hormone and thyroxine, undoubtedly play roles separately and in concert, in conditioning cellular responses to $1,25(OH)_2D_3$, PTH and calcitonin (Fraser,1980).

Vitamin D_3 or cholecalciferol (a secosteroid) is the main form of vitamin D found in the body, it is obtained from the diet, but is mainly formed from 7-dehydrocholesterol by the action of ultraviolet light in the skin. It is transported in the blood by a vitamin D-binding protein (Edelstein,1974) to the liver, where 25-hydroxy vitamin D_3 ($25OHD_3$, the most abundant circulating

form of vitamin D_3) is produced by 25-hydroxylation. In the kidneys further hydroxylation occurs, releasing the major active metabolite, 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$, Norman et al,1982; Brommage and Deluca,1985) and also, 24,25-dihydroxy vitamin D_3 ($24,25(OH)_2D_3$, see MacIntyre,1986).

It is clear that hypocalcaemia and hypophosphatemia can stimulate the production of $1,25(OH)_2D_3$ without any hormonal intervention (Buskinsky et al,1985). PTH, growth hormone and prolactin all seem to stimulate $1,25(OH)_2D_3$ production, being especially active in pregnancy when the demand for calcium is high (Spanos et al,1976;1978). Human chorionic gonadotrophin also seems to be involved in the regulation of fetoplacental vitamin D metabolism (Kidroni et al,1984). The mechanism of action of $1,25(OH)_2D_3$ seems to be similar to that of a steroid hormone. This involves binding to an intracellular receptor protein, translocation of the complex to the nucleus and induction of mRNA which codes for specific functional proteins (Clemens and Holick,1983), e.g. CaBP's. Receptors for $1,25(OH)_2D_3$ have been well characterized (Haussler,1986) and observed in many tissues (Braidman and Anderson,1985). $1,25(OH)_2D_3$ acts on the intestine to increase active transport of calcium (see Chapter 1, Section D5). It reduces intracellular calcium concentrations in many cells, possibly via stimulation of Ca^{2+}-ATPase activity (Bar et al,1986; Lidor and Edelstein, 1987). In bone, it has two actions :1) A direct action on the mobilization of calcium, dependent in part on the presence of PTH. $1,25(OH)_2D_3$ increases the activity of osteoclast cells, suppressing osteoblast cells (Raisz et al,1972). 2) It

indirectly causes facilitation of bone mineralization (Tanaka et al,1972), probably by supplying adequate amounts of calcium to the mineralizing bone matrix by means of its effect on the intestinal absorption of calcium.

PTH is an 84 amino acid single chain polypeptide (molecular weight = 9500), full activity requiring positions 1-34 (Rosenblatt et al,1980). PTH is synthesized as an 115 amino acid peptide chain (pre-proPTH) and cleavage produces the 84 amino acid form. This is incorporated into secretory granules ready for release (Habener et al,1984), along with many other PTH fragments (Brown,1983). The main factor controlling PTH secretion is plasma calcium concentration, low extracellular calcium levels stimulating a calcium-sensitive magnesium-dependent adenylate cyclase, with the consequent generation of cAMP, protein phosphorylation and peptide secretion. Calcium-dependent PTH release may also occur via inositol trisphosphate (Dean et al,1986) and protein kinase C (Muff and Fischer,1986). Apart from calcium, a number of other factors can directly modulate PTH release, e.g. β-adrenergic agonists (Brown,1983).

PTH has 3 separate effects on the kidney :1) it markedly enhances phosphate excretion, 2) it increases calcium resorption and 3) it accelerates the conversion of $25OHD_3$ to $1,25(OH)_2D_3$ (Bourdeau et al,1986). These three actions are brought about by cAMP and changes in intracellular calcium, producing the basic action of PTH, i.e. the enhancement of plasma calcium levels (see MacIntyre,1986). PTH also indirectly enhances osteoclastic activity in the skeleton, its primary action being the suppression of osteoblast activity (Brown,1983). In the gut, PTH

enhances calcium and phosphate absorption, but only indirectly, by increasing production of $1,25(OH)_2D_3$ (Omadahl et al,1971). It is interesting to note that human placental tissue itself can synthesize PTH *in vitro* (Balabanova et al,1987A) and, like the kidney and liver, it also has the ability to degrade PTH (Balabanova et al,1986).

Calcitonin, a 32 amino acid peptide, is produced by the parafollicular or C-cells in the thyroid. Its release involves an adenylate cyclase/cAMP system (Care et al,1970A; Care et al, 1971), with calmodulin probably playing an important role (Cooper and Borosky,1986). Again, it has been shown that human placental tissue secretes calcitonin *in vitro* (Balabanova et al, 1987B). The major factor controlling its secretion from the C-cells is certainly the plasma calcium level, with a rise in calcium concentration increasing calcitonin release, but other substances may be involved too, e.g. adrenaline (Care and Bruce, 1971), glucagon (Care et al,1970B) and oestrogen (Catherwood et al,1983; Greenberg et al,1986). The main direct action of calcitonin is on the osteoclast, inhibiting bone resorption, hence antagonizing the action of PTH (Chambers et al,1985). Indeed, abundant receptors have been observed in isolated rat osteoclasts (Nicholson et al,1986) and in human placental tissue (Nicholson et al,1988). Calcitonin also acts on the kidney, enhancing the production of $1,25(OH)_2D_3$ (Galante et al,1972; Jaeger et al,1986).

These three hormones, $1,25(OH)_2D_3$, PTH and calcitonin, obviously all act closely together to maintain calcium levels within a narrow physiological range. Augmented extracellular

fluid volume in pregnancy, along with increased renal function, means that additional calcium is required if the calcium ion concentration is to be maintained within its relatively narrow physiological range. This mainly comes from increased intestinal calcium absorption (Schacter et al,1960) and although total serum calcium seems to fall during pregnancy, ionized calcium levels remain constant (Pitkin and Gebhardt,1977). PTH secretion tends to rise and a state of 'physiological hyperparathyroidism' is seen in pregnancy (Cushard et al,1972). Measurement of maternal calcitonin levels has given a variety of results, but overall, calcitonin seems to increase throughout gestation (rat-Garel and Jullienne,1977; sheep-Garel et al,1974). It has been suggested that gestational hypercalcitonemia serves a function in counteracting hyperparathyroidism, by protecting the maternal skeleton from excessive resorption, while at the same time permitting the gut and kidney actions of PTH to provide the extra calcium needed (Taylor et al,1975). The circulating concentrations of $25OHD_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ also change with gestation, the latter rising, whilst the others fall (Halloran et al,1979). The increase in $1,25(OH)_2D_3$ (Pike et al, 1979) is most likely mediated by the rising PTH levels, although prolactin, placental lactogen and steroids, may contribute to the stimulation of 1α-hydroxylase activity (Spanos et al,1976). Indeed, the placenta itself seems capable of producing $1,25(OH)_2D_3$ (Tanaka et al,1979; Weisman et al,1979; Zerwekh and Breslau,1986), but what role it plays is unclear. It is also not clear whether these changes are important in the control of transplacental calcium flux. They may simply make sufficient

calcium available for the fetus when it requires it, whilst maintaining maternal calcium homeostasis. In order to unravel what is a complex picture, it is easier to look at the opportunities for control of placental calcium transfer from either the maternal side or the fetal side.

a) Maternal Control Of Placental Calcium Transfer?

The existence of correlations between maternal and cord blood concentrations of $25OHD_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$, are well known in man (Hillman and Haddad,1974; Weisman et al,1978A), and rats (Weisman et al,1976). However, great caution must be observed when interpreting these single point measurements of vitamin D metabolites in cord, maternal and fetal blood, especially with relation to control of placental transport. It must also be remembered that nearly 99% of vitamin D metabolites are transported bound to vitamin D-binding protein (DBP, Bouillon and Van Baelen,1981). Indeed, it is the fraction of 'free' metabolite that is thought to be the physiologically active form. Hence, this is the important fraction with respect to cellular activity and placental transport (Bouillon et al, 1981). DBP is a 56-58000 molecular weight α_2-glycoprotein and it has been detected on the surface of human cytotrophoblast, where it may play a role in placental transfer of vitamin D metabolites and possibly IgG (Nestler et al,1987). The total levels of vitamin D metabolites seem to be higher in the maternal circulation in man, but the 'free' metabolite concentration seems to be higher in the fetal circulation (Weisman et al, 1978A; Wieland et al,1980). Direct evidence for

placental transfer of vitamin D_3 and $25OHD_3$ came from injection of tritiated molecules into the rat, on day 18 of gestation (Haddad et al,1971) and $1,25(OH)_2D_3$ transfer has now been observed in sheep (Ross et al,1979) and man (Ron et al,1984). This is in direct contrast to PTH and calcitonin which do not appear to cross the placenta. Experiments involving PTH injection in the rat (Garel and Dumont,1972), sheep (Erenberg et al,1978) and monkey (Northrop et al,1977), and calcitonin injection into the rat (Garel et al,1969), all suggest that these hormones do not cross the placenta in either direction. However, a more recent report (Balabanova et al,1982) suggests that a small amount of biologically active PTH or calcitonin can cross the perfused human placenta in both directions.

It is well known that vitamin D deficiency is a common problem among Asian immigrants, partly because dark skin pigmentation decreases the amount of vitamin D_3 formed after exposure to sunlight (Clemens et al,1982). Vitamin D supplements to these women during pregnancy have been shown to be beneficial to mother and baby in preventing excessive maternal calcium loss or fetal rickets (Brooke et al,1980; Delvin et al,1986; Mallet et al,1986), but how this effect is brought about is unknown.

The placenta certainly has receptors for $1,25(OH)_2D_3$ (Pike et al,1980; Stumpf et al,1983) and a vitamin D-dependent CaBP which changes throughout gestation (Bruns et al,1978). However, dietary produced vitamin D deficiency in the rat (Marche et al, 1978) or $1,25(OH)_2D_3$ supplementation in mice (Bruns et al,1982), caused a reduction or increase respectively in intestinal CaBP, but had no effect on placental CaBP. So placental CaBP may not

respond to maternal $1,25(OH)_2D_3$, but be controlled by fetal $1,25(OH)_2D_3$ (Ross et al,1981). Similarly, vitamin D deficiency in pregnant rats, had no effect on the maternofetal calcium concentration gradient or on net fetal calcium accretion (Halloran and Deluca,1981; Brommage and Deluca,1984). However, the administration of $1,25(OH)_2D_3$ in guinea-pigs (Durand et al, 1983A) and sheep (Durand et al,1983B) significantly increased fetal calcium content (i.e. net calcium flux), whilst causing a small (but not significant) increase in J_{mf} calcium. So it may be that placental calcium transfer can be moderately increased by maternal $1,25(OH)_2D_3$, but transfer is protected in some way (possibly by fetal control) from the effect of maternal vitamin D deficiency. It is impossible to conclude that these effects are due to a direct action on the placenta and it is probably more likely that they are due to gut, bone or kidney effects.

PTH can directly control the renal production of $1,25(OH)_2D_3$ and therefore, Garel et al (1981B) investigated the role of maternal parathyroid glands and $1,25(OH)_2D3$ in controlling levels of CaBP in rat placentas. After maternal thyro-parathyroidectomy (TPTX), CaBP content of all tissues including maternal intestine, was reduced by 50%, whilst CaBP content of the placenta was reduced by 20%, both being restored by administration of $1,25(OH)_2D_3$. The relevance of this data is not certain because, as described previously (Section D5), it is not yet even clear whether placental CaBP is involved in placental calcium tranport. Other workers have used the maternal TPTX model, for example in sheep (Ross et al,1981), where the resulting maternal hypocalcaemia had no effect on fetal plasma

total calcium concentration. This evidence makes it less likely that there is a role for maternal PTH and calcitonin in the maintenance of the transplacental calcium gradient, but does not exclude a role for maternal $1,25(OH)_2D_3$, since despite the removal of the stimulatory effect of PTH, the ensuing hypocalcaemia may have a direct effect on maternal plasma $1,25(OH)_2D_3$. However, maternal TPTX, followed by calcium infusion to maintain normocalcaemia, also had no effect on fetal plasma total calcium concentration, suggesting that in sheep at least, the placental transfer of calcium is largely independent of maternal PTH, calcitonin and $1,25(OH)_2D_3$ (Weatherley et al, 1983). Ross et al (1980B) showed that the marked reduction in maternal plasma $1,25(OH)_2D_3$ concentration seen with maternal TPTX in sheep, led to only a small fall in fetal plasma $1,25(OH)_2D_3$ concentration. The transplacental calcium gradient could be abolished by procedures which reduce fetal plasma $1,25(OH)_2D_3$ concentration, e.g. fetal nephrectomy or TPTX, but this might not necessarily result from a direct effect on the placenta.

In man, clinical studies have shown that maternal hypoparathyroidism is associated with newborn parathyroid hyperplasia (Aceto et al,1966; Landing and Kamoshita,1970). It has been postulated that the maternal hypocalcaemia caused by the maternal condition, results in a decrease in maternofetal calcium transport (Anast,1976). Fetal parathyroid hyperplasia has also been recorded in the pregnant rat after maternal parathyroidectomy (Sinclair,1941;1942). Maternal TPTX in rats also reduced fetal serum calcium levels and fetal body weight

(Gilbert et al,1980; Garel et al,1981C), with maternal $1,25(OH)_2D_3$ replacement being able to recover the fetal serum calcium levels. However, when Ibrahim et al (1984) repeated these experiments, no fall in fetal weight and only a small fall in fetal serum calcium concentration was observed. It is important to remember that even though fetal hypocalcaemia was seen in Garel's (1981C) and Gilbert's (1980) experiments, the maternofetal calcium gradient was not abolished, suggesting that maternal factors are not involved in the maintenance of this gradient.

It has been suggested that maternal calcitonin plays a role in protecting the maternal skeleton from excessive demineralization (Stevenson et al,1979). Barlet (1985A) showed that maternal calcitonin deficiency produced by maternal TP on day 30 of gestation (with thyroxine replacement), had no significant effect, either on fetal total calcium content or on maternofetal placental calcium transfer, in 77 day gestation fetal lambs. But calcitonin deficiency for 110 days (i.e. on day 140 of gestation) was associated with an increased total fetal calcium content and increased maternofetal calcium transfer, not seen in TP controls supplemented with thyroxine and calcitonin. Also, an increase in fetal plasma total calcium concentration was observed, when maternal hypocalcaemia was induced in pregnant guinea-pigs by maternal injection of calcitonin (Crawford et al, 1969). This suggests that calcitonin might modulate placental calcium transfer during the period of intense mineralization of the fetal skeleton and prevents excessive loss from the maternal body. Barlet (1985B) has also shown that prolactin can stimulate

placental calcium transport in sheep, but this may be an indirect effect due to PTH (Magliola and Forte,1984) and/or $1,25(OH)_2D_3$ (Spanos et al,1976). Other modulators like corticosteroids (Kimberg et al,1971), growth hormone (Braithwaite,1975), etc., have also been implicated in the maintenance of calcium homeostasis in pregnancy.

In conclusion therefore, changes in maternal $1,25(OH)_2D_3$, PTH and calcitonin concentrations certainly provide the milieu in which large amounts of calcium become available for transfer to the fetus, whilst maintaining calcium homeostasis in the maternal organism. However, the degree of control of placental calcium transfer by the mother is still unclear. The main problem being that very few studies directly measure placental transfer and so it is hard to separate gut, bone, kidney and placental effects.

b) Fetal Control Of Placental Calcium Transfer?

Although the fetus is completely dependent on its dam for calcium supply, fetal plasma calcium concentration appears to be controlled relatively independently of maternal plasma calcium concentration (see Garel,1987).

At each stage studied, plasma ionized calcium concentration was higher in the fetal rat than in the mother, during the last 6 days of gestation (Pic,1969). A small decrease in fetal plasma calcium concentration occurs in the rat fetus between 16.5 and 17.5 days of gestation, with the onset of fetal ossification (Jost et al,1960). The subsequent increase in fetal plasma calcium concentration after day 17.5, being attributed to the

onset of fetal parathyroid gland function (Pic,1969). Finally, the small decrease in fetal plasma calcium between 19.5 and 21.5 days, suggests the onset of thyroid C-cell function (Garel et al,1981A). Similar trends in fetal plasma calcium concentration were seen in the guinea-pig (Graham and Scothorne,1970).

The role of vitamin D in the fetus is complicated by the several sources of fetal vitamin D metabolites. The fetus can derive vitamin D metabolites from the mother by placental transfer (Haddad et al,1971) and presumably from the placenta itself (Tanaka et al,1979; Whitsett et al,1981). The relative concentrations of the various metabolites in mother and fetus seem to show species differences. In the rat fetus, the concentration of $1,25(OH)_2D_3$ in tissue and plasma is very low, despite the increase in maternal plasma $1,25(OH)_2D_3$ concentration during pregnancy (Weisman et al,1976; Noff and Edelstein,1978). The main circulating metabolite in fetal tissue and plasma being $24,25(OH)_2D_3$ (Lester et al,1978; Noff and Edelstein,1978). In man, the level of cord blood $1,25(OH)_2D_3$ is also very low, the major circulating metabolite being $25OHD_3$ (Weisman et al,1978A). Among the possible causes of the differences between species in plasma vitamin D metabolite concentration, is a variation in the fetal production of these sterols. <u>In vitro</u> studies have shown that fetal kidneys from 18 day gestation rats (Weisman et al,1976), 61 day gestation guinea-pigs (Fenton and Britton,1980) and rabbits (26th day of gestation to birth; Sunaga et al,1979) are able to convert $25OHD_3$ to $1,25(OH)_2D_3$.

With the aim of finding a physiological role for fetal

$1,25(OH)_2D_3$, Ross et al (1980B) nephrectomized sheep fetuses. This eliminated the maternofetal calcium gradient, which was restored by administering $1,25(OH)_2D_3$ to the fetus. This effect of the fetal $1,25(OH)_2D_3$ concentration was confirmed by administration to the fetus of an ovine antiserum to $1,25(OH)_2D_3$ (Ross et al,1980B), which also eliminated the maternofetal calcium concentration gradient. However, these results again do not differentiate between a $1,25(OH)_2D_3$ effect on fetal bone or a direct effect on the putative calcium pump in the placenta. In fact, Moore et al (1985) suggest that ovine fetal $1,25(OH)_2D_3$ controls fetal phosphate homeostasis, perhaps via the placenta, which in turn regulates fetal plasma ionized calcium concentration. In rats, fetal nephrectomy performed on day 19.5 of gestation did not change the fetal plasma total or ionized calcium concentration. This indicates, at least in rats, that fetal kidneys are not involved in the control of fetal plasma calcium levels, perhaps because there is a relatively greater flux of $1,25(OH)_2D_3$ across the placenta (Chalon and Garel, 1985B).

The hypercalcaemic effect of $1,25(OH)_2D_3$ in fetuses is well established in sheep, but is less clear in rats. Intravenous administration of $1,25(OH)_2D_3$ into 125-135 day gestation sheep fetuses caused a rise in fetal plasma total calcium concentration within 24 hours (Barlet et al,1978). A hypersensitivity to $1,25(OH)_2D_3$ was described in rat fetuses from TPTX mothers (Chalon and Garel,1983), causing a marked fetal hypercalcaemia, which was not seen in fetuses from normal mothers.

Autoradiographic localization of target cells for $1,25(OH)_2D_3$ in bones from 18-20 day gestation fetal rats, revealed a nuclear concentration of radioactivity in osteoprogenitor cells and osteoblasts (Narbaitz et al,1983) and receptors for $1,25(OH)_2D_3$ were identified in fetal rat calvaria (bone cells from the upper part of the skull; Kream et al,1977). Binding sites for $1,25(OH)_2D_3$ have since been observed in the fetal rat skeleton, lungs and kidney (Nguyen et al,1987). Also, $1,25(OH)_2D_3$ significantly increased the ^{45}Ca release from prelabeled rat fetal long bones in vitro (Mahgoub and Sheppard,1977) and tritiated vitamin D_3 was rapidly sequestered by the fetal rat liver, probably as a source of $25OHD_3$ (Martial et al,1985). Therefore, fetal $1,25(OH)_2D_3$ whether derived from the mother, placenta or fetus, seems to play an important role in fetal calcaemia, at least in the sheep (Ross,1983) and the rat (Lester,1983). Whether it acts directly on fetal bone or kidney, or via stimulation of the putative placental calcium pump, is unknown. The production and turnover of $1,25(OH)_2D_3$ is presumably very fast, being reflected in the low circulating levels of fetal $1,25(OH)_2D_3$ in comparison to greater concentrations of $25OHD_3$ and $24,25(OH)_2D_3$.

Turning now to the role of fetal PTH, Leroyer-Alizon et al (1981) showed evidence for PTH in human fetal parathyroids by 10 weeks gestation, but it is not until several days after birth that the fetal parathyroids respond to hypocalcaemia (Schedewie et al,1979). In the rat, the fetal parathyroids seem to be functional during the last 4 days of gestation and appear to be regulated by circulating calcium concentrations (Thomas et al,

1981). The rat visceral yolk sac also seems to be a target organ for PTH in the last 9 days of pregnancy (Gügi et al,1986) and indeed it may secrete its own PTH (Evain-Brion et al,1984).

Several lines of evidence suggest that fetal parathyroid glands may partly control fetal plasma calcium in the rat. A fall in plasma total calcium concentration occurs in the rat fetus after fetal TPTX (Pic et al,1965; Pic,1973) or injection of antibovine PTH serum (Garel,1970) and this fall is corrected by parathyroid extract injection (Garel et al,1971). Higher plasma total calcium concentrations in the fetus than in the mother, in TPTX rat fetuses from TPTX mothers, demonstrated that the plasma calcium level in the rat depends primarily on the calcium flux through the placenta (Pic,1968). In the sheep (like the rat; Pic,1973), fetal TPTX alone completely abolishes the maternofetal calcium concentration gradient (Care and Ross, 1984). All these observations strengthen the suggestion that fetal PTH is involved in the maintenance of hypercalcaemia in the fetus. Indeed, injection of synthetic bovine PTH (1-34) into conscious sheep fetuses in utero induced a rise in fetal plasma total calcium concentration, 2-3 hours later (Barlet et al, 1978). However, this action of PTH may simply be an effect on fetal bone. In 21.5 day gestation rat fetuses, 12 hours after the injection of parathyroid extract, the number of osteoclasts per unit area in the femur and femur bone resorption were increased (Legrand and Phouc,1970). Also, PTH is readily metabolized (Freitag et al,1979) and stimulates bone resorption (Stern and Krieger,1983) in fetal rat calvaria in vitro. Of course, all these effects of PTH could be due to the PTH-

stimulated release of $1,25(OH)_2D_3$ (Fraser and Kodicek,1973). However, it has been suggested that it is not fetal PTH itself that is important in controlling maternofetal calcium transfer (Abbas et al,1987A) and fetal plasma calcium levels (Abbas et al,1988). They suggest that this is brought about by a substance with PTH-like bioactivity, which seems to be produced by fetal sheep and pig parathyroid glands, but this substance has not yet been positively identified.

Finally turning to calcitonin, immunochemical and radio-immunological studies have revealed a large number of C-cells and a high content of calcitonin in human thyroid glands as early as the 14th week of pregnancy (Wolfe et al,1975; Leroyer-Alizon et al,1980). The effects of calcitonin upon amino acid, glucose and fat metabolism and the influence of this hormone on neonatal calcium levels, led to the hypothesis that at this stage in life calcitonin may be involved in the regulation of nutrient absorption from the alimentary tract, especially calcium. Garel et al (1981A) reported the presence of small amounts of calcitonin in thyroid glands of 17.5 day gestation rat fetuses, with a further exponential increase in calcitonin content at later developmental stages. Jarzab et al (1984) detected calcitonin in fetal rat thyroids as early as day 15.5, increasing linearly with gestational age.

Intravenous calcium infusion into the fetal pig increases fetal plasma calcitonin concentration, but an infusion of calcitonin does not produce fetal hypocalcaemia (Littledike et al,1972). However, a fetal hypocalcaemic response to fetal infusion of calcitonin has been seen in monkeys (Reynolds et al,

1975) and sheep (Barlet et al,1978). Also, in 19.5-21.5 day gestation rat fetuses, a subcutaneous injection of porcine calcitonin produced fetal hypocalcaemia one hour later (Garel et al,1968), whilst injection of an anti-human calcitonin serum caused a slight fetal hypercalcaemia (Garel and Barlet,1978). Fetal calcitonin may therefore also have a role in controlling fetal plasma calcium levels at the end of gestation in the rat, promoting the mineralization of the fetal skeleton by stimulation of the placental calcium pump and/or directly increasing bone accretion or inhibiting bone resorption.

In conclusion therefore, it appears that there is no correlation between maternal and fetal plasma calcium concentrations in most species. From which it may be concluded that fetal calcium homeostasis is largely independent of the mother, except in so far that the mother provides an adequate supply of calcium for fetal growth (Care and Ross,1984). This fetal calcium homeostasis essentially involves two processes : 1) the control of net calcium transfer across the placenta to the fetus, and 2) the distribution of that calcium between the extracellular fluid and the fetal skeleton. Thus, all three hormones involved in calcium homeostasis in the adult, are present in the fetus and all three can be associated with changes in fetal plasma total calcium concentration. However, as with the maternally derived hormones, it is generally unclear whether they can directly control placental transfer or whether their main locus of action is elsewhere.

Introduction To The Present Work

The aim of this thesis was to study the control of placental calcium transfer, by directly measuring transplacental calcium fluxes under various conditions.

For this study we adapted the recently reported rat placental perfusion model of Štulc and Štulcová (1986). The more widely used *in situ* perfused guinea-pig placenta was not used, as its permeability to a range of hydrophilic molecules is increased in this preparation as compared to the intact guinea-pig placenta (Hedley and Bradbury,1980). Hence, our initial experiments as described in Chapter 2 were carried out to investigate the permeability of the perfused rat placenta and extend the results of Štulc and Štulcová (1986) concerning the validity of the model.

The presence of active maternofetal calcium transfer across the *in situ* perfused rat placenta, has been investigated in Chapter 3. It was then possible to study the control of placental calcium transfer, by looking at maternal (Chapter 4) and fetal (Chapter 5) control mechanisms separately. The final discussion of this thesis appears in Chapter 6.

CHAPTER 2

PERMEABILITY OF THE NEAR TERM RAT PLACENTA TO HYDROPHILIC MOLECULES

SECTION A : INTRODUCTION

The major aim of this permeability study was to confirm and extend the observations of Štulc and Štulcová (1986), on the permeability of the intact and *in situ* perfused rat placenta. They found that the permeability of the rat placenta was like that of the other haemochorial types, despite there being three layers of trophoblast. They also found, that unlike the guinea-pig (Hedley and Bradbury,1980), perfusion had no effect on permeability.

Steady state transplacental clearance measurements were made *in vivo* using the method of Flexner and Pohl (1941). Here a radioactive tracer is injected into the mother and, after a known period, a fetus is removed and the amount of radioactivity in the carcass measured. If the average concentration of tracer in the maternal plasma during the period of the experiment is known, and if placental transfer achieves a steady state rapidly after tracer injection (the fetal plasma tracer concentration must remain low throughout the experiment), then the unidirectional maternofetal clearance, K_{mf}, of the tracer per gram of placenta can be calculated. For molecules transferred by simple membrane-limited diffusion, such as many inert tracers, K_{mf} is the same as the permeability surface area product (PS) (Meschia et al,1967; Faber,1973; see Chapter 1, Section B). In the *in situ* placental perfusion model, K_{mf} was simply measured by applying Fick's law to the perfused fetal circulation. The electron microscopic appearance of the perfused rat placenta was investigated for us, by Dr.C.Jones (Department of Pathology)

and her results are also presented here for completeness. As already described (Chapter 1, Section A), in the rat, as in other rodents, the yolk sac placenta co-exists with the chorioallantoic placenta right up to term (Jollie,1964). The yolk sac appears to be important in the transfer of immunoglobulin (Brambell and Halliday,1956; Thornburg and Faber,1977), but whether it subserves other functions is unclear. We have therefore also investigated the contribution the yolk sac may make to the transfer of hydrophilic molecules at term in the rat.

SECTION B : MATERIALS AND METHODS

1) Permeability of the intact placenta

Female Sprague Dawley rats maintained on a normal rat pellet diet were mated on day 1 and then used for experimentation on day 21 of gestation (term is 23 days). The mothers were anaesthetized with 110mgkg^{-1} i.p. sodium thiobutabarbital (Inactin, BYK Gulden, Hamburg, FRG). The trachea, a jugular vein (for the administration of tracers) and a carotid artery (for blood sampling) were cannulated. The maternal carotid artery cannula was connected to a pressure transducer (Gould) and then to a 4-channel Electromed chart recorder. The cannulae were kept viable throughout the experiment by occasional flushing with 10 U/ml Heparin saline (0.9%). Lignocaine hydrochloride (1% w/v) was used during the neck surgery and the animals were kept warm throughout the experiment using a dissection lamp. This method was not ideal, as the animals were not maintained at a specific

body temperature. Unfortunately, maternal body temperature was not always monitored, however, in those animals where it was monitored, rectal temperature was found to range from 34-37°C. Tracers were injected at time zero and maternal carotid blood samples (0.5ml) were taken throughout the experiment for radioactivity analysis. At various time intervals, one fetus was removed from each horn after making a small incision in the uterus at the appropriate point. The time of fetal harvesting depended on the tracers used, fetuses being taken only at times when the fetal plasma radioactivity concentration was certain to be less than 10% of the maternal plasma radioactivity concentration. This was determined for each tracer in preliminary experiments where blood samples (usually from four fetuses) were taken and pooled to obtain sufficient volume. This was carried out at each of several increasing times after injection of tracer to the mother and plasma radioactivity counted as described below. It was thus found that for ^{22}Na fetuses should be taken before 15 min, for ^{14}C-mannitol, ^{14}C-sucrose and ^{51}Cr-EDTA before 60 min, for ^{3}H-inulin before 80 min and for ^{125}I-albumin before 110 min.

Each harvested fetus was either: a) homogenized in a Waring blender for 20 seconds with 2.5ml 0.5N $HClO_4$ (perchloric acid). The volume was then made up to 10ml with $HClO_4$ and samples were centrifuged for 10 minutes at 4000 rpm, the supernatant being used for radioactivity analysis (see below); or b) for ^{125}I-albumin only, the homogenization was carried out in distilled water and the supernatant was analysed as above. 1ml $HClO_4$ was then added to the remaining supernatant and pellet, causing any

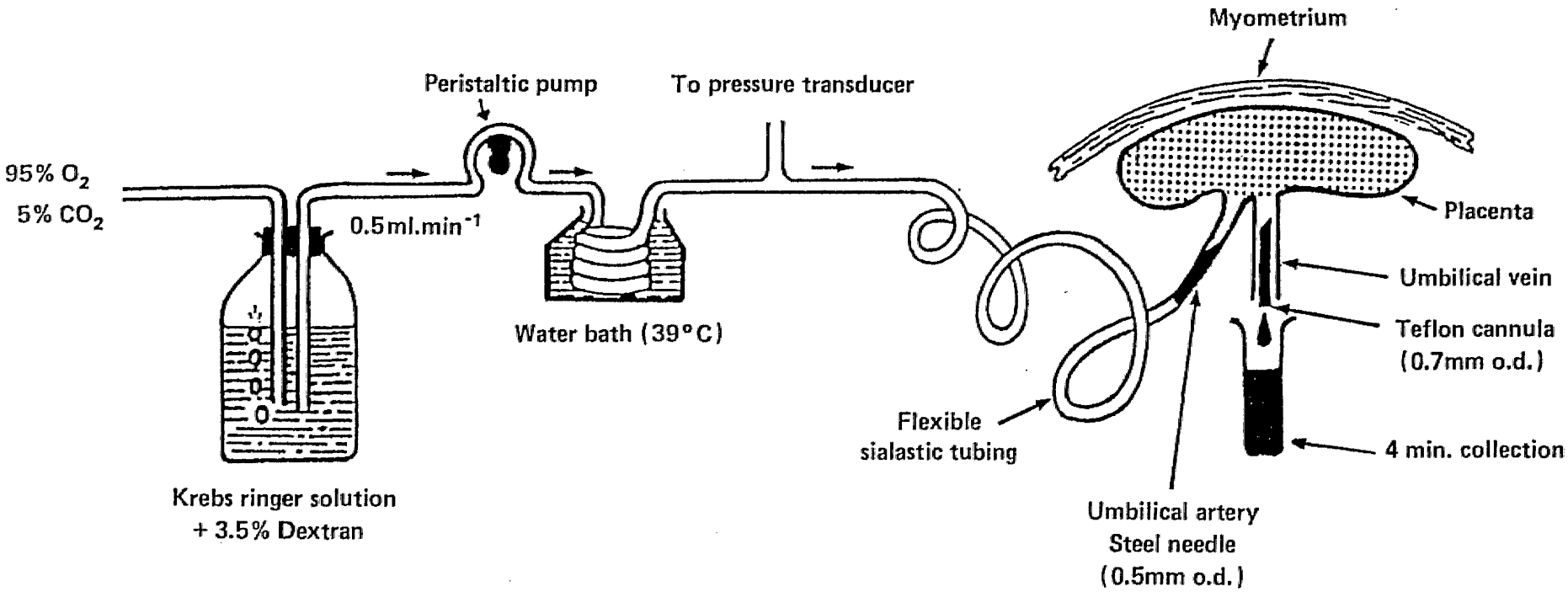
^{125}I-albumin to precipitate out. After further centrifugation for 10 minutes at 4000 rpm, the new supernatant was analysed for radioactivity, giving a measure of any non-protein bound ^{125}I present. Recovery of isotope from fetuses was checked by analysing a fetus which had been injected deeply after killing, using a known amount of isotope and then treated in the way described above. Recovery of radioisotopes from fetal mince was as follows : ^{22}Na 83.7 ± 1.9%, ^{125}I-albumin 90.6 ± 2.6, ^{14}C-mannitol 70.3 ± 2.4, ^{3}H-inulin 95.6 ± 1.4, ^{51}Cr-EDTA 74.7 ± 5.7, ^{14}C-sucrose 90.7 ± 3.1 (mean ± s.e.m., n = 8 fetuses for each isotope). These values were used to correct the experimental value of total fetal radioactivity content.

The uteroplacental unit probably cannot autoregulate and consequently the maternal blood pressure is very important (Wallenburg,1981). Hence, we chose to reject all animals where the mean maternal blood pressure during any of the experiments described in this chapter, fell below 65-70mmHg (8.6-9.2 kPa).

2) Permeability of the perfused placenta.

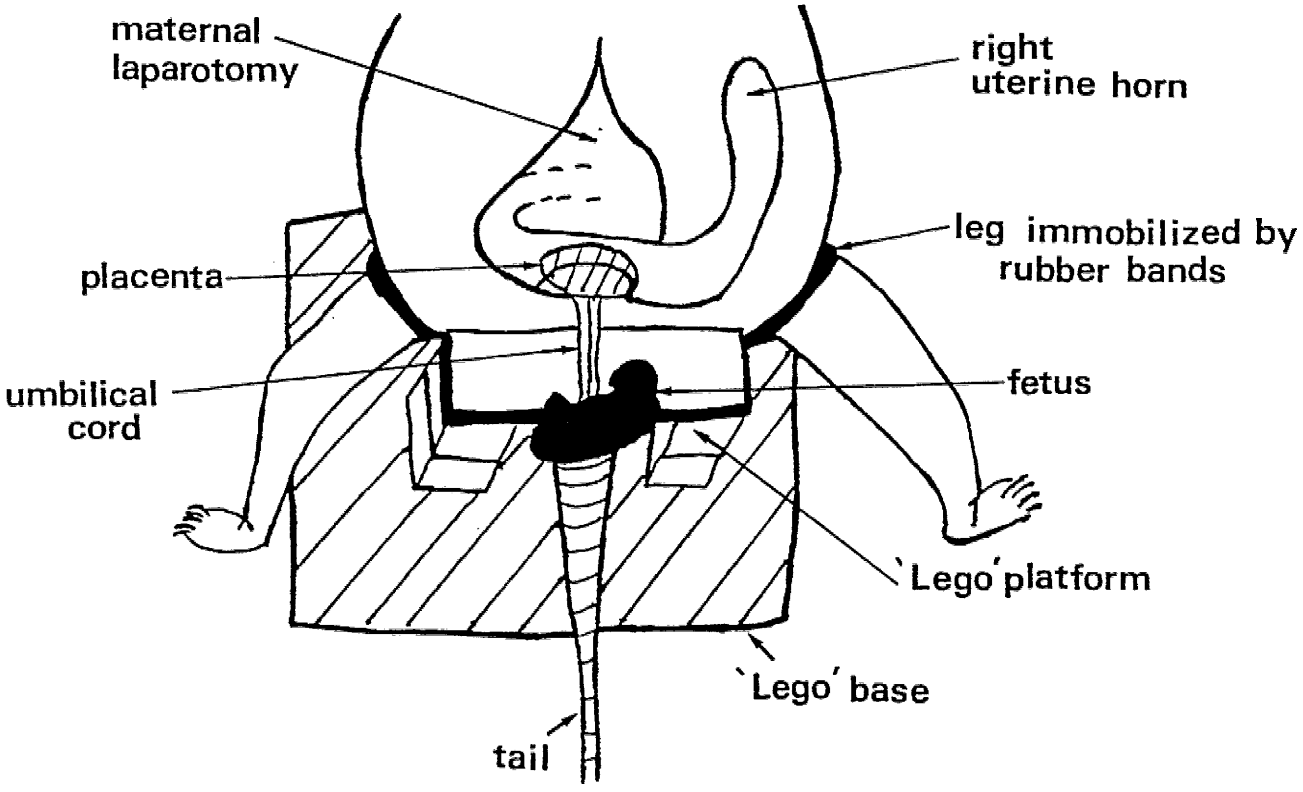
A method modified from that of Štulc and Štulcová (1986) was used to perfuse the rat placenta through its fetal circulation (Fig.2.1). Female Sprague Dawley rats maintained on a normal rat pellet diet were anaesthetized as above on day 21 of gestation and the trachea, a jugular vein (for administration of tracers) and a carotid artery (for monitoring maternal blood pressure and blood sampling) were cannulated. The rat was then immobilised on its back and kept warm using a dissection lamp. After laparotomy and hysterotomy by a small incision in the uterus,

Figure 2.1 The 'in situ' perfused rat placental model.



one fetus was delivered in such a way that the uterus retracted and enveloped the placenta, so preventing placental separation. Care was taken not to touch the umbilical cord as this leads to rapid constriction of the two umbilical vessels. The umbilical cord was gently supported over a platform (Fig.2.2) constructed from plastic bricks ('Lego').

Figure 2.2 The 'Lego' platform for securing the rat and allowing cannulation of the umbilical vessels.



The umbilical artery (there is only one in the rat) was cannulated using a steel needle (0.5mm O.D.) attached to Silastic tubing (0.63mm O.D., 0.3mm I.D., Dow Corning Corporation, Michigan, USA) and the umbilical vein was then cannulated using a 24G Jelco catheter (Critikon, Tampa, USA). The cannulae in both vessels were tied together and incorporated in a suture used to occlude the vitelline vessels. The fetal

circulation was perfused with a modified Kreb's Ringer solution containing (mM) NaCl 118, KCl 4.7, $NaHCO_3$ 24.9, $CaCl_2-2H_2O$ 1.25, KH_2PO_4 1.18, $MgSO_4-7H_2O$ 1.18, glucose 0.2%, dextran (40,000 MW) 3.5%, pH 7.4. This solution was gassed with 95% O_2/5% CO_2, warmed to 37°C and perfused at a rate of 0.5ml/min. Venous effluent was collected at 4-minute intervals after a single passage through the placenta. Perfusion pressure was monitored via a side arm in the arterial catheter. In some experiments maternal haematocrit, maternal blood gases and perfusate gases were checked throughout. For permeability measurements, tracers were injected at time zero and 3 maternal blood samples (0.5ml) were taken at 3, 13 and 23 minutes. Four 4-minute perfusate collections were made at 4-8, 8-12, 12-16 and 16-20 minutes. Both blood and perfusate were analysed for radioactivity content, the precipitation method described above was used in an attempt to separate free and protein-bound ^{125}I in perfusate samples.

3) Experiments

Permeability measurements were initially made as described above in intact placentas and using the in situ perfused placenta in separate animals. In further experiments, 'intact' and 'perfused' measurements were made in the same animal, by injection of tracers into the mother as normal, followed by perfusion of one placenta and then harvesting of fetuses. Because of the time required to perfuse and then harvest, these experiments could only be carried out with tracers whose transfer was relatively slow and ^{14}C-mannitol, ^{51}Cr-EDTA and ^{3}H-

inulin were used.

In a final set of intact measurements, one uterine horn was used as a control, whilst in the other horn the vitelline artery and vein supplying each conceptus were cauterized using an electrocautery, without opening the uterus. Three isotopes were used in this part of the study, ^{125}I-albumin, ^{51}Cr-EDTA and ^{22}Na, and maternal blood samples and fetuses were obtained and prepared for analysis as described above.

Fetal and placental wet weight was measured routinely in all experiments.

4) Analysis of radioactivity

All the radioisotope labelled tracers used were obtained from Amersham International plc, Bucks. The tracers were studied singly in each animal or in appropriate pairs (Table 2.1). When an isotope loses a 'β' or 'ɣ' particle it is emitted at a specific energy value (KeV), which can be picked up by a range of channel settings in the machine. A 'β' emitter actually produces a photon in the scintillant, which is detected. However, the energy produced by the emission of a 'ɣ' particle can release electrons (i.e. 'β' particles) from other molecules and these can then react with the liquid scintillant in the same way. An emission spectrum can be produced for each isotope, enabling one to choose the most efficient channel settings and to observe any overlap between emissions from a mixture of isotopes. Spillover of any energy emission from one isotope into the channel setting of the other isotope in a pair, was adjusted for by counting standards of the appropriate 'overlapping'

isotope each time or by using the external standards ratio of the machine.

Table 2.1 Radioisotope activities and measurements
This table shows the pairs of isotopes used and how their energies were separated on counting. 'Spillover' energy from one radioisotope into the channel of another, was corrected for as described in the text.

Isotope pairs	Decay modes	Particle energy (keV)		Separation (keV)	
		β	γ	β counter	γ counter
^{22}Na	(β+) γ	(545.5)	300,1275	--	433-1417
^{125}I	γ		35	--	15-80
^{3}H	β-	18.6		0-12	--
^{14}C	β-	156		12-156	--
^{51}Cr	γ		320	--	240-400
^{125}I	γ		35	--	15-80
^{14}C	β-	156		0-75	--
^{22}Na	(β+) γ	(545.5)	300,1275	75-500	433-1217
^{51}Cr	γ		320	0-5	240-400
^{14}C	β-	156		5-260	--

For example (Table 2.1), the pairs ^{22}Na/^{125}I and ^{51}Cr/^{125}I were only counted in the Packard Autogamma 800, with the ^{22}Na and ^{51}Cr emissions 'spilling-over' into ^{125}I emissions. Single standards of ^{22}Na or ^{51}Cr were counted each time and the percentage of overlap was taken into account when calculating cpm. ^{3}H and ^{14}C are 'β' emitters only, with the ^{14}C emission 'spilling-over' into the ^{3}H channel and they were counted in the LKB Wallac Rackβeta or the Packard Tricarb 2000CA. If one isotope is mainly a 'β' emitter, e.g. ^{14}C, whilst the other is a

'γ' emitter, e.g. ^{22}Na or ^{51}Cr, then both 'β' and 'γ' counters have to be used. For the pairs $^{14}C/^{22}Na$ and $^{51}Cr/^{14}C$, the 'γ' emissions from ^{22}Na and ^{51}Cr were counted in the Packard Autogamma 800. However, this 'γ' emission also released electrons which could be picked up by the 'β' counters, with ^{22}Na and ^{51}Cr emission overlapping into the ^{14}C-channel. This was corrected for by counting single standards of ^{22}Na or ^{51}Cr (LKB Wallac Rackβeta) or by use of the external standards ratio (Packard Tricarb 2000CA). Quench correction was made by comparison to appropriate quench curves.

Radioactivity in duplicate experimental samples was counted for 10 minutes, with background radioactivity and overlap being taken into account each time. 1ml samples were used for gamma counting (i.e. 0.4ml perfusate/supernatant or 0.05ml plasma, made up to 1.0ml with distilled water). Aquasol (New England Nuclear) a universal LSC 'cocktail', was used as scintillant for beta emitting isotopes (i.e. 0.4ml perfusate/supernatant or 0.05ml plasma plus 0.35ml water, mixed with 4ml scintillant).

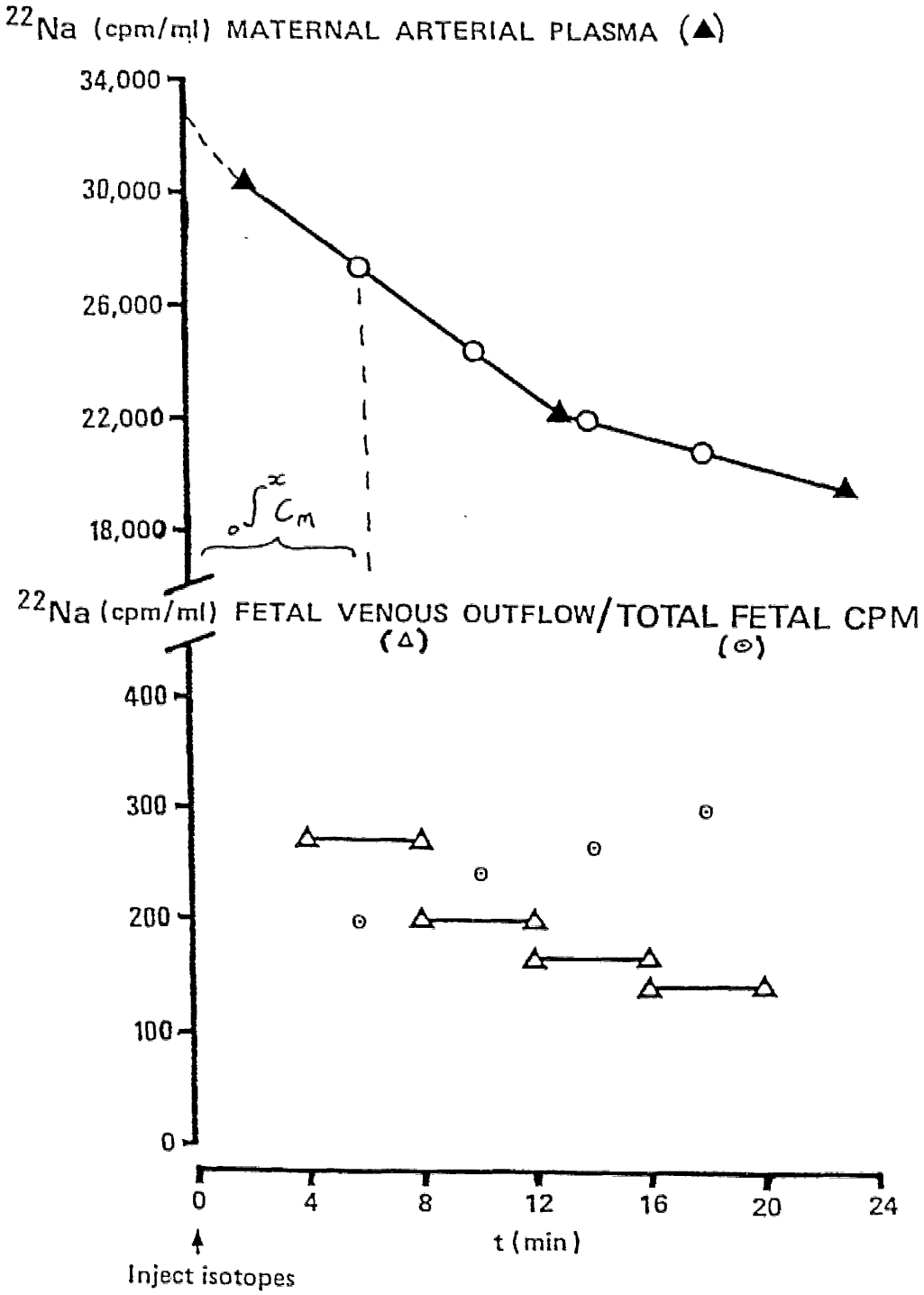
5) Calculations and Statistics

The unidirectional maternofetal clearance (K_{mf}) for each tracer was calculated as follows (see Fig.2.3). In the intact situation, K_{mf} was calculated from

$$K_{mf} = \frac{Nx}{W_o \int^x C_m(t)dt} \quad \mu l min^{-1} g^{-1} \qquad (5)$$

where N is the total radioactivity content of the fetus taken at x minutes from the time of tracer injection (shown as '⊙' in Fig.2.3), W is the wet weight of the placenta, $_o\int^x C_m$ is the time integral of radioactivity concentration in the maternal

Figure 2.3 An example of clearance (K_{mf}) calculation in the intact or 'in situ' perfused rat placenta.
The top half of the graph shows the decline in maternal arterial plasma radioactivity with time (▲-▲), extrapolated back to time zero using a computer programme. The bottom half of the graph shows the radioactivity concentration (cpm/ml) in the fetal venous outflow (Δ-Δ), from the perfused model and the total fetal cpm (⊙), from the intact model. K_{mf} can be calculated (see text), using the maternal radioactivty concentration at the mid-time point of perfusate collection (o), for the perfused model and the time integral of radioactivity concentration in the maternal plasma ($_0\int^x C_m$), for the intact model.



blood plasma (' ▲-▲ ' on Fig.2.3) from time 0 to x minutes. A computer programme written by Dr.W.G.Bardsley (Department of Obstetrics and Gynaecology) was used for this calculation. The ratio of K_{mf} for each molecule divided by its diffusion coefficient in water (D_w) was also calculated. For the perfused placentas K_{mf} was calculated from:

$$K_{mf} = \frac{[v]}{[A]\ W} \ . \ Q \quad \mu l min^{-1} g^{-1} \qquad (6)$$

where [v] is the concentration of radioisotope in the fetal venous outflow (' △-△ ' in Fig.2.3), Q is the perfusion flow rate, [A] is the tracer concentration in the maternal arterial blood shown as ' ▲-▲ ' on Fig.2.3 (extrapolated from the graph of isotope decay in the maternal blood, taken at the mid-time point of each perfusate collection, 'o' in Fig.2.3), and W is the wet weight of the placenta.

Data is shown in all cases as mean ± the standard error of the mean (s.e.m.), expressed per wet weight of placenta unless otherwise specified. Statistical comparisons have been made using the Student's 't' test, paired or unpaired as appropriate.

6) Electron Microscopy

In separate experiments from those described above, three control 21 day gestation rat placentas were prepared for electron microscopy by perfusion fixation for 20 minutes through the fetal circulation (without prior Ringer perfusion), with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.4. Three other placentas were initially perfused as normal with Krebs Ringer solution for 20 minutes and were then perfusion

fixed as above for a further 20 minutes. A final three placentas were perfused with Krebs Ringer for 1 hour, prior to fixation. The following procedure was carried out by Dr.C.Jones. The placentas were diced into 1mm^3 cubes and post fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer, pH 7.4, at 4°C for 1 hour. After rinsing in buffer, the tissue was dehydrated in graded alcohols and after two 15-minute changes of propylene oxide, was soaked in a 1:1 mixture of propylene oxide and Taab resin (Taab Laboratories Equipment Limited, Reading, UK) for 1 hour at room temperature. This was then replaced with a 1:3 propylene oxide/resin mixture overnight and the next day tissues were given 3 changes of fresh resin, embedded in gelatin capsules and polymerised at 60°C for 72 hours. Ultrathin sections of appropriate areas were cut on a Reichert OMU III ultramicrotome, mounted on uncoated grids and stained with uranyl acetate and lead citrate, prior to examination in a Philips 301 electron microscope, at an accelerating voltage of 60 KV.

SECTION C : RESULTS

1) Placental and fetal wet weights

The mean (± s.e.m.) wet weight of placentas and fetuses from animals used in this permeability study, on day 21 of gestation, was 0.54 ± 0.004g (n=524) and 3.42 ± 0.02g (n=522) respectively. There was a significant correlation ($p<0.01$) between placental and fetal wet weight.

2) Permeability of the intact placenta

As can be seen from Table 2.2, K_{mf} values for the tracers were generally related to their diffusion coefficients in water (D_w).

Table 2.2 K_{mf} and K_{mf}/D_w ratios for the intact rat placenta and K_{mf} for the perfused rat placenta (mean ± s.e.m.; n = number of placentas)

Isotope	D_w (37°C) $cm^2 sec^{-1}$		Intact placenta n	K_{mf} ($\mu l min^{-1} g^{-1}$)	K_{mf}/D_w ($cm g^{-1}$)	Perfused placenta n	K_{mf} ($\mu l min^{-1} g^{-1}$)
^{22}Na	17.0×10^{-6}	a	59	19.3 ± 0.69	19.0 ± 0.68	16	14.9 ± 1.13**
^{14}C-mannitol	9.9×10^{-6}	c	73	4.44 ± 0.17	7.48 ± 0.30	13	3.82 ± 0.35
^{14}C-sucrose	7.5×10^{-6}	d	93	4.33 ± 0.15	9.62 ± 0.33	8	3.83 ± 0.57
^{51}Cr-EDTA	7.0×10^{-6}	b	94	3.09 ± 0.13	7.37 ± 0.29	12	2.67 ± 0.35
^{3}H-inulin	2.6×10^{-6}	d	39	1.30 ± 0.07	8.31 ± 0.46	12	1.76 ± 0.35
^{125}I-albumin	0.91×10^{-6}	d	115	0.072 ± 0.006	1.33 ± 0.1	19	<0.05 e ±0.006

a Pappenheimer, 1953
b Paaske and Sejrsen, 1977
c Štulc et al, 1969
d Schneider et al, 1985
e value not corrected for presence of non-precipitable ^{125}I (see text)
** $p<0.01$ (unpaired 't' test) v. intact placenta.

Non-precipitable ^{125}I was found to have a mean 34.4 ± 2.7% (n=12) of the total ^{125}I present in the fetuses and the non-

precipitable ^{125}I counts in each fetus were subtracted from the total ^{125}I present before calculation of the K_{mf} for ^{125}I-albumin (Table 2.2). There was no correlation between time of harvesting of fetuses and K_{mf} for any tracer and it was therefore assumed that these measurements approached steady state values. K_{mf}/D_w values (Table 2.2) for ^{14}C-mannitol, ^{14}C-sucrose, ^{51}Cr-EDTA and ^{3}H-inulin were comparable, however K_{mf}/D_w for ^{22}Na was significantly ($p<0.01$) higher and for ^{125}I-albumin significantly ($p<0.01$) lower than for the other tracers.

3) Permeability of the perfused placenta.

a) Viability of preparation

Again animals were rejected if the mean maternal blood pressure fell below 65-70mmHg (8.6-9.2kPa). Also, a very high perfusion pressure suggests resistance to flow and was usually reflected in poor perfusate recovery (<70%) and the placenta often went white. Therefore, experiments were only accepted if maternal blood pressure was >65-70mmHg (8.6-9.2kPa), perfusate recovery was 95-100%, perfusion pressure was around 20mmHg (2.63 kPa) and the placenta was still bright red at the end. Table 2.3 summarizes some general physiological measurements made in the experiments that were used for this permeability study. The time from start of anaesthesia to start of perfusion was approximately 30 minutes. The 'start of experiment' values shown in Table 2.3 are those 3 minutes after injection of isotope and 'end of experiment' values are those obtained 20 minutes later. There was a slight, but significant ($p<0.05$) decline in haematocrit by the end of the experiments, probably due to blood

sampling and this was therefore kept to a minimum compatible with accurate measurement of K_{mf} (generally three maternal blood samples were taken during a 20 minute perfusion). Maternal blood pH and pCO_2 was unchanged and there was a slight, but significant increase in pO_2 ($p<0.001$) by the end of the experiments. Mean maternal blood pressure also decreased significantly during the experiments ($p<0.001$), which was a further reason for keeping blood sampling to a minimum. Perfusion pressures in the fetal circulation were relatively constant throughout (Table 2.3).

Table 2.3 Perfusion experiment parameters

(mean ± s.e.m.; n = number of animals)

Parameter	Start of experiment			End of experiment		
Maternal blood haematocrit(%) n = 15	34.4 ± 0.82			31.0 ± 1.0*		
	pH	pCO_2 (kPa)	pO_2 (kPa)	pH	pCO_2 (kPa)	pO_2 (kPa)
Maternal blood gases n = 27	7.314 ±0.008	6.17 ±0.19	12.2 ±0.26	7.325 ±0.01	5.67 ±0.29	14.6 ±0.43**
Maternal blood pressure n = 32 (kPa)	14.5 ± 0.43			10.9 ± 0.52**		
Perfusion pressure[a] n = 28 (kPa)	1.84 ± 1.8			2.20 ± 0.28		

* $p<0.05$) paired 't' test v. start of experiment
** $p<0.001$)

a. corrected for pressure drop between transducer and umbilical artery.

Figure 2.4 Ultrastructural appearance of the rat placenta after perfusion fixation through the fetal circulation (2.4a, mag.= x12,425); after 20 minutes Ringer perfusion, followed by fixation (2.4b, mag.= x2,375) and after 1 hour of Ringer perfusion followed by fixation (2.4c, mag.= x6,750). The numerals mark the three trophoblast layers numbered from the maternal lacuna (M) side towards the fetal capillary (F) side. E =fetal capillary endothelium.

Figure 2.4a

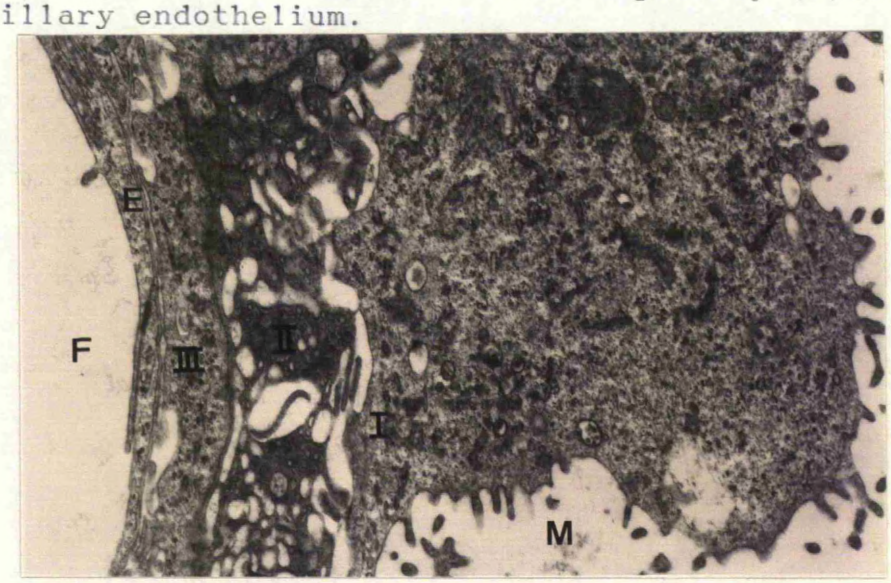


Figure 2.4b

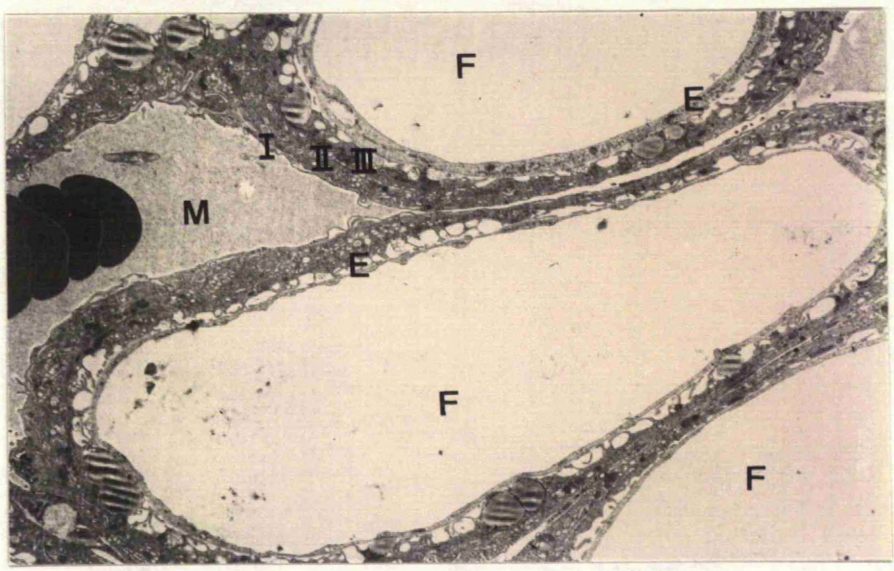
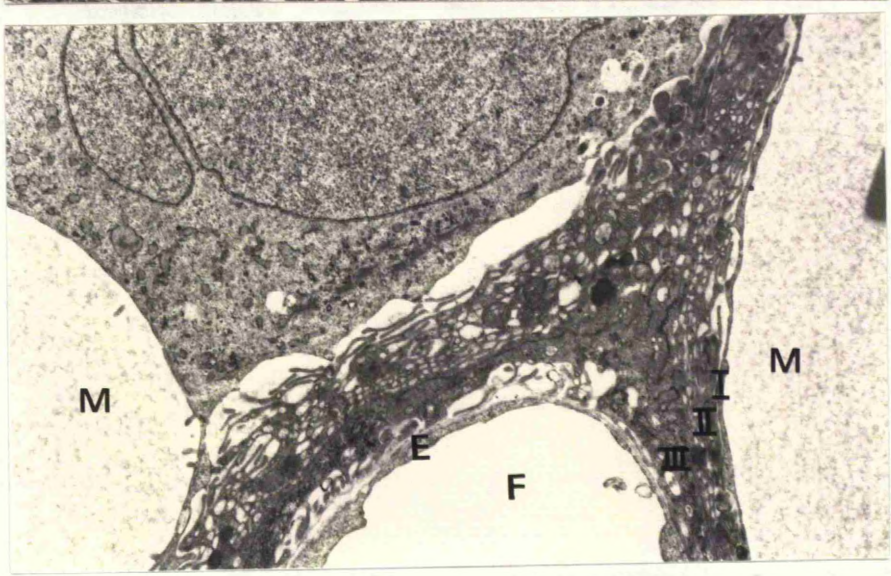


Figure 2.4c



The results of the electron microscopy performed by Dr.C.Jones are given in Figure 2.4 and show micrographs of the rat placenta perfusion-fixed without prior Ringer perfusion (Fig.2.4a), with 20 minutes of Ringer perfusion (Fig.2.4b) and with 1 hour of Ringer perfusion (Fig.2.4c). The three trophoblastic layers (I, II, III) were found to be intact in all cases, with no obvious clefts in the tissue.

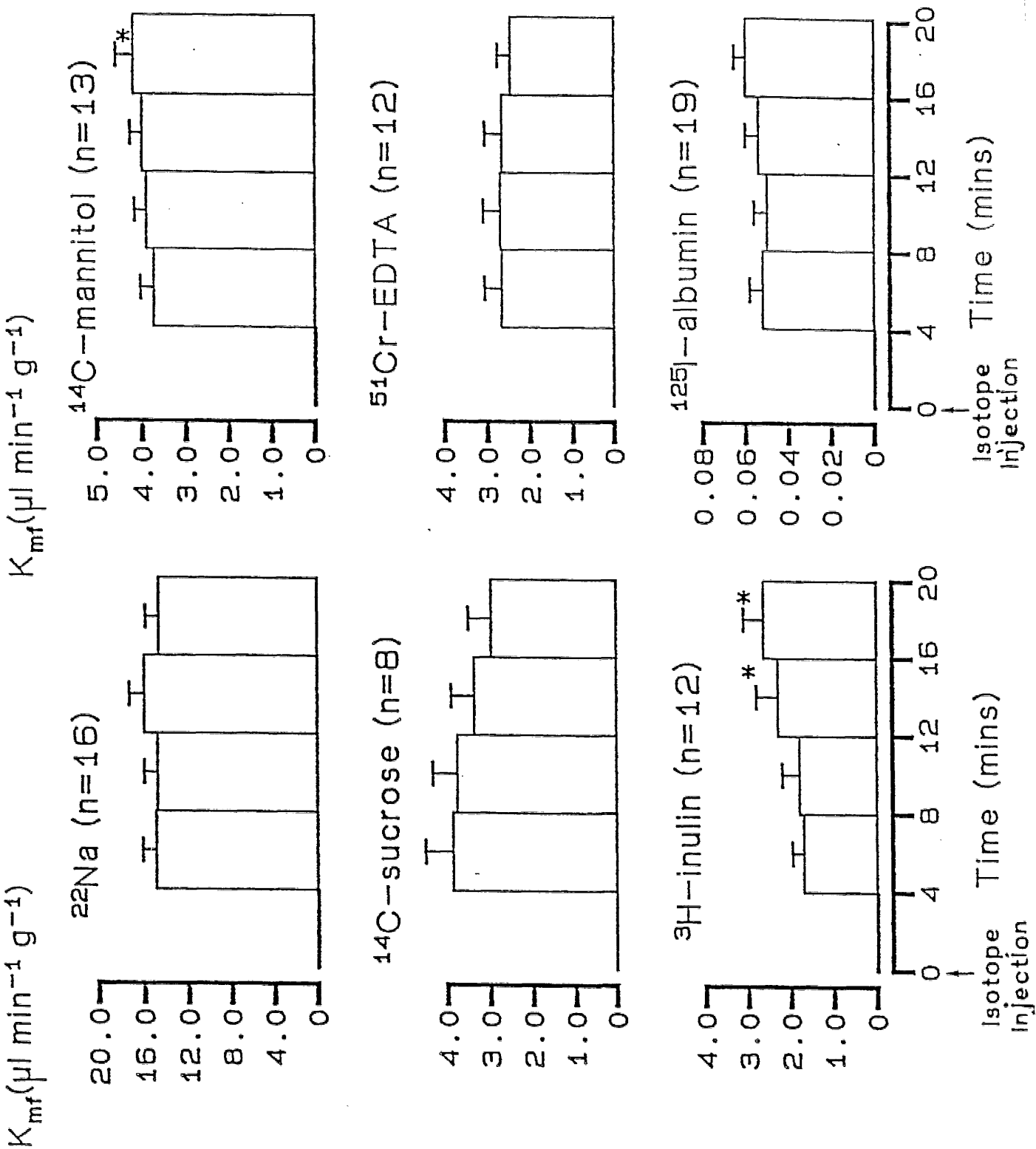
Mitochondria and other organelles were also well preserved in all three situations and no red blood cells were noted in the fetal circulation. In general, an excellent degree of ultrastructural preservation was found throughout, with no differences to that reported for the rat by other authors (e.g. Aoki et al,1978; Metz et al,1978).

b) Permeability measurements

Figure 2.5 shows the K_{mf} data for each tracer, for the four successive 4-minute perfusate collection periods. There was no significant difference (paired 't' test) between K_{mf} values obtained from the first collection period (4-8 min) compared to subsequent ones, for ^{22}Na, ^{14}C-sucrose, ^{51}Cr-EDTA and ^{125}I-albumin. For ^{14}C-mannitol and ^{3}H-inulin there were slight, but significant increases in K_{mf} towards the end of perfusion.

The further K_{mf} data from perfusion experiments as presented, was calculated using the mean K_{mf} of the first two 4-minute collection periods, to give the K_{mf} value for that individual placenta. No measurable ^{125}I was precipitable from the perfusates, suggesting that either only free ^{125}I crossed the placenta in these experiments or that the precipitate formed was

Figure 2.5 Unidirectional clearance (K_{mf}) across the perfused placenta in four successive perfusate collection periods. Note that this perfusion K_{mf} ^{125}I-albumin may be an overestimate (see text). Bars are mean ± s.e.m. (n = number of placentas).
* = $p<0.05$ v. 4-8 minute collection period (paired 't' test).



too small (in the absence of any other protein) to be centrifuged down. Thus, the perfusion K_{mf} ^{125}I-albumin must be regarded as an overestimate.

Table 2.2 shows the mean K_{mf} values for the perfused placentas; K_{mf} for Na^+ and ^{125}I-albumin was significantly ($p<0.01$) lower in the perfused placentas, compared to the intact placentas. The K_{mf}/D_w ratio for Na^+ was significantly higher than that for mannitol, EDTA and sucrose ($p<0.01$), but not inulin. The K_{mf}/D_w ratio for ^{125}I-albumin was significantly lower than that for all the other tracers ($p<0.01$).

4) Comparison of permeability in the intact placenta and with perfusion

Figure 2.6 shows K_{mf} data obtained from intact and perfused placentas in the same animal. One placenta from each animal was perfused and as several intact measurements were made using the remaining fetuses in each animal, these intact K_{mf} values were pooled and a mean K_{mf} value obtained for comparison to the perfusion K_{mf} for that animal. Paired Student's 't' test showed no significant difference between the two groups for any of the tracers.

5) Comparison of permeability in the intact situation, with or without a yolk sac placenta

Figure 2.7 shows K_{mf} data for intact placentas with or without the presence of a fully vascularized yolk sac. Paired Student's 't' test showed that cautery of the yolk sac vessels caused a small, but highly significant ($p<0.001$) reduction in the K_{mf} for ^{22}Na. Neither of the other two tracers investigated was affected.

Figure 2.6 Unidirectional clearance (K_{mf}) for each of three tracers across intact and perfused placentas in the same mother. Bars are mean ± s.e.m. (n = number of animals, see text). There were no significant differences (paired 't' test).

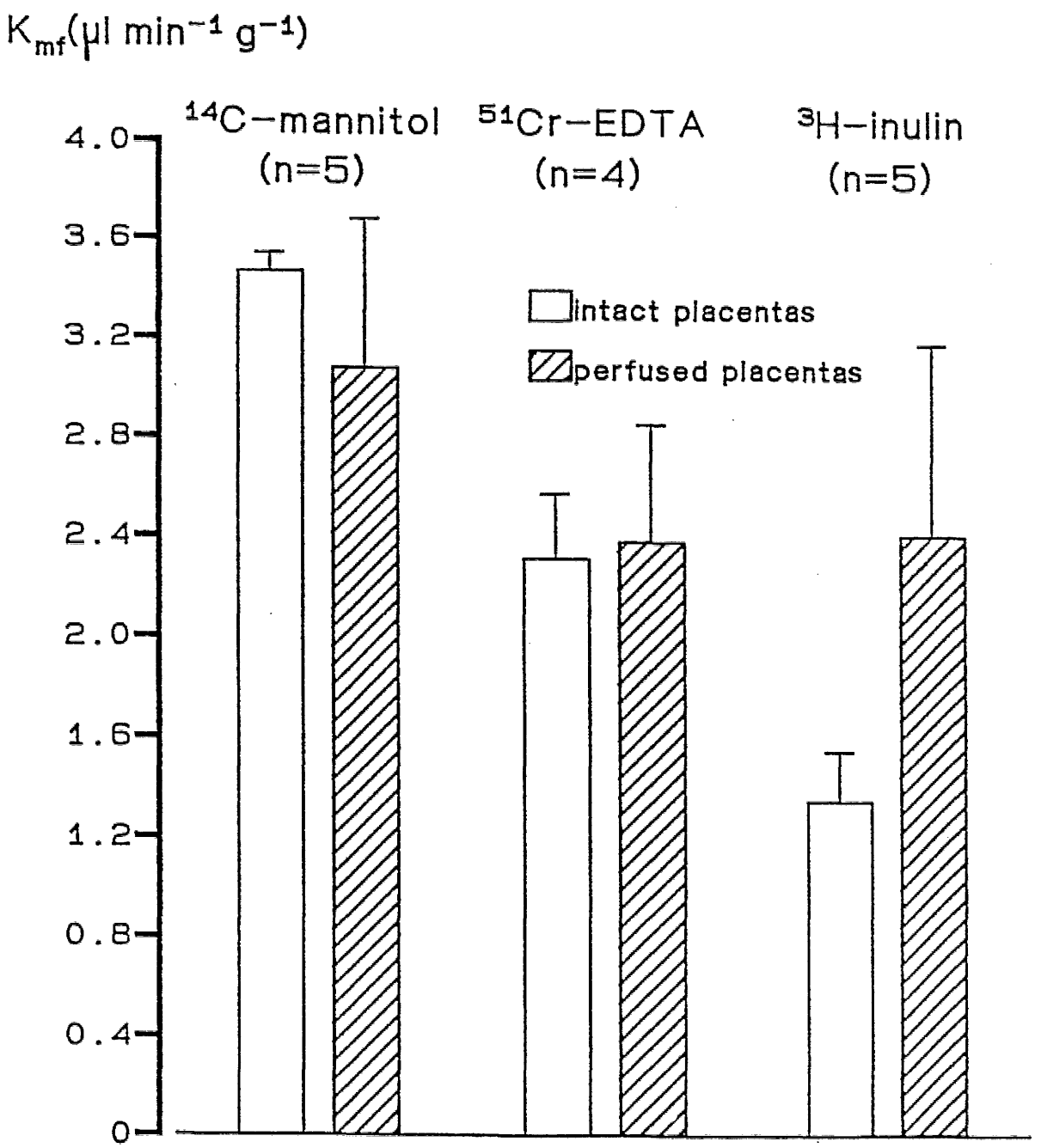
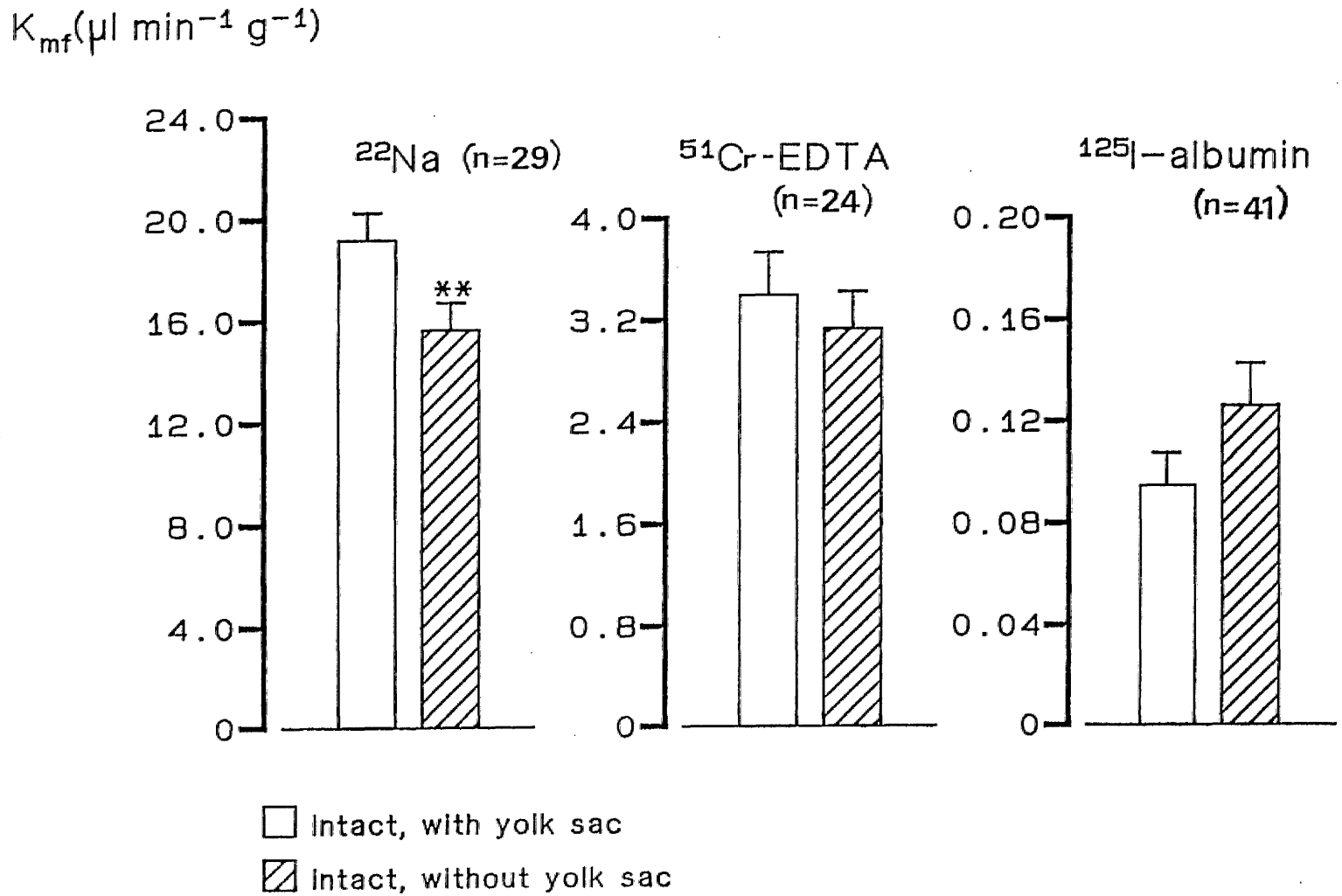


Figure 2.7 Unidirectional clearance (K_{mf}) for each of three tracers, across intact placentas and across intact placentas in which the vitelline vessels had been cauterized.
Bars are mean ± s.e.m. (n = number of placentas)
** = $p<0.001$ v. intact with yolk sac (paired 't' test).



SECTION D : DICUSSION

In a range of species studied at specific times in gestation, there is a close relationship between fetal growth, placental size and maternal uterine blood flow (pig - Wootton et al,1977; guinea-pig - Jones and Parer,1983; sheep - Harding et al,1985). In the rabbit, fetal weight, placental weight and placental blood flow are greatest in the position adjacent to the ovary (Bruce and Abdul-Karim,1973). In the rat, uterine artery ligation has been shown to reduce both placental blood flow, fetal and placental weights. Furthermore, a linear correlation has been demonstrated between rat fetal weight and placental weight (Gilbert and Leturque,1982). It was therefore not surprising, that there was a significant correlation ($p<0.01$) between placental and fetal wet weight in this study. The actual correlation coefficient was quite small however (0.34), suggesting that placental weight is a poor predictor of fetal weight on day 21 of gestation and vice versa.

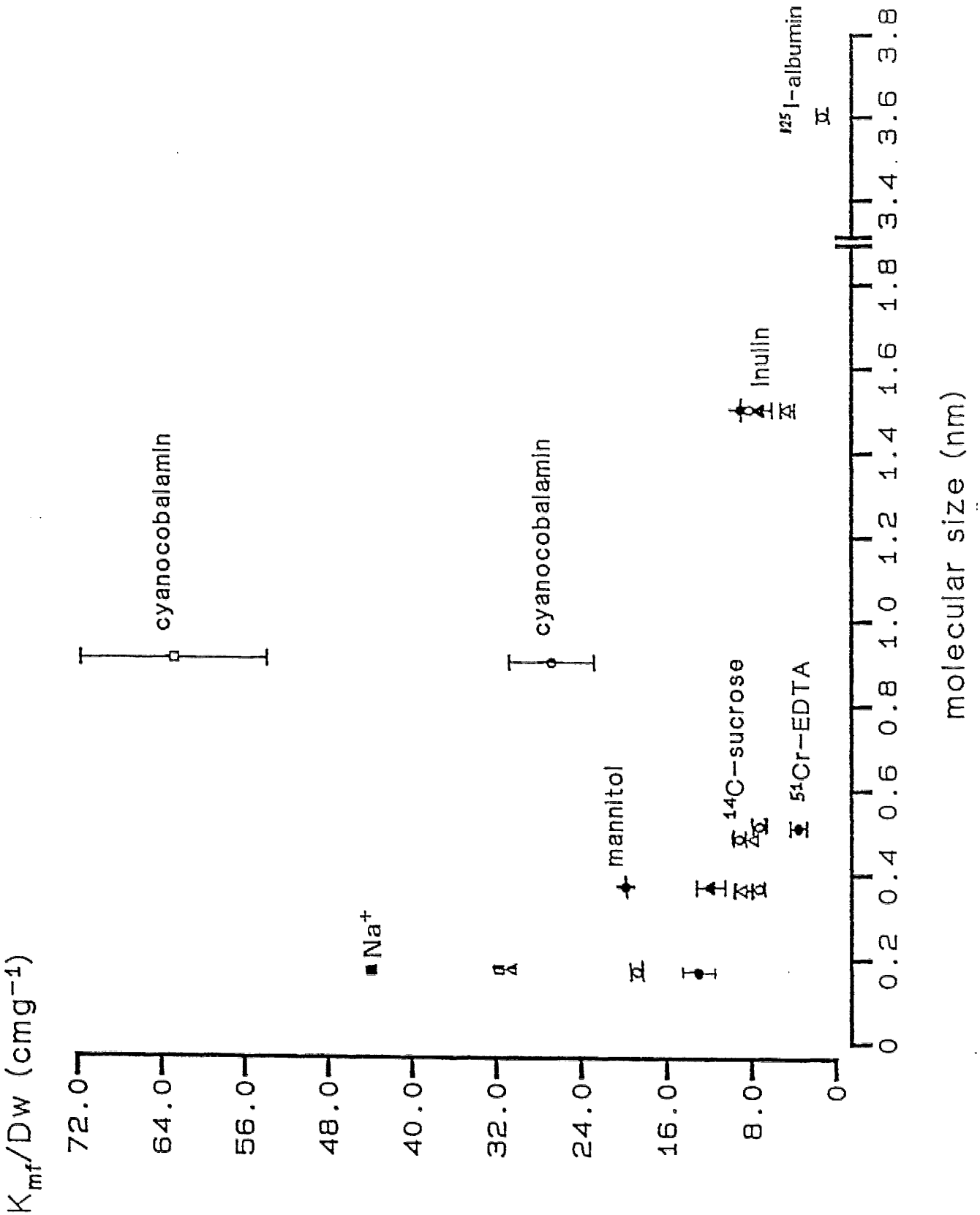
In the absence of flow limitation and of differential lipid solubility, the steady state K_{mf} value for each of a series of inert, electrically neutral, hydrophilic tracers passing across the placenta by simple diffusion through large water-filled channels, will equal its permeability surface area product and should be directly related to its diffusion coefficient in water (D_w). It follows that the ratio of K_{mf}/D_w will be a constant (related to the diffusion distance) irrespective of molecular size, if the above conditions are met. The data obtained here for the intact rat placenta, in general shows this relationship

between K_{mf} and D_w, as the K_{mf}/D_w ratios are similar for ^{14}C-mannitol, ^{14}C-sucrose, ^{51}Cr-EDTA and ^{3}H-inulin. However, K_{mf}/D_w for $^{22}Na^+$ appears to be at least twice that of these four tracers. This may be partly explained by sodium transfer across the yolk sac; there is some direct evidence for this in vitro (Chan and Wong,1978; Gibson and Ellory,1984) and in the 20% drop in K_{mf} ^{22}Na after vitelline vessel cautery (Fig.2.7). It could also be explained by electrical effects (Canning and Boyd,1984; Faber et al,1987), by transtrophoblastic as well as paracellular sodium transfer (Sibley et al,1985), by heterogenous pore size (Štulc et al,1969) or by any combination of these.

The intact placenta K_{mf} for ^{125}I-albumin may be an over-estimate, as precipitable ^{125}I counts may not be solely due to diffusional transfer of this tracer. Even so, K_{mf}/D_w for ^{125}I-albumin is markedly lower than that for all the other tracers and this is most likely to be due to restricted diffusion, i.e. the size of the albumin molecule (Einstein-Stokes radius =3.6nm) approaches that of the narrowest part of the water-filled channels through which diffusion probably occurs.

For comparison of the data here with that of Štulc and Štulcová (1986) and with that obtained for the intact guinea-pig and human placenta from several sources, the K_{mf}/D_w against molecular radius has been plotted in Fig.2.8. It can be seen that our results are close to those of Štulc and Štulcová (1986) and that there is considerable similarity between the three placental types. It may be concluded that the two extra trophoblast layers in the rat placenta have little effect on its permeability. This also suggests that transport occurs via wide

Figure 2.8 The ratio of the unidirectional clearance to the diffusion coefficient in water at 37°C (K_{mf}/D_w) for a number of tracers has been plotted against molecular radius. Data for three species, rat (■ Flexner et al,1948; △ Štulc and Štulcová,1986; ○ this study), guinea-pig (▲ Flexner et al, 1948; ▲ Thornburg and Faber,1977; ● Hedley and Bradbury,1980; ◑ Willis et al,1986), and human (◧ Flexner et al,1948; ✦ Bain et al,1988; □ Willis et al,1986) is shown, measurements made in intact placentas.



aqueous channels, having an estimated pore radius (Renkin,1954) of 15nm and a pore area per unit length of pores of 10cm (Štulc and Štulcová,1986).

There are two other points to note from Fig.2.8. Firstly, the K_{mf}/D_w values for sodium in all three species appear to be relatively high, probably for one or more of the reasons already described (although there is no yolk sac in the human at term). Secondly, the values for cyanocobalamin in the guinea-pig and human appear to be anomolously high; the reason for this is not clear, as Willis et al (1986) appeared to have taken adequate precautions to prevent artefact due to receptor-mediated transfer.

In general, the perfused rat placenta showed very similar permeability properties to the intact placenta, both by comparison of all data and from the experiments where intact and perfused measurements were made in the same animal. The fetal perfusate flow rate (0.5ml/min) was an arbitrary figure, because no measurements of fetal placental blood flow have been made. Bruce (1976) measured a maternal placental blood flow of 1.2 $ml\,min^{-1}\,g^{-1}$placenta in 22 day gestation rats, whilst Atkinson (unpublished) in our laboratory measured a maternal placental flow of 0.806 ± 0.3 $ml\,min^{-1}\,g^{-1}$placenta (n=8 rats) in 21 day gestation rats. Therefore, a flow rate slightly lower than these maternal placental flow measurements was chosen and it certainly did not seem to damage the placenta, as there was a good electron microscopic appearance of the placentas, even after an hour of Ringer perfusion through the fetal circulation. Consequently, the <u>in situ</u> perfused placental model seems to be a

good representation of the in vivo situation. However, as Figure 2.5 suggests, we may not have reached a steady state for placental transfer of inulin and perhaps mannitol, or it could be that this represents a small increase in permeability in some preparations as perfusion proceeds. Hedley and Bradbury (1980), using different animals for 'intact' and 'perfused' measurements, found marked increases in the permeability to most tracers studied (including ^{24}Na and ^{51}Cr-EDTA), when the guinea-pig placenta was perfused through the fetal circulation. The only effects of perfusion which we have found on the rat placenta, were a small (25%) decrease in K_{mf} for Na^+, which might easily be explained by the absence of the yolk sac when perfusing and, perhaps, a decrease in K_{mf} for ^{125}I-albumin. It is possible, though highly unlikely, that perfusion could decrease maternal placental blood flow sufficiently to exactly mask a perfusion induced increase in permeability for each tracer; further experiments are required to investigate this point. Why the rat placenta appears to be resistant to perfusional increase in hydrophilic permeability, whilst the guinea-pig placenta is not, are unclear at present.

There were some problems with the yolk sac cautery experiments, in that it was not possible to be certain that all the vitelline vessels had been cauterized. However, the significant fall in $K_{mf}{}^{22}Na$ and the lack of effect on $K_{mf}{}^{51}Cr$-EDTA and $K_{mf}{}^{125}I$-albumin, obtained after this procedure does suggest some sodium transfer via this route. This was backed up by the observation that $K_{mf}{}^{22}Na$ in the perfused placenta where the yolk sac is absent, was lower than that in the intact

situation.

Both the intact and the in situ perfused rat placental models, with their respective methods for calculation of placental clearance, have been used in the experiments to be described in the following chapters, where placental calcium transfer has been investigated in detail.

CHAPTER 3

CALCIUM TRANSFER ACROSS THE RAT PLACENTA

SECTION A : INTRODUCTION

Placental calcium transport has already been discussed in detail in Chapter 1 (Section D5). The mother seems capable of transporting calcium in large quantities across the placenta to the near term fetus against a concentration gradient (Bogert and Plass 1923; Chef,1969A). It is generally assumed, although not proven, that it is ionized calcium which is physiologically important in this transfer, since the protein-bound calcium is only poorly diffusible through plasma membranes (Racker,1980) and that low intracellular calcium levels are maintained throughout placental transfer. As already described, the detailed mechanisms involved in placental transfer are not clear, but the work of Twardock and Austin (1970) in the guinea-pig and Štulc and Štulcová (1986) in the rat, has demonstrated active placental calcium transfer.

This chapter reports work which was aimed at confirming and extending that of Štulc and Štulcová (1986) and in particular to compare calcium transfer across the intact and *in situ* perfused rat placenta. Thus, maternofetal calcium flux (J_{mf}) and clearance (K_{mf}) was measured in both preparations and compared with calcium accretion by the fetus, which gives a measure of net flux.

It has been reported that calcium transfer increases markedly in the last third of gestation (Feaster et al,1956; Romeu,1986; Romeu et al,1986) and this has been investigated here by using animals on two gestation dates (day 18 and 21, the day of mating being day 1). The possible role of the yolk sac in maternofetal

calcium transfer has also been studied. To confirm active carrier-mediated placental calcium transfer in the *in situ* perfused rat placenta, the effects of maternal body temperature and fetally administered metabolic inhibitors was investigated. Also, an attempt was made to analyse the kinetics of placental calcium transfer in the *in situ* perfused model, by measuring changes in maternofetal calcium flux with variations in maternal blood ionized calcium concentration.

Therefore, from these experiments we hoped to gain a good understanding of placental calcium transfer in the rat, prior to investigating the control of calcium transfer.

SECTION B : MATERIALS AND METHODS

1) Plasma calcium pools and calculation of maternofetal calcium flux

In previous studies it has been assumed that all ^{45}Ca injected into the maternal circulation is free to cross the placenta and that only the 'free' ^{45}Ca does actually cross. In this part of the study, these assumptions were tested.

Measurement of total calcium concentration (mM), using atomic absorption spectrophotometry and ionized calcium concentration (mM), using the ICA1 Radiometer (Copenhagen), was carried out on maternal plasma and blood respectively. Blood samples were collected at the end of most experiments reported in this thesis. From these measurements, the ratio of ionized to total plasma calcium concentration was calculated.

A Perkin-Elmer atomic absorption spectrophotometer was used to

measure total calcium concentration. This has a hollow cathode lamp for Ca^{2+}-Mg^{2+}-Zn^{2+} analysis, used at a wavelength for calcium of 422.7nm, with an air/acetylene flame. The instrument shows a sensitivity of about 0.08μg/ml calcium for 1% absorption. Substances that can interfere with the degree of sensitivity include silicon, aluminium, phosphate and sulphate. This interference can be reduced by diluting all samples immediately in 0.5% lanthanum chloride (Spectrosol, BDH Chemicals Ltd.) and using this solution as a 'blank' reference, instead of distilled water. Samples were measured against a calibration curve of calcium concentration versus absorption. Calcium standards were made from calcium nitrate solution (Spectrosol, BDH Chemicals Ltd.; 1ml≈1mg calcium, 24.9 mmol calcium per litre). Four standards were used, 0.1245mM (4.98μg/ml), 0.0996mM, 0.0747mM and 0.0498mM calcium; the curve being linear below 5μg/ml calcium. Maternal plasma samples were diluted 1:20 (i.e. 100μl up to 2ml) and were counted in triplicate. Ionized calcium concentration was measured immediately after blood collection into a Pasteur pipette, which had been previously aspirated in a recommended heparin solution (Radiometer, Copenhagen), this reduces the problem of chelation of the ionized calcium, whilst preventing clotting. The ionized calcium measurements had a reproducibility accurate to within 0.01mM, results having been corrected to pH 7.4 and 37°C (at lower pH values, the equilibrium for binding is altered, such that less calcium is bound).

To investigate whether ^{45}Ca was rapidly distributed between the three plasma calcium pools in the same way as 'cold'

calcium, ^{45}Ca was injected into mothers at time zero and then after 1 minute a 3ml maternal carotid blood sample was taken. The plasma was spun off and half of it was saved for total calcium analysis and ^{45}Ca content. The rest was pipetted into two ultrafiltration tubes (Centrifree Micropartition System, Amicon Ltd.) and spun for either 10 or 15 minutes, at 3000rpm and 25°C. The ultrafiltrate was saved for total calcium analysis and ^{45}Ca content.

At the same time, a placenta was perfused in the usual way and two 4-minute collections of perfusate were made. Similar to above, half of the perfusate sample was ultrafiltered and subsequently spun for 10 or 15 minutes. Total calcium and ^{45}Ca content was measured in the stock perfusate and ultrafiltrate. For the total calcium measurement, perfusate was diluted 1:10 in 0.5% lanthanum chloride and the plasma was diluted 1:20 or 1:40. Because of the small volume of sample available, it was often not possible to do duplicate measurements. From these experiments, the specific activity of calcium in the stock and ultrafiltrates of plasma and perfusate, have been calculated as $cpmmM^{-1}$ total calcium.

Initial experiments confirmed that ultrafiltration of stock ^{45}Ca and ^{125}I-albumin solutions in water, at 3000rpm, 25°C, for 10-15 minutes, gave a recovery of isotope in the ultrafiltrate of 99% and 3.4% respectively. This agrees well with the manufacturers specifications and other reports (D'Costa and Cheng,1983), that all 'free' material will be filterable, whilst protein-bound material will not. This data allowed analysis of the correct way to calculate maternofetal calcium flux.

2) Maternofetal calcium clearance measured in the 'in situ' perfused and intact placenta, and the effect of maternal body temperature

Female Sprague Dawley rats, maintained on a normal rat pellet diet, were anaesthetized on day 21 of gestation with 110mgkg^{-1} sodium thiobutabarbital (Inactin, BYK Gulden, Hamberg, FRG). In one group of animals, the fetal circulation of a placenta was perfused as described in Chapter 2. The Kreb's Ringer was modified, having a calcium concentration of 1.25mM, similar to maternal and fetal blood ionized calcium concentrations. 10μCi ^{45}Ca was injected into the maternal jugular vein at time zero. Three maternal carotid blood samples (0.5ml) were taken at 3, 13 and 23 minutes. Four 4-minute perfusate samples were collected at 4-8, 8-12, 12-16 and 16-20 minutes. The $K_{mf}{}^{45}Ca$ and the $K_{mf}{}^{45}Ca/D_w$ ratio were then calculated as described in Chapter 2 (equation 6), using the mean of the first two perfusate collections for each placenta (with 'n' therefore being the number of placentas, which is equivalent to the number of animals).

In another group of animals, $K_{mf}{}^{45}Ca$ was measured in intact placentas, using the method of Flexner and Pohl (1941), as described in Chapter 2. 10μCi ^{45}Ca was injected into the maternal jugular vein at time zero and two fetuses were taken (one from each horn) at 2, 3, 4 and 5 minutes. Several maternal carotid blood samples (0.5ml) were taken throughout the experiment. Preliminary experiments had shown that fetuses needed to be taken before 6 minutes, to ensure that fetal plasma ^{45}Ca concentration was less than 10% of maternal plasma ^{45}Ca concentration, i.e. when fetomaternal backflux is assumed to be

negligable. Each harvested fetus was homogenized in a Waring blender for 20 seconds with 2.5ml 0.5N $HClO_4$. The volume was made up to 10ml with $HClO_4$ and samples were then centrifuged for 10 minutes at 4000rpm, the supernatant being used for radioactivity analysis (see Chapter 2). Recovery of ^{45}Ca from fetal mince was 86.4 ± 4.3% (mean ± s.e.m., n=8) and this value was used to correct the experimental value of total fetal radioactivity content. $K_{mf}{}^{45}Ca$ and $K_{mf}{}^{45}Ca/D_w$ were calculated as described in Chapter 2 (equation 5). Because of the time required to perfuse and then harvest fetuses, these experiments unfortunately could not be carried out in the same group of animals.

Criteria for rejection of all experiments described in this chapter were as mentioned in Chapter 2, i.e. mean maternal blood pressure >65-70mmHg (8.6-9.2kPa), perfusion pressure of approx. 20mmHg (2.63kPa), perfusate recovery of 95-100% and bright red placentas at the end of the experiment.

In some experiments animals were kept warm only with a dissection lamp, resulting in a maternal body temperature ranging from 34-37°C. In later experiments, for reasons that will be made clear in the results, animals were placed on a metal hotplate as well, through which warm water was circulated. This ensured that the maternal body temperature could be accurately maintained at exactly 37°C or higher as required. Control and experimental animals within each group, were always at the same temperature and this is noted in each section.

A separate group of experiments was designed specifically to reveal the effect of maternal body temperature on maternofetal

calcium transfer. In these animals, the clearance of both ^{45}Ca and ^{51}Cr-EDTA (used as an extracellular marker) was measured. ^{51}Cr-EDTA is a 'γ' emitter (320keV), whilst ^{45}Ca emits both 'γ' (12keV) and 'β' (258keV) particles. Samples were counted in the Packard Autogamma 800, between 240-400keV, to collect ^{51}Cr-EDTA emission only. Then, they were also counted in the LKB Wallac Rackβeta or the Packard Tricarb 2000CA, to measure ^{45}Ca emission. ^{51}Cr-EDTA emissions overlap into the ^{45}Ca channel in these 'β' counters (but only with a low efficiency) and this spillover was measured each time by counting ^{51}Cr-EDTA standards or by using the external standards ratio. Before the isotopes were injected into the mother, the maternal rectal temperature was carefully monitored. Three groups of pregnant rats were taken on day 21 of gestation, for the measurement of $K_{mf}{}^{45}Ca$ and $K_{mf}{}^{51}Cr$-EDTA using the <u>in situ</u> perfused placental model (data being given as the mean K_{mf} of the first two perfusate collections). One group was maintained exactly at 34°C, the second group at 37°C and the third at 40°C. Similarly, another group of rats were taken for intact measurement of $K_{mf}{}^{45}Ca$ only, being maintained exactly at 37°C, for comparison with those intact measurements made in animals with maternal body temperatures ranging from 34-37°C (see above).

3) <u>Net calcium flux across the near term rat placenta</u>

Three female Sprague Dawley rats were taken on each of 3 consecutive days of gestation, i.e. the 19th, 20th and 21st day. The mothers were anaesthetized with ether and 8 fetuses were harvested from each animal, 4 from each horn. The fetuses were

weighed (wet) and then frozen, prior to total calcium analysis.

After thawing, each fetus was ashed by baking in a crucible in the Gallenkamp Hotspot furnace, at 700°C, for 8-10 hours. The ash was dissolved in 3ml 3N hydrochloric acid (HCl) and the solution was made up to 10ml with distilled water. This solution was then diluted 1:120 (i.e. 40μl up to 4.8ml) with 0.5% lanthanum chloride, ready for total calcium analysis by atomic absorption spectrophotometry. Samples were measured in triplicate and results were expressed as mg calcium per gram wet weight of fetus.

From the difference in total calcium accretion between day 19 and 20, or day 20 and 21, it was possible to estimate the net flux of calcium to the fetus on day 20 and 21 respectively. This is the net flux of calcium which occurs at a normal maternal body temperature of 37.5°C (Baker et al,1979). Unfortunately the placental weights for these experiments are not available, so the net calcium flux is expressed as nmolmin^{-1}g^{-1}fetus. However, it is possible to express this data as nmolmin^{-1}g^{-1}placenta, using the mean placental weight obtained (n=524) in the permeability study, in Chapter 2 (i.e. 0.54 ± 0.004g).

4) Role of the yolk sac in calcium transfer

In a set of intact measurements of $K_{mf}{}^{45}Ca$, one uterine horn was used as a control, whilst in the other horn the vitelline artery and vein supplying each conceptus was cauterized using electrocautery without opening the uterus. Maternal blood samples and fetuses were obtained and prepared for analysis as described previously (Chapter 2). In these animals, the maternal

body temperatures ranged from 34-37°C.

5) Maternofetal calcium clearance on day 18 of gestation

Female Sprague Dawley rats were taken on day 18 of gestation and the fetal circulation of a placenta was perfused as described previously (Chapter 2). ^{45}Ca and ^{51}Cr-EDTA (used as an extracellular marker) were injected into the maternal jugular vein at time zero. Three maternal carotid blood samples and four 4-minute perfusate collections were taken. The K_{mf} ^{45}Ca and K_{mf} ^{51}Cr-EDTA were calculated as described previously, for direct comparison with data obtained on day 21 of gestation (using the mean K_{mf} of the first two perfusate collections). The animals in this study were maintained accurately at a maternal body temperature of 37°C.

6) The effect of perfusate flow rate on maternofetal calcium transfer

Fetal perfusion of placentas was carried out as normal, but the perfusate flow rate was altered. Four animals were used with a control flow rate of 0.5mlmin^{-1}, another four animals had a reduced flow rate of 0.25mlmin^{-1}, whilst three others had an increased flow rate of 0.74mlmin^{-1}. Both ^{45}Ca and ^{51}Cr-EDTA were injected into the mother and the clearance of these markers across the placenta was calculated (using the mean K_{mf} of the first two perfusate collections). These animals had maternal body temperatures ranging from 34-37°C.

7) The effect of potassium cyanide on maternofetal calcium transfer

Again, the in situ perfused rat placental model was used to measure $K_{mf}{}^{45}Ca$ and $K_{mf}{}^{51}Cr$-EDTA, but in the presence of potassium cyanide (KCN). The isotopes were injected at time zero and two 4-minute control perfusate collections were made (i.e. A and B). Then either 1.0mM (final concentration) KCN (BDH Chemicals Ltd.) or saline diluent, was introduced into the perfusate via a side-arm in the perfusion tubing, at a rate of $0.05ml min^{-1}$, for 28 minutes. Because of the lag time between the start of infusion and KCN entering the umbilical artery, the next two perfusate collections were not analysed (i.e. C and D). Then, the following five 4-minute collections were analysed (i.e. E-I), in order to see whether KCN or its diluent had any effect on $K_{mf}{}^{45}Ca$ or $K_{mf}{}^{51}Cr$-EDTA. The percentage change in K_{mf} with KCN or saline diluent, from control values, for each of the five experimental collections, was calculated. Data was also expressed as the mean K_{mf} of the first two perfusates (for the control K_{mf}), for comparison with the mean K_{mf} of the 5 perfusate collections with KCN or diluent. In a few experiments, several perfusates were analysed after the removal of KCN from the perfusion fluid, to check for any signs of recovery. These animals had maternal body temperatures ranging from 34-37°C.

8) The effect of acute maternal blood ionized calcium manipulation on maternofetal calcium clearance and flux

Placental perfusion was carried out as normal using 21 day gestation rats. Variation in maternal blood ionized calcium concentration was achieved by infusion throughout the experiment

into the maternal jugular vein, of either 10%, 7.5% or 5% calcium gluconate, 25-50mg/ml ethylenediaminetetra-acetic acid (EDTA) in 0.9% saline, or 0.9% saline, at a rate of 0.018 $ml min^{-1}$. Maternal infusion was started approximately 15 minutes before injection of ^{45}Ca and ^{51}Cr-EDTA into the maternal jugular vein, ensuring that a steady state maternal blood ionized calcium concentration had been achieved. Four 4-minute perfusate collections were made at 4-8, 8-12, 12-16 and 16-20 minutes. Three maternal carotid blood samples (0.5ml) were taken for radioisotope analysis at 3, 13 and 23 minutes. Also, four small (200μl) maternal carotid blood samples were taken at 6, 10, 14 and 18 minutes (i.e. at the mid-perfusate collection time) for ionized calcium measurement (mM; ICA1 Radiometer, Copenhagen). Each perfusate K_{mf} value was used as a single point for data analysis, with its associated maternal blood ionized calcium concentration. J_{mf} calcium was also calculated, using a method discussed later (Section C1). This group of animals were maintained accurately at 37°C throughout the experiment.

Regression analysis was performed between maternal blood ionized calcium concentration and K_{mf} ^{51}Cr-EDTA, K_{mf} ^{45}Ca and J_{mf} calcium. Finally, the calcium flux data was expressed as a Lineweaver Burk plot, the 'best-fit' line being derived using a computer programme. The Lineweaver Burk plot makes use of the fact that the reciprocal of the equation of a rectangular hyperbola, is the equation of a straight line (see Morris,1968). It allows the determination of values for K_m (i.e. Michaelis constant/affinity constant; the substrate concentration at which the response is $\frac{1}{2}V_{max}$) and V_{max} (i.e. the maximum initial

response/velocity of response).

Hence, $1/R = K_m/V_{max} \cdot 1/[S] + 1/V_{max}$ (7)

This is the equation of a straight line, crossing the y axis at $1/V_{max}$ and the x axis at $-1/K_m$, with 'R' being the response, [S] being the substrate concentration and K_m/V_{max} being the gradient of the line. Therefore, by plotting $1/J_{mf}$ calcium (1/R) versus 1/maternal blood ionized calcium concentration (1/[S]), one can calculate K_m and the V_{max} of maternofetal calcium transfer in this system.

9) Statistics

All data is given as the mean ± s.e.m., expressed per wet weight of placenta unless otherwise specified. Statistical comparisons were made using the Student's 't' test, unpaired or paired as appropriate.

SECTION C : RESULTS

1) Unidirectional maternofetal clearance and flux of calcium across the rat placenta

The mean cumulative maternal plasma total calcium and maternal blood ionized calcium concentration from a selection of experiments reported in this thesis, was 1.97 ± 0.04 and 1.11 ± 0.02 mM (n=297) respectively. From this, the calculated mean ratio of ionized to total calcium concentration in maternal blood, was 58.0 ± 0.71 % (n=297). This ratio was not significantly different between any of the various experimental groups studied. It is noteworthy, that the mean fetal blood

ionized calcium concentration (as measured in experiments to be described in Chapter 4 and 5), was 1.42 ± 0.02 mM (n=22), consequently there was an 'uphill' maternofetal ionized calcium concentration gradient.

Table 3.1 : Total, ultrafilterable and ionized calcium concentrations in maternal blood and fetal perfusate, including specific activities (mean ± s.e.m.; n = number of animals)

Calcium species measured	Maternal plasma (n=13)	Fetal perfusate (n=8)
Total calcium (mM)	1.65 ± 0.07	1.34 ± 0.009
Ultrafilterable (UF) calcium (mM)	1.08 ± 0.04	1.24 ± 0.015
Ionized calcium (mM)	1.04 ± 0.02	1.11 ± 0.007
% UF/Total calcium	66.6 ± 3.5	92.3 ± 1.1
% Ionized/Total calcium	64.9 ± 3.4	83.1 ± 0.59
Specific activity of ^{45}Ca ($cpmmM^{-1}$ total calcium) In stock sample	353.0 ± 29.8	8.66 ± 1.0
In UF sample -after 10 min.	349.9 ± 30.0	10.4 ± 1.2
-after 15 min.	348.4 ± 24.1	10.2 ± 1.2

Table 3.1 shows the data for total, ultrafilterable and ionized calcium concentrations in maternal plasma and fetal perfusate, in experiments where the specific activity of calcium was also calculated. This gives an indication of whether ^{45}Ca is rapidly distributed between the 3 pools of 'cold' calcium. The ratio of total to ionized calcium concentration in maternal blood in this group of experiments was similar to the cumulative data given above, even though the total calcium concentration

was rather low. The specific activity of ^{45}Ca did not change significantly when comparing stock plasma to the ultrafiltrate, suggesting that ^{45}Ca rapidly equilibrates between the three plasma pools of 'cold' calcium. A significant rise in the specific activity of ^{45}Ca in ultrafiltered plasma would have suggested that more ^{45}Ca distributes only in the 'free' calcium fraction. A fall in specific activity would have suggested that ^{45}Ca is more avidly bound to proteins than 'cold' calcium.

In the perfusate, measured total calcium concentration was 1.34mM (Table 3.1) and the ultrafilterable fraction was somewhat lower than this, suggesting some high molecular weight binding. The perfusate ionized calcium concentration was slightly lower than the ultrafilterable calcium concentration, suggesting some binding to inorganic substances, phosphates, etc.; this being a common occurrence in most physiological buffers (McKercher et al,1982). However, the ratios of ultrafilterable and ionized calcium to total calcium are high, suggesting that protein-bound calcium does not cross the placenta in large amounts. As with plasma, the specific activity of ^{45}Ca did not significantly change from the stock perfusate to the ultrafiltrate. This again suggests that ^{45}Ca rapidly distributes between the various pools of calcium present, the difference here (cf. plasma) being that most of the calcium is ultrafilterable, with only a small protein-bound fraction.

This data strongly confirms that it is the ultrafilterable, mainly ionized calcium moiety, which is important in placental transfer. Also, that ^{45}Ca once injected into the maternal circulation, rapidly distributes between the three pools of

plasma 'cold' calcium. In these experiments, this equilibration was complete within about 15 minutes (i.e. blood samples being taken 1 minute after isotope injection, plasma removed in approx. 1 minute and centrifugation started by 2-3 minutes, followed by a 10-15 minute spin), which is well within the time course of the shortest experiment that is reported here.

Thus, not all of the ^{45}Ca present in maternal blood is available for placental transfer. Hence, clearance of calcium should be calculated using the concentration of 'free' ^{45}Ca in maternal blood, rather than the total ^{45}Ca concentration and in order to calculate maternofetal calcium flux (for the perfused preparations) the following equation should be used :

$$J_{mf}\ \text{calcium} = \frac{[v]}{[*A]} \cdot \frac{Q}{W} \cdot M_i \quad \text{nmol min}^{-1}\text{g}^{-1} \qquad (8)$$

Where [v] is the concentration of ^{45}Ca in the fetal venous perfusate outflow (cpm ml^{-1}), Q is the perfusion flow rate (ml min^{-1}), [*A] is the concentration of 'free' ^{45}Ca (i.e. not protein-bound) in maternal arterial blood (cpm ml^{-1}), W is the wet weight of the placenta (g) and M_i is the maternal blood ionized calcium concentration (mM). Maternal ionized calcium is used rather than total plasma calcium concentration, as again it is the ionized calcium which is the important species in placental transfer. Most reports in the literature have not taken into consideration that only a proportion of the ^{45}Ca is available for transfer. It was not possible to measure the distribution of ^{45}Ca in maternal plasma in every experiment, therefore for ease of comparison with the literature, $K_{mf}{}^{45}Ca$ in this thesis has been calculated as described previously (Chapter

2; equation 6), i.e. using the total ^{45}Ca concentration in maternal plasma. In most studies, where there was no difference in maternal plasma ionized calcium concentration between experimental groups, $K_{mf}{}^{45}Ca$ was an adequate simple measure of calcium transfer. Nevertheless, in some experiments it was necessary to calculate maternofetal calcium flux (J_{mf}).

As described previously, the data in Table 3.1 shows that ^{45}calcium distributes between bound and free fractions in maternal plasma, exactly as 'cold' calcium does. Therefore, because the distribution of ^{45}Ca was not measured in every experiment, the mean ionized to total plasma calcium concentration ratio, 58.0 ± 0.71%, obtained from a very large sample (297 pregnant rats), was used to correct the maternal plasma ^{45}Ca concentration for bound ^{45}Ca, consequently equation 8 becomes :

$$J_{mf}\ \text{calcium} = \frac{[v]}{[A]\ .\ 0.58}\ .\ \frac{Q}{W}\ .\ M_i \quad \text{nmolmin}^{-1}\text{g}^{-1} \qquad (9)$$

Where [A] is the total ^{45}Ca concentration in maternal arterial blood and 0.58 is the ratio of ionized to total maternal plasma calcium. Equation 9 has thus been used for J_{mf} calcium calculation throughout this thesis.

The $K_{mf}{}^{45}Ca$ measured in the <u>in situ</u> perfused and intact rat placenta (in animals with a maternal body temperature between 34-37°C) was 57.0 ± 3.3 (n=43) and 46.7 ± 1.8 (n=60) μlmin^{-1}g^{-1} respectively. Unfortunately, the maternal blood ionized calcium concentration was not measured in the intact placenta study, however, it is assumed that it was not significantly different to that measured in the perfused placenta study (1.06 ± 0.018mM, n=42). The $K_{mf}{}^{45}Ca$ in the perfused placenta was thus

significantly higher ($p<0.01$) than in the intact, despite the lack of a yolk sac in this preparation. In both preparations a steady state seemed to have been achieved, as there were no differences in $K_{mf}{}^{45}Ca$ with time. Figure 3.1 shows the graph of K_{mf}/D_w ratio versus the molecular radius for a range of hydrophilic molecules, measured in the intact and perfused placenta (see Chapter 2; animals having maternal body temperatures ranging from 34-37°C), with the K_{mf}/D_w ratio for calcium added to it. It is clear that the K_{mf}/D_w ratio for calcium is significantly higher than for all the other molecules studied. It is noteworthy, that if the concentration of 'free' ^{45}Ca in maternal arterial plasma had been used instead, then the K_{mf}/D_w ratio for calcium would have shown an even greater difference from that of the other hydrophilic molecules investigated.

Figure 3.2 shows the data from perfused placentas for $K_{mf}{}^{51}Cr$-EDTA and $K_{mf}{}^{45}Ca$ ($\mu l min^{-1} g^{-1}$), measured with the animals maintained exactly at three different maternal body temperatures. There was a significant increase in $K_{mf}{}^{45}Ca$ at both 37°C ($p<0.05$; $K_{mf}{}^{45}Ca = 76.0 \pm 10.9$) and 40°C ($p<0.01$; $K_{mf}{}^{45}Ca = 97.9 \pm 12.0$), when compared to 34°C ($K_{mf}{}^{45}Ca = 48.7 \pm 2.9 \mu l min^{-1} g^{-1}$). However, the $K_{mf}{}^{45}Ca$ at 40°C was not significantly different from that at 37°C, and $K_{mf}{}^{51}Cr$-EDTA was not affected by any of these temperature changes. Similarly, measurement of $K_{mf}{}^{45}Ca$ in intact placentas, showed a significant increase ($p<0.001$) from 46.7 ± 1.8 (n=60; with maternal body temperatures ranging from 34-37°C) to 142.5 ± 5.2 $\mu l min^{-1} g^{-1}$ (n=50) at 37°C. Maternal ionized calcium concentration did not

differ from one experimental group to another (i.e. perfused data at 34°C =1.04 ± 0.02, 37°C =1.01 ± 0.04, 40°C =1.02 ± 0.03; intact data at 37°C =0.97 ± 0.05 mM). It is noteworthy, that with maintenance of maternal body temperature at exactly 37°C, comparison of $K_{mf}{}^{45}Ca$ in the perfused and intact preparations now shows a significantly higher value in the intact case ($p<0.05$). It is now possible to quote the cumulative data for measurements of $K_{mf}{}^{45}Ca$ in the perfused placenta at 37°C, i.e. 62.8 ± 2.44 $\mu l min^{-1} g^{-1}$ (n=83). This converts into a J_{mf} calcium (using equation 9) of 116.3 ± 3.86 $nmol min^{-1} g^{-1}$ (n=83; maternal blood ionized calcium = 1.09 ± 0.01mM), compared to the J_{mf} calcium measured in intact placentas, at 37°C, of 236.2 ± 8.0 $nmol min^{-1} g^{-1}$ (n=50).

Figure 3.1 The K_{mf}/D_w ratio for a range of hydrophilic molecules measured in the intact and perfused rat placenta on day 21 of gestation (mean ± s.e.m.)

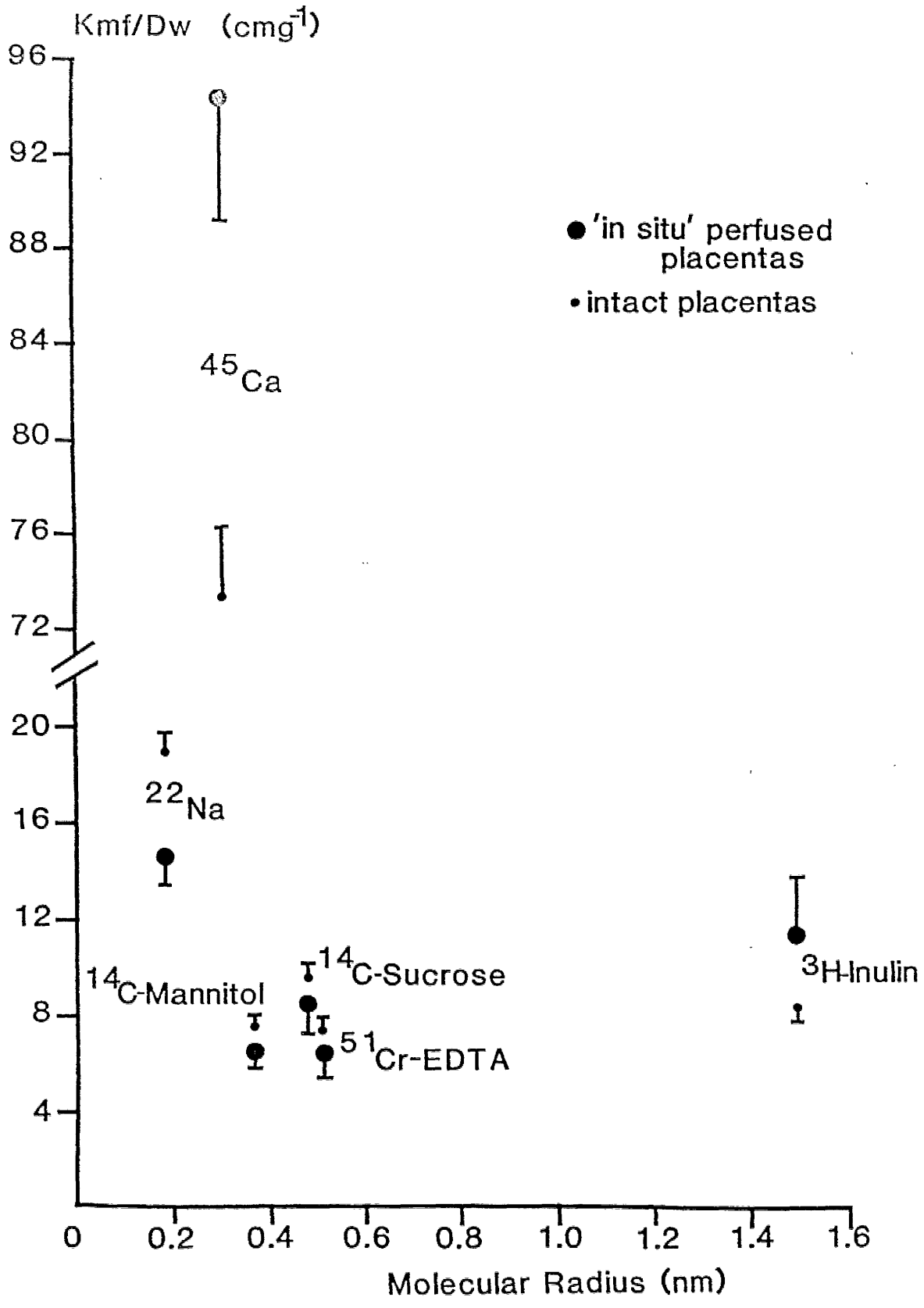
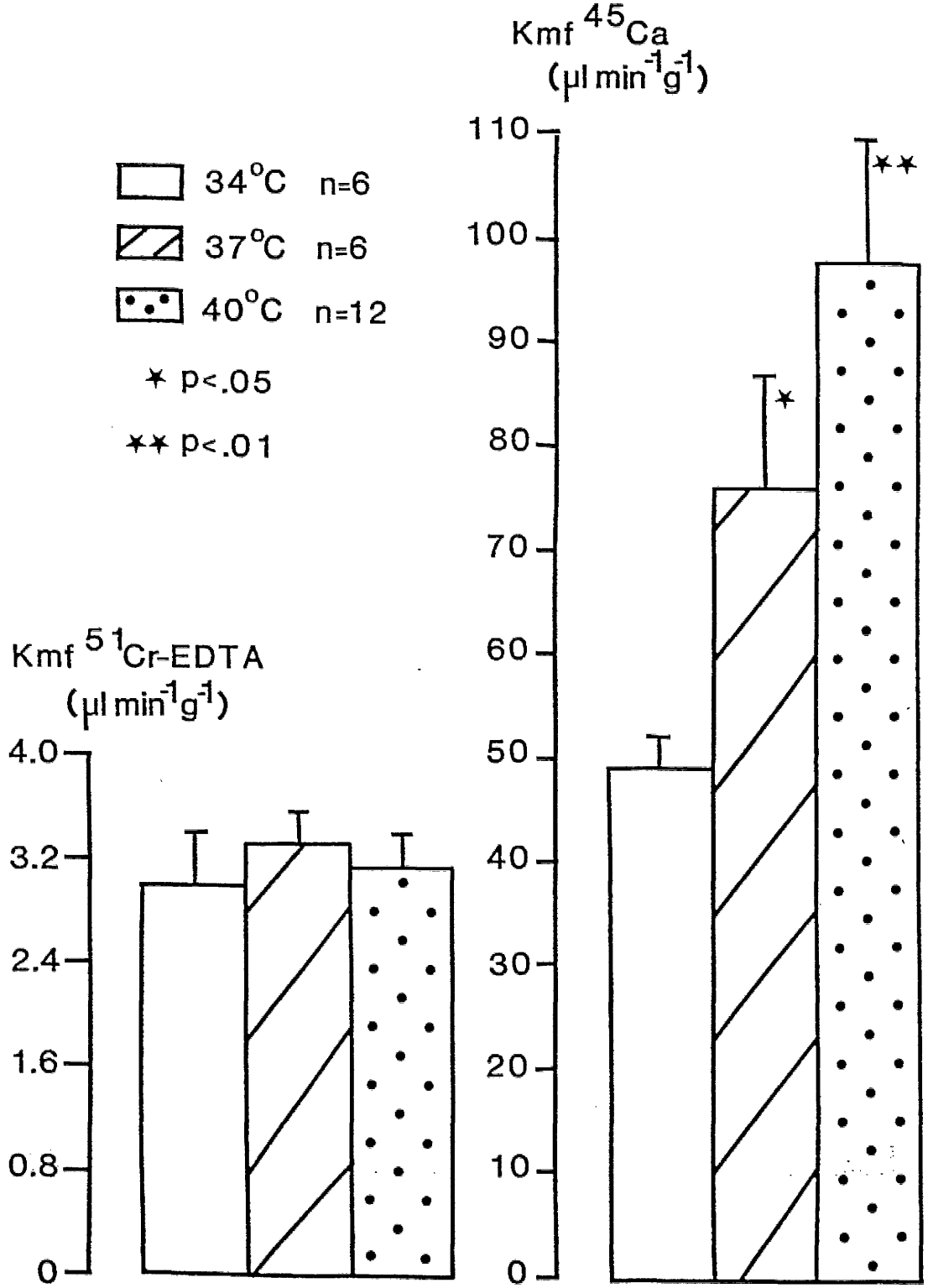


Figure 3.2 The effect of maternal body temperature on maternofetal calcium transfer across the perfused rat placenta on day 21 of gestation. Bars are given as mean ± s.e.m. An unpaired Student's 't' test, compared data at 37°C or 40°C, with that at 34 °C. $K_{mf}{}^{45}Ca$ at 40°C was not significantly different from $K_{mf}{}^{45}Ca$ at 37°C. (n=number of placentas)



2) Net calcium flux across the rat placenta

Table 3.2 shows the total fetal calcium accretion (mg calcium g^{-1} fetus), on days 19, 20 and 21 of gestation and the calculated net flux ($nmolmin^{-1}g^{-1}$), on day 20 and 21. There is clearly a marked increase in net calcium flux from day 20 to 21. We could only express net flux per gram of placenta for day 21 and this can thus be compared to J_{mf} calcium measured in intact and perfused placentas at 37°C.

Table 3.2 : Fetal calcium accretion on three consecutive days of gestation and the calculated net calcium flux across the rat placenta on day 20 and 21 (mean ± s.e.m.; n = number of fetuses; using the mean placental wet weight of 0.54 ± 0.004g, n=524, see Chapter 2)

Parameter	Gestation date 19	20	21
Fetal calcium accretion (mg calcium g^{-1} fetus)	0.56 ± 0.02	1.00 ± 0.02	1.89 ± 0.04
Net calcium flux			
$nmolmin^{-1}g^{-1}$fetus	-	7.65	15.5
$nmolmin^{-1}g^{-1}$placenta	-	-	128.7

3) Role of the yolk sac in calcium transfer

The intact $K_{mf}{}^{45}Ca$ measurements with or without the presence of a yolk sac placenta (in rats at between 34-37°C), were 47.7 ± 3.1 and 36.0 ± 3.2 $\mu l min^{-1}g^{-1}$ (n=25) respectively. There was a significant fall ($p<0.01$; paired Student's 't' test) in the $K_{mf}{}^{45}Ca$ without a viable yolk sac. This was similar to the result found for sodium (see Chapter 2). Maternal blood ionized

calcium concentration was unfortunately not measured in this study.

4) Gestational changes in maternofetal calcium transfer

Perfusion of the rat placenta on day 18 of gestation was very difficult, because the umbilical vessels were very fragile and consequently the results should be viewed with some caution. Figure 3.3 shows the comparison of K_{mf} ^{51}Cr-EDTA and K_{mf} ^{45}Ca measured on day 18 and day 21 of gestation (rats maintained exactly at 37°C throughout the experiment). There was a significant increase ($p<0.0001$) in the clearance of both ^{51}Cr-EDTA and ^{45}Ca between day 18 and 21. K_{mf} ^{51}Cr-EDTA increased by 104.9%, whilst K_{mf} ^{45}Ca increased by 930.0%. The maternal blood ionized calcium concentration was significantly higher in the 18 day gestation animals ($p<0.05$; 18day =1.10 ± 0.02mM, 21day =1.01 ± 0.04mM). However, this will only slightly alter the differences seen between the two gestation dates for J_{mf} calcium (J_{mf} calcium, 18 day = 15.3 ± 2.9, 21 day = 126.7 ± 13.0 nmolmin^{-1}g^{-1}; calculated using equation 9).

5) The effect of perfusate flow rate on K_{mf} ^{51}Cr-EDTA and K_{mf} ^{45}Ca

Table 3.3 shows that the raising or lowering of fetal perfusate rate outside the usual range (ie: ≈0.5mlmin^{-1}), had no significant effect on K_{mf} ^{51}Cr-EDTA or K_{mf} ^{45}Ca. Maternal blood ionized calcium concentration was similar in the three groups (see Table 3.3). However, it should be acknowledged that the size of the experimental groups in this study are small.

Figure 3.3 Gestational changes in $K_{mf}{}^{45}Ca$ and $K_{mf}{}^{51}Cr$-EDTA (mean ± s.e.m.). Unpaired Student's 't' test showed a significant ($P<0.0001$) increase in both measurements, when comparing day 18 to day 21 (g.a.=gestational age; n=number of placentas)

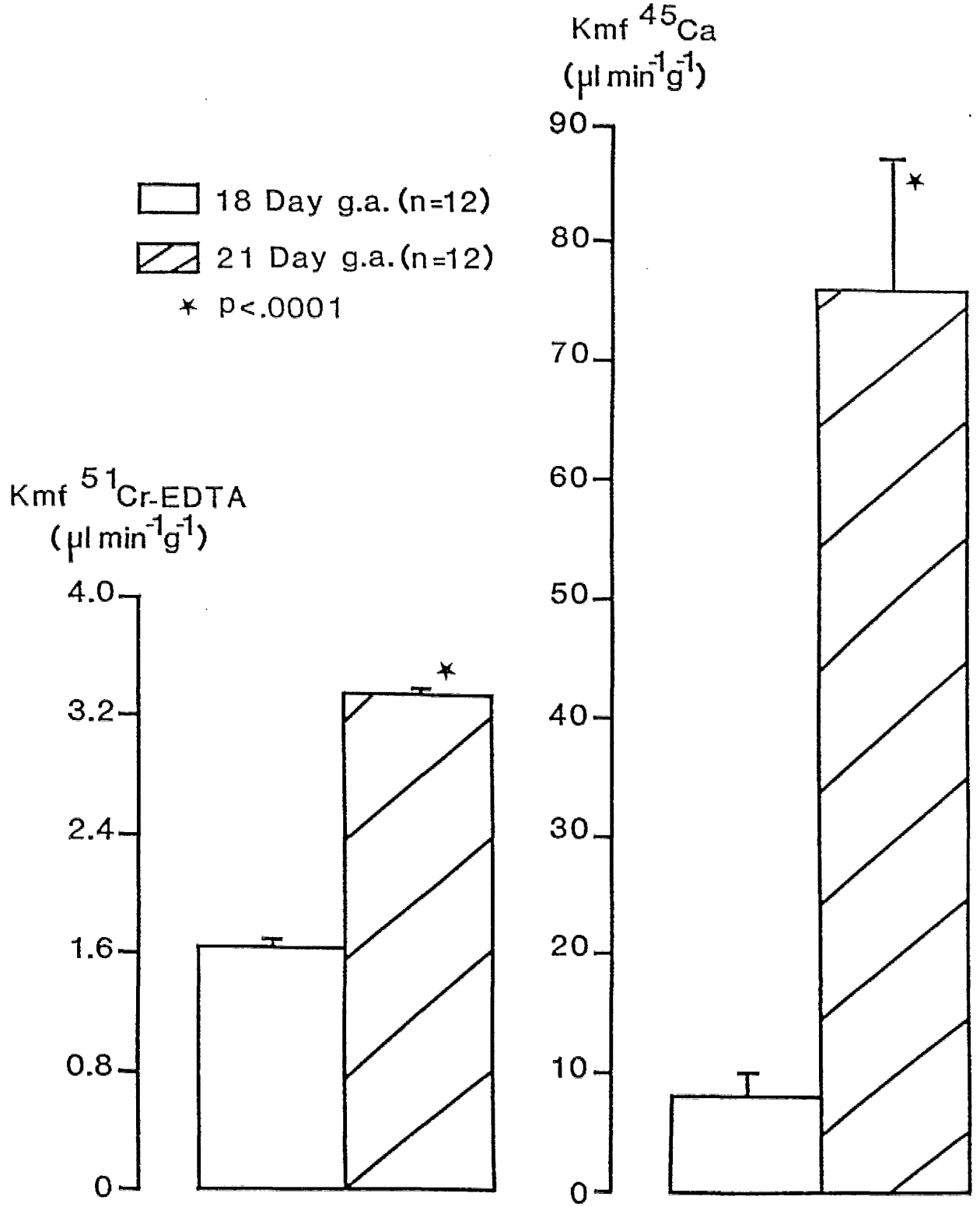


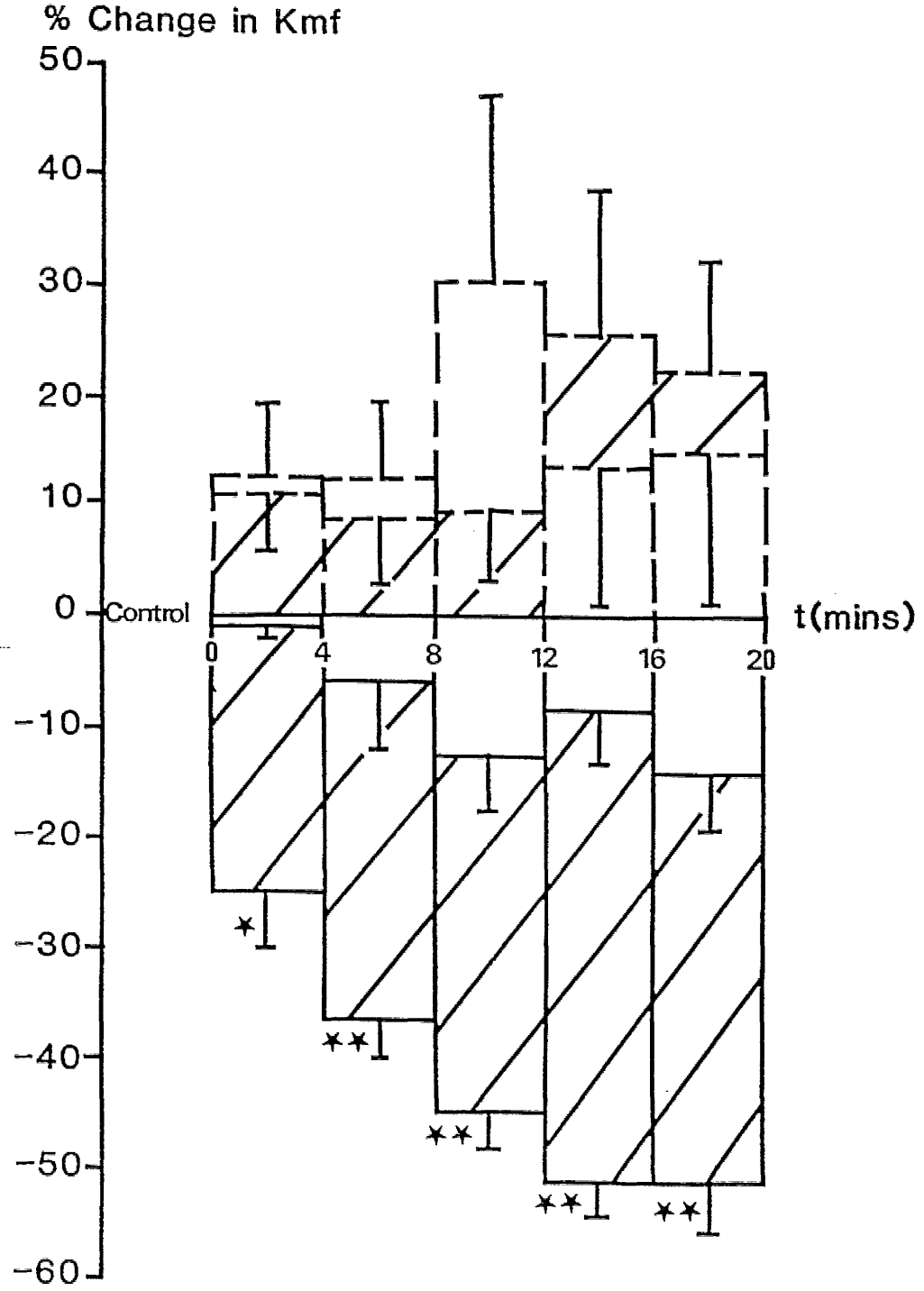
Table 3.3 : The effect of perfusate flow rate on $K_{mf}{}^{51}$Cr-EDTA and $K_{mf}{}^{45}$Ca (mean ± s.e.m.; n=number of placentas)

Perfusate flow rate ($ml min^{-1}$)	$K_{mf}{}^{51}$Cr-EDTA ($\mu l min^{-1} g^{-1}$)	$K_{mf}{}^{45}$Ca ($\mu l min^{-1} g^{-1}$)	Maternal ionized calcium (mM)	n
0.25 ± 0.03	2.76 ± 0.2	56.1 ± 7.7	1.06 ± 0.02	4
0.50 ± 0.02	3.03 ± 0.5	48.7 ± 4.6	1.05 ± 0.02	4
0.74 ± 0.01	3.66 ± 0.4	50.1 ± 3.3	1.02 ± 0.04	3

6) The effect of potassium cyanide on maternofetal calcium transfer

The percentage change in K_{mf} with KCN or saline diluent (for each of the five experimental perfusate collection periods), from control values (i.e. mean of the first two perfusates samples), is shown in Figure 3.4. There was a significant difference between the percentage change in $K_{mf}{}^{45}$Ca obtained with KCN perfusion, as compared to that with saline diluent. Whereas the percentage change in $K_{mf}{}^{51}$Cr-EDTA was comparable for KCN and saline diluent perfusion. Indeed, addition of KCN had a rapid inhibitory effect on calcium transfer, causing $K_{mf}{}^{45}$Ca to fall significantly ($p<0.001$; paired Student's 't' test) from a control value of 50.6 ± 6.1 to 29.4 ± 3.7 $\mu l min^{-1} g^{-1}$ (mean of the 5 experimental perfusate collections). However, if you take the maximal effect of KCN on $K_{mf}{}^{45}$ for each experiment, then $K_{mf}{}^{45}$Ca was reduced to a maximal value of 23.8 ± 3.2 $\mu l min^{-1} g^{-1}$. Even after the removal of KCN, the $K_{mf}{}^{45}$Ca continued to fall slowly, plateauing off, but with no signs of recovery, even after 40 minutes. The clearance of ^{51}Cr-EDTA was not affected by KCN and maternal blood pressure was not significantly lowered by

Figure 3.4 The effect of perfusion with potassium cyanide or its diluent on $K_{mf}{}^{51}Cr$-EDTA and $K_{mf}{}^{45}Ca$, on day 21 of gestation (mean ± s.e.m., n = 8, n=number of placentas). Unpaired Student's 't' test was carried out between KCN data and saline diluent data, for the % change in both $K_{mf}{}^{51}Cr$-EDTA and $K_{mf}{}^{45}Ca$, for each 4-minute perfusate collection.



% change in Kmf ^{45}Ca with saline diluent (n=8)

% change in Kmf ^{45}Ca with KCN (n=8)

% change in Kmf ^{51}Cr-EDTA with saline diluent (n=8)

% change in Kmf ^{51}Cr-EDTA with KCN (n=8)

any KCN which may have crossed into the maternal circulation. Maternal blood ionized calcium concentration was again similar in the two experimental groups (KCN= 1.07 ± 0.06mM, saline diluent= 0.99 ± 0.04mM) and maternal body temperatures ranged from 34-37°C.

7) The effect of acute maternal blood ionized calcium manipulation on K_{mf} ^{51}Cr-EDTA, K_{mf} ^{45}Ca and J_{mf} calcium

K_{mf} ^{51}Cr-EDTA was not altered by changes in maternal blood ionized calcium concentration (iCa; see Figure 3.5; animals maintained exactly at 37°C), suggesting that this manipulation had no effect on placental permeability. Regression analysis of maternal blood ionized calcium concentration (iCa) versus K_{mf} ^{45}Ca showed the expected significant correlation ($p<0.001$; corr. coeff.= -0.76), simply reflecting a change in specific activity of calcium (Figure 3.6). J_{mf} calcium (calculated as above, equation 9) did not correlate with maternal blood ionized calcium concentration (iCa; Figure 3.7), perhaps suggesting that the transport mechanism was largely saturated. However, it was difficult to get measurements at very low maternal blood ionized calcium concentrations, as the animals died and without these it was impossible to be certain that saturation was indeed occurring. To attempt further analysis, a Lineweaver Burk plot was used (Figure 3.8). There was only a poor correlation between $1/J_{mf}$ calcium and 1/maternal blood ionized calcium concentration ($p=0.14$, corr.coeff.=0.15) and although the line of 'best-fit' suggested a V_{max} of 68.03 nmolmin^{-1}g^{-1} and a K_m of 0.149mM, these cannot be accurate. It seems clear however, that the putative calcium transporter may have a very high affinity.

Figure 3.5 Regression plot(R) of K_{mf} ^{51}Cr-EDTA with a variable maternal blood ionized calcium concentration (single perfusate data points plotted).

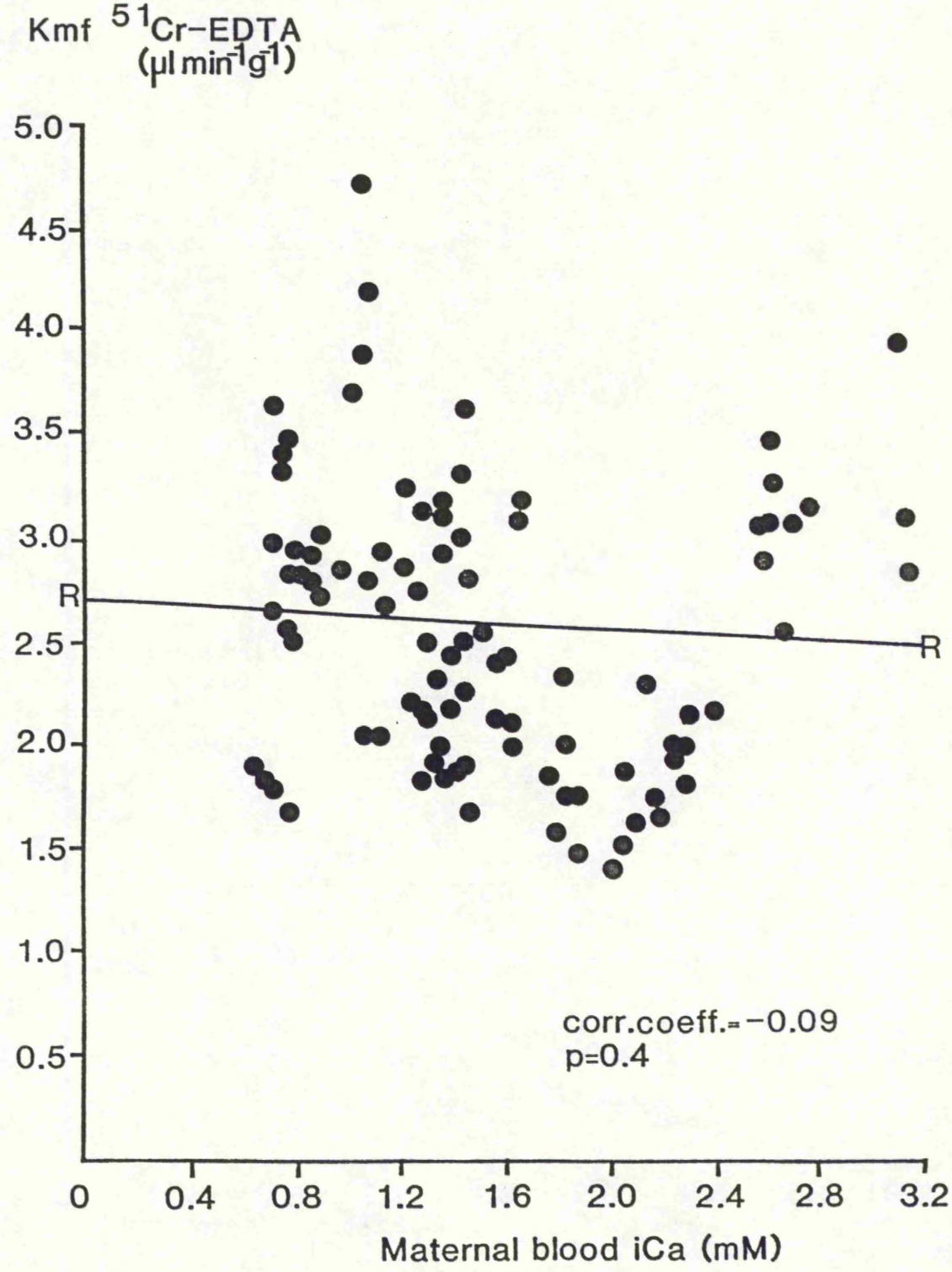


Figure 3.6 Regression plot (R) of $K_{mf}{}^{45}Ca$ with a variable maternal blood ionized calcium concentration (single perfusate data points plotted)

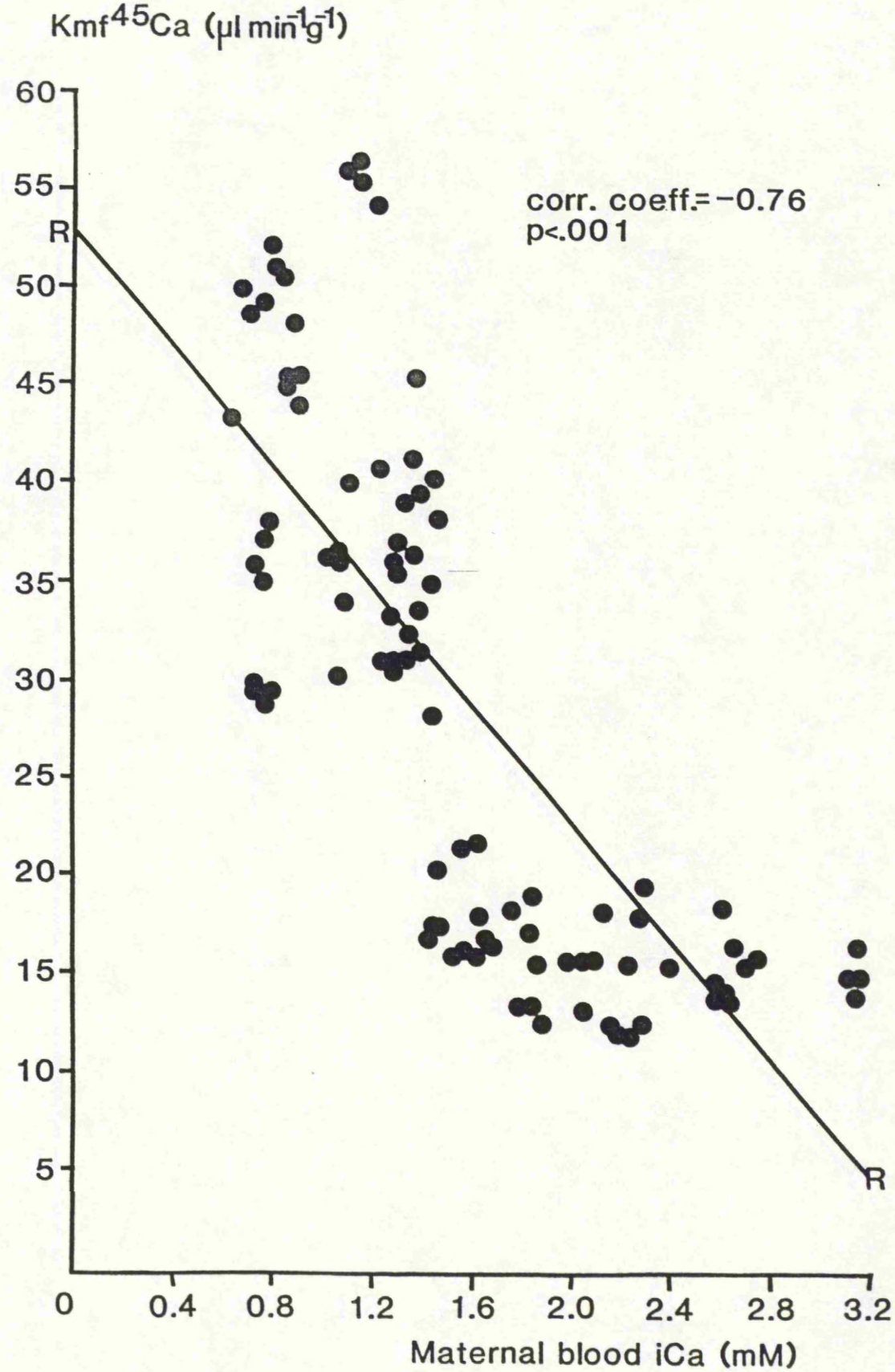


Figure 3.7 Regression plot (R) of J_{mf} calcium with a variable maternal blood ionized calcium concentration (single perfusate data points plotted)

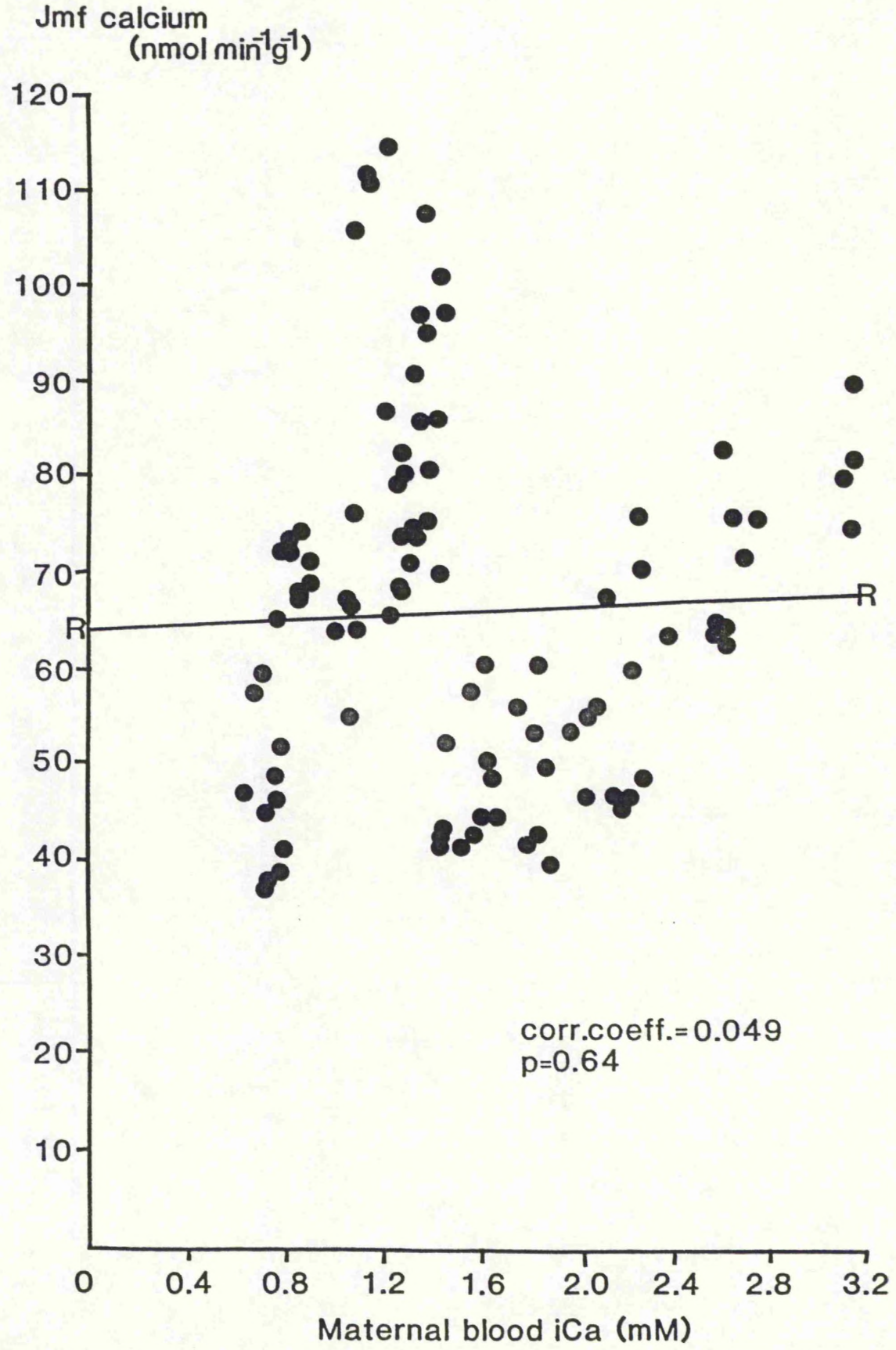
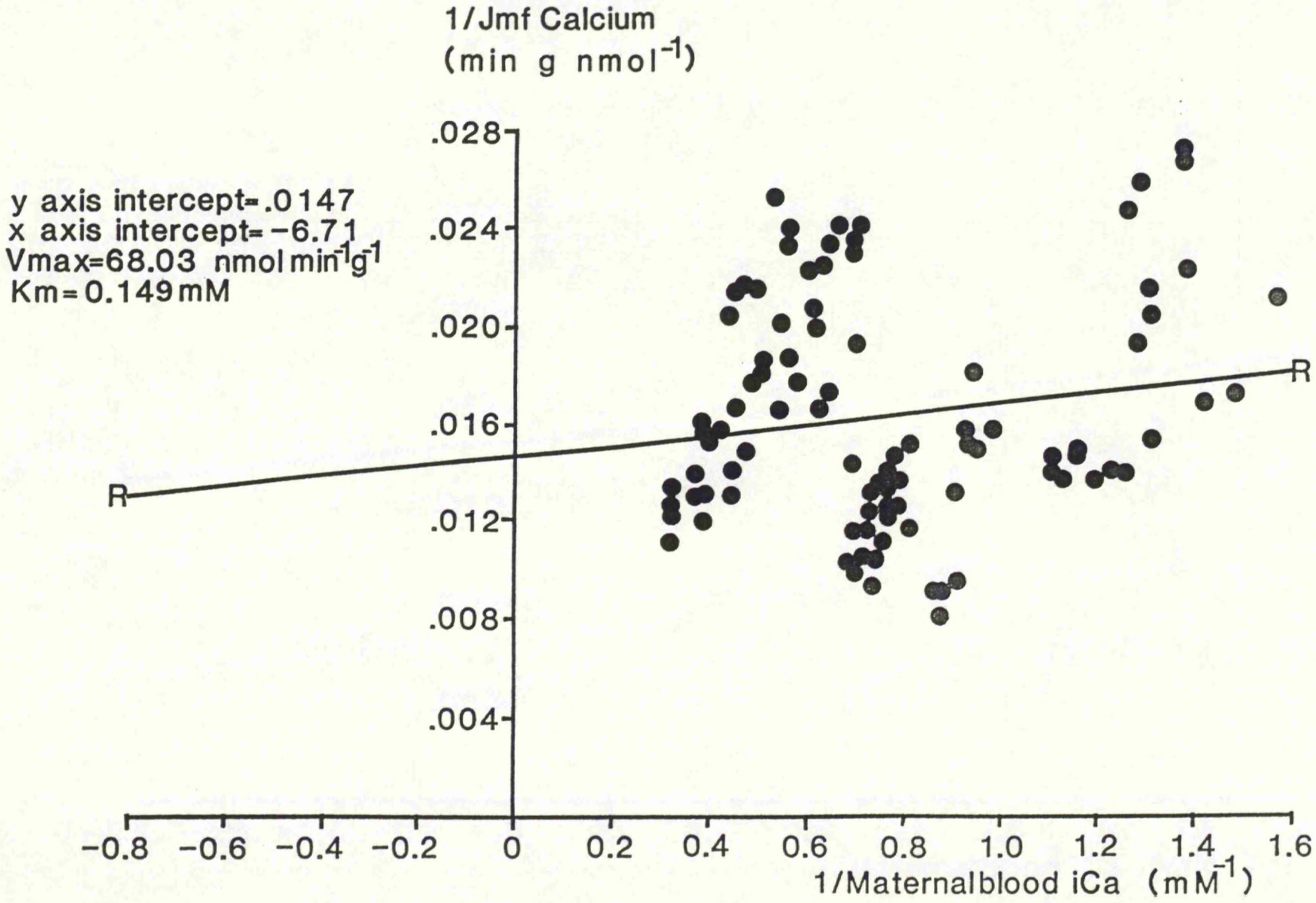


Figure 3.8 Lineweaver Burk plot showing the regression plot (R) of $1/J_{mf}$ calcium versus 1/maternal blood ionized calcium concentration. (see text for calculation of V_{max} and K_m)



SECTION D : DISCUSSION

1) Bidirectional and net calcium fluxes

Net calcium flux across the rat placenta on day 21 of gestation was 128.7 nmolmin^{-1}g^{-1}placenta (assuming a placental weight of 0.54g), as measured by fetal calcium accretion. This may be compared to the J_{mf} calcium (at 37°C, in the intact placenta; as calculated from equation 9), of 236.2 ± 8.0 nmol min^{-1}g^{-1}placenta (n=50). This suggests that about 54% of the unidirectional maternofetal calcium flux is actually retained by the fetus, although the accretion method will only give an approximation of net calcium flux on day 21.

Chef (1969A) measured a net calcium flux on day 21 of gestation, from accretion studies, of 225.0 nmolmin^{-1}g^{-1}placenta (assuming a placental weight of 0.5g). Similarly, Štulc and Štulcová (1986) observed a net calcium flux of 200.0 ± 0.8 nmolmin^{-1}g^{-1}placenta (assuming a placental weight of 0.5g), as compared to a J_{mf} calcium (in intact placentas) of 194.0 ± 14.0 (n=12), again suggesting that J_{fm} is very small. They also measured J_{fm} calcium directly in the perfused rat placenta, estimated from the steady state extraction of ^{45}Ca from the umbilical perfusate and found it to be about 20% of the maternofetal transfer. Their flux measurements therefore do not add up and this probably demonstrates the difficulties involved, particularly as regards the accretion method for net flux determination. Indeed, our measurements of net calcium flux are clearly lower than those of Chef (1969A) and Štulc and Štulcová (1986), whereas our measurements of J_{mf} calcium, in both the

intact and the perfused rat placenta, are comparable to those of Štulc and Štulcová (1986; although the methods of calculation are rather different).

Table 3.4 : Transplacental fluxes of calcium (mean values)

Species	Placental calcium flux (mg calcium $day^{-1}kg^{-1}$fetus)		
	J_{mf}	J_{fm}	J_{net}
Rat			680_a
	973_b		572_b
	799_c	181_c	823_c
Sheep	215_d	12_d	203_d
	255_e		
	277_f		
Guinea-pig	374_g		
	380_h		
Monkey	391_i	326_i	65_i
Human			148_j
			26_k

a Chef,1969A (assume fetal weight=3.5g; 21 g.a.)
b Our data (assume fetal weight=3.5g; 21 g.a.)
c Štulc and Štulcová,1986 (assume fetal weight=3.5g; 21 g.a.)
d Ramberg et al,1973
e Durand et al,1983B (140 g.a.)
f Barlet, 1985A (140 g.a.)
g Durand et al,1983A (assume fetal weight=89g; 66 g.a.)
h Symonds et al,1978 (assume fetal weight=80g; 61 g.a.)
i MacDonald et al,1965
j Shaw,1973
k Comar,1956
g.a. = gestational age

However, it is clear that the flux of calcium across the rat placenta is bidirectional, with the relative magnitude of the fetomaternal flux being somewhat uncertain at the present time. In the sheep (Symonds et al,1972; Ramberg et al,1973), J_{fm} calcium has been reported to be comparatively small, but by contrast in monkeys (MacDonald et al,1965), J_{mf} and J_{fm} are

comparable. Table 3.4 attempts a comparison of J_{mf}, J_{fm} and net calcium flux across the placenta, in a range of species. However, it does contain many assumptions and uncertainties, because of the various techniques used.

As already mentioned, many workers like Štulc and Štulcová (1986) have calculated J_{mf} calcium by simply multiplying the $K_{mf}{}^{45}Ca$ (obtained using the total ^{45}Ca concentration of maternal plasma) by the total calcium concentration of maternal plasma. This gives the same final result as does the calculation of flux using equation 9. However, equation 9 does take into consideration the correct pool of radiolabelled calcium, i.e. the 'free' ^{45}Ca, which seems to be the important species in placental transfer. Several years ago there were reports suggesting that ^{45}Ca injected intravenously into an animal, rapidly distributes between the three pools of 'cold' blood calcium (Wiester et al,1963; Giese and Comar,1964). However, this fact seems to have been largely ignored when calculating clearance of calcium and avoided when calculating calcium flux by using total calcium as the species of calcium in placental transfer; the data here demonstrates that this is not the case (at least in the placental perfusion model).

The cumulative data for the total calcium concentration of maternal plasma reported here, is in agreement with measurements by Green and Hatton (1988), who used the same source of pregnant Sprague Dawley rats (1.95 ± 0.05mM; n=10); but unfortunately they did not measure plasma ionized calcium concentrations. Ideally, in the present study, total and ionized calcium concentration in maternal blood should have been measured in

every experiment; therefore when calculating J_{mf} calcium, each experiment would have its own specific ionized to total blood calcium concentration ratio. However, we are confident that the cumulative mean ratio of ionized to total calcium concentration used in our calculation of J_{mf}, is a true representation of the amount of calcium usually available for placental transfer and that it does not change between experimental groups. Many other workers have measured the percentage of ultrafilterable calcium in plasma from pregnant animals, e.g. 56.8% -man, 66.4% -sheep (Delivoria-Papadopoulos et al,1967), 56.6% -guinea-pig (Twardock and Austin,1970); and these are similar to the present findings.

2) Viability of the perfused rat placental preparation for calcium studies

The Kreb's Ringer perfusate calcium concentration used in these experiments was approximately 1.25mM (all of which was expected to be 'free' calcium, as there was no protein present), which is similar to the maternal blood ionized calcium concentration (≈ 1.25mM), rather than the fetal blood ionized calcium concentration (≈ 1.40mM). However, it has been shown that changes in (fetal) perfusate ionized calcium concentration have no effect on maternofetal calcium transfer (Twardock and Austin,1970; Štulc and Štulcová,1986).

Initial experiments were performed with maternal body temperatures of between 34-37°C and gave a significantly higher $K_{mf}{}^{45}Ca$ in the perfused, than in the intact placenta. This suggests that maternofetal calcium clearance was susceptible to the effects of perfusion. As already mentioned, in the guinea-

pig passive placental transfer of several molecules was increased by perfusion (Hedley and Bradbury,1980). However, we did not see this effect with any other molecule studied (Chapter 2) and data obtained from animals maintained at exactly 37°C showed the opposite effect, i.e. the $K_{mf}{}^{45}Ca$ in intact placentas was highest. Ideally, measurements of $K_{mf}{}^{45}Ca$ by both methods would have been made in the same animal, but this was not possible. It was concluded therefore, that perfusion does have an effect on calcium transfer across the rat placenta. This was not related to perfusate flow rate, as this had no effect on $K_{mf}{}^{45}Ca$ or $K_{mf}{}^{51}Cr$-EDTA. Unfortunately, umbilical blood flow has not been measured in the rat, so there is no <u>in vivo</u> data for comparison. The perfusate flow rate used was slightly less than that through the maternal circulation of the placenta reported by Bruce (1976) to be 1.21 $ml\,min^{-1}g^{-1}$placenta, in 22 day gestation rats and by Atkinson (unpublished; using University of Manchester, pregnant Sprague Dawley rats) to be 0.806 ± 0.3 $ml\,min^{-1}g^{-1}$ placenta (n=8 rats), in 21 day gestation rats.

The perfusion model used here is a modification of that reported by Štulc and Štulcová (1986) and our data is very similar. They measured a $K_{mf}{}^{45}Ca$ of 74.2 ± 1.6 (n=49) and 92.2 ± 6.7 $\mu l\,min^{-1}g^{-1}$ (n=12; assuming a placental weight of 0.5g; rats maintained at 37°C), for perfused and intact placentas respectively; also suggesting some effect of perfusion on the calcium transfer mechanism. Nevertheless, as detailed below, there is a large proportion of the normal calcium transfer retained in the perfused placental preparation and strong evidence that this is an active carrier-mediated process. It

must be noted that there is a large inter-animal variation in the $K_{mf}{}^{45}Ca$ measurements, which is compounded by the fact that the exact maternal body temperature is not known for all animals and consequently some of the variability may be due to temperature effects.

3) Evidence for active transport of calcium across the rat placenta

As already discussed (Chapter 1), there is evidence in the literature that placental transfer of calcium involves a carrier-mediated active mechanism (Twardock and Austin,1970; Van Dijk,1981). We therefore sought evidence of carrier-mediated active maternofetal calcium transfer across both the intact and *in situ* perfused rat placenta.

i) The first piece of confirmatory evidence, is simply the presence of an 'uphill' maternofetal calcium concentration gradient. The mean maternal blood ionized calcium concentration being 1.11 ± 0.02 mM (n=297), whilst the mean fetal blood ionized calcium concentration was 1.42 ± 0.02 mM (n=22). This calcium gradient seems to be present in all species that have been studied (see Chapter 1, Table 1.1).

ii) The K_{mf}/D_w ratio for ^{45}Ca measured in either intact or perfused placentas, was around 10-fold that found for the inert, neutral hydrophilic molecules that were investigated previously (see Chapter 2 and Figure 3.1). The diffusion coefficient used here, was that of ionized calcium in aqueous solution (D_w = 10.1 x 10^{-6} $cm^2 s^{-1}$; American Institute Handbook of Physics,1957), but it might have been more useful to use the free diffusion

coefficient in cytoplasm, as calcium may be crossing transcellularly. However, this diffusion coefficient is smaller than the previous one (D_w = 5.3 x 10^{-6} $cm^2 s^{-1}$; Donahue and Abercrombie,1987), which would make the K_{mf}/D_w ratio even larger. Also, $K_{mf}{}^{45}Ca$ was calculated using the total maternal plasma ^{45}Ca concentration, consequently the K_{mf}/D_w for 'free' ^{45}Ca would have been even greater. Although this very high K_{mf}/D_w ratio for ^{45}Ca could be explained by heterogenous pore size or the effect of a transplacental p.d., the fact that it was also at least 4-5 times greater than the ratio for sodium, provides strong evidence that it was not crossing by diffusion alone and that transcellular, probably carrier-mediated transfer was involved.

iii) Maternofetal calcium transfer was highly susceptible to maternal body temperature, both in the intact and perfused preparations, as might be expected for an energy-requiring process; transfer of the extracellular marker ^{51}Cr-EDTA being unaffected. Mean maternal rectal temperature for an unanaesthetized rat has been quoted as 37.5°C (with a range from 35.8-37.6°C; Baker et al,1979). A small sample of our unanaesthetized pregnant rats showed a similar range; therefore, it is obviously important to measure placental calcium transfer at a physiological maternal body temperature.

iv) There was a significant ($p<0.001$) reduction of $K_{mf}{}^{45}Ca$, but not $K_{mf}{}^{51}Cr$-EDTA, with addition of the metabolic inhibitor, KCN, to the fetal perfusate. This is consistent with the hypothesis that metabolic energy is involved in placental calcium transfer, perhaps for the putative Ca^{2+}-ATPase (Shami

and Radde,1971; Tuan and Kushner,1987). However, the actions of KCN are by no means limited to Ca^{2+}-ATPase and therefore more specific reagents, such as antibodies, are clearly needed to define the extent of Ca^{2+}-ATPase participation in placental calcium transfer. In the perfused guinea-pig placenta, no inhibition of maternofetal calcium transfer was noted with other inhibitors of Ca^{2+}-ATPase, e.g. diuretics (McKercher et al, 1983), however, this may be a perfusion artefact as the guinea-pig placenta seems to be very prone to an increase in permeability upon perfusion (Hedley and Bradbury,1980). Our data agrees well with the findings of Štulc and Štulcová (1986), who also showed a marked inhibition of calcium transfer with KCN. Similarly, Abramovich et al (1987B) demonstrated a marked inhibitory effect of DNP on calcium accumulation in the perfused human placenta.

v) Raising or lowering maternal plasma calcium levels had very little effect on J_{mf} calcium. Štulc and Štulcová (1986) similarly showed that a doubling of maternal blood ionized calcium concentration, caused a 60% reduction in $K_{mf}{}^{45}Ca$ from control values, but only a 30% increase in J_{mf} calcium. This change in J_{mf} calcium, may have been missed here, as control and experimental data was not obtained in the same animal (as Štulc and Štulcová, 1986, had done), consequently any change may have been masked by inter-animal variation. This data suggests the predominance of a saturable calcium transport mechanism in the rat and that the affinity of the calcium transport system to calcium is very high. A similar conclusion was reached by Štulc and Štulcová (1986) and Chalon and Garel (1985C), the latter

from studies in an intact preparation. The K_m of the calcium transporter in the placental perfusions in this study, was calculated to be 0.149mM. Although this number must be viewed with considerable caution (as it was not possible to achieve very low maternal blood ionized calcium concentrations), it is clear that the fetus can still receive adequate amounts of calcium from the mother, even in the face of drastic maternal hypo-calcaemia. Similarly, the fetus is protected from receiving excess calcium, when the mother is hypercalcaemic. Derewlany and Radde (1985) would not agree with this, as their in situ perfusion of the guinea-pig did not show a saturation of maternofetal calcium transfer. However, the artificially high passive permeability of the perfused guinea-pig placenta (Hedley and Bradbury,1980) might lead to a masking of the active calcium transport; in fact, Derewlany and Radde (1985) did not demonstrate active calcium transport in their preparation.

This data therefore suggests that maternofetal calcium transfer across both the in situ perfused and intact rat placenta, occurs through the operation of a saturable carrier-mediated active transport mechanism.

4) Gestational changes in placental calcium transport and the role of the yolk sac

Having established the presence of a carrier-mediated calcium transfer across the rat placenta on day 21 of gestation, it was interesting to look at calcium transport earlier on in gestation and across the yolk sac placenta.

Maternofetal calcium transfer across the chorioallantoic rat

placenta, is certainly dependent on gestational age (Chef,1969A: Romeu,1986; Romeu et al,1986). The $K_{mf}{}^{45}Ca$ was increased by 930% from day 18 to day 21 in the present study. The $K_{mf}{}^{51}Cr$-EDTA was also increased, although to lesser extent, which suggests that the diffusional permeability of the placenta increases, as well as the possible 'switching-on' of an active transport mechanism specifically for calcium. Results obtained from the intact rat placenta on day 18 of gestation (Atkinson,unpublished), are much higher than those measured here ($K_{mf}{}^{51}Cr$-EDTA = 2.46 ± 0.2, n=34; $K_{mf}{}^{45}Ca$ = 25.5 ± 5.5, n=17; animals at 37°C; n=number of placentas). This could suggest that the yolk sac on day 18 of gestation plays a more important role in maternofetal calcium transfer (the yolk sac being absent in the in situ perfused preparation). However, it could also be that perfusion is too much of a disturbing influence at day 18, the umbilical vessels being very fragile. Atkinson et al (1988) have also measured $K_{mf}{}^{45}Ca$ in intact placentas on day 15 and day 22 of gestation. $K_{mf}{}^{45}Ca$ showed a similar increase between day 15 and 18, to that between day 18 and 22. These results correlate well with the sudden increase in net calcium accretion by the rat fetus from day 17 onwards (Romeu,1986). It may be that calcium crosses the placenta by simple diffusion until day 17, and thereafter active transport mechanisms markedly increase calcium transfer. This would fit well with the onset of fetal ossification (Jost et al,1960), increased fetal growth (Bruns et al,1978), appearance of fetal hypercalcaemia (Pic,1969), a sudden increase in placental Ca^{2+}-ATPase activity (Legrand et al,1978) and placental CaBP levels (Bruns et al,1978).

Cautery of the yolk sac blood vessels certainly seemed to reduce $K_{mf}{}^{45}Ca$ (cf.sodium, Chapter 2). Also, the $K_{mf}{}^{45}Ca$ measured in the perfused placenta was lower than that in the intact situation (at least, at a maternal body temperature of 37°C), possibly partly reflecting the lack of a yolk sac in the perfusion model. This is in agreement with work by Derewlany et al (1983), who measured bidirectional calcium flux across the in vitro visceral yolk sac of the guinea-pig (mounted in an Ussing chamber) and found net calcium flux to be in the maternofetal direction. Other evidence that suggests a role for the yolk sac in calcium transport, includes the presence of receptors for $1,25(OH)_2D_3$ (Danan et al,1981), the localization of a vitamin D-dependent CaBP in yolk sac endodermal cells (Delorme et al,1983) and the suggestion that the rat visceral yolk sac is a target organ for PTH (Gügi et al,1986). Therefore, the yolk sac could be another route for maternofetal calcium transfer, but whether the amount transported via this route is important quantitatively or varies throughout gestation, is unclear.

5) Conclusions

In conclusion, data from both the perfused and intact rat placenta tend to support a carrier-mediated, saturable, high affinity, placental calcium transport mechanism. This transport mechanism is sensitive to maternal body temperature and inhibitable by potassium cyanide, suggesting energy dependence. Furthermore, maternofetal calcium transfer seems to increase rapidly during the last few days of gestation, with the yolk sac possibly contributing to this transfer throughout gestation.

There appears to be bidirectional movement of calcium across the rat placenta, maternofetal predominating with a smaller fetomaternal component, giving a net flux of calcium from mother to fetus. Although the perfused rat placenta does not appear to transport as much calcium as the intact organ, it is clear that much of the active transport mechanism is preserved in this preparation and therefore this is a useful model for studies into the control of placental calcium transfer (see Chapter 4 and 5).

CHAPTER 4

MATERNAL CONTROL OF PLACENTAL CALCIUM TRANSPORT IN THE RAT

SECTION A : INTRODUCTION

As already mentioned in Chapter 1 (Section E2), the control of calcium transport across the placenta presents a complex picture. Research in man suggests that maternal vitamin D deficiency (especially prevalent in Asians) causes fetal rickets and neonatal hypocalcaemia, which can be alleviated by vitamin D supplements during pregnancy (Brooke et al,1980; Mallet et al, 1986). Similarly, in guinea-pigs, maternal injection of $1,25(OH)_2D_3$ significantly increased fetal total calcium content, but without any significant increase in ^{45}Ca transfer from mother to fetus (Durand et al,1983A). Studies using rats however, suggest that interference with the maternal parathyroid /vitamin D endocrine system, has no effect on fetal calcaemia or net placental calcium transfer (Halloran and DeLuca,1981; Brommage and DeLuca,1984; Ibrahim et al,1984).

The aim of the work reported in this chapter, was to carry out similar experiments in the rat, by manipulating the maternal parathyroid/vitamin D system. This was partly achieved by using dietary-induced vitamin D deficient pregnant rats, which are relatively easy to raise. A major problem with interpreting previous work, is that in most cases the degree of vitamin D deficiency has not been assessed (Brommage and DeLuca,1984) and to overcome this, $1,25(OH)_2D_3$ levels in maternal and fetal blood were measured. As well as dietary manipulations, maternal thyroparathyroidectomy (TPTX) was also used, hence removing the hormonal influence of parathyroid hormone and calcitonin, and the stimulation for $1,25(OH)_2D_3$ production.

Three groups of pregnant rats were used for these experiments, the first group being rendered vitamin D deficient by maintenance on a high calcium, vitamin D deficient diet (i.e. 'pink dough only'). The second group was again maintained on this diet, but also lacked the maternal parathyroids and calcitonin (i.e. 'pink dough + TPTX'; simplified to 'TPTX only'). The third group, was similar to the second group, but with daily replacement injections of $1,25(OH)_2D_3$ (i.e. 'pink dough + TPTX + $1,25(OH)_2D_3$'; simplified to 'TPTX + $1,25(OH)_2D_3$'). Using this variety of maternal PTH/vitamin D manipulations, the maternal control of placental calcium transfer was investigated.

Whereas most previous studies have simply involved measuring the maternofetal calcium concentration gradient or net placental calcium transfer, with the in situ perfused rat placental model it is possible to actually measure maternofetal calcium clearance and flux. Such measurements, together with estimation of net calcium flux, from fetal total calcium accretion, could give a clear picture of where any controlling mechanisms have their effect.

SECTION B : MATERIALS AND METHODS

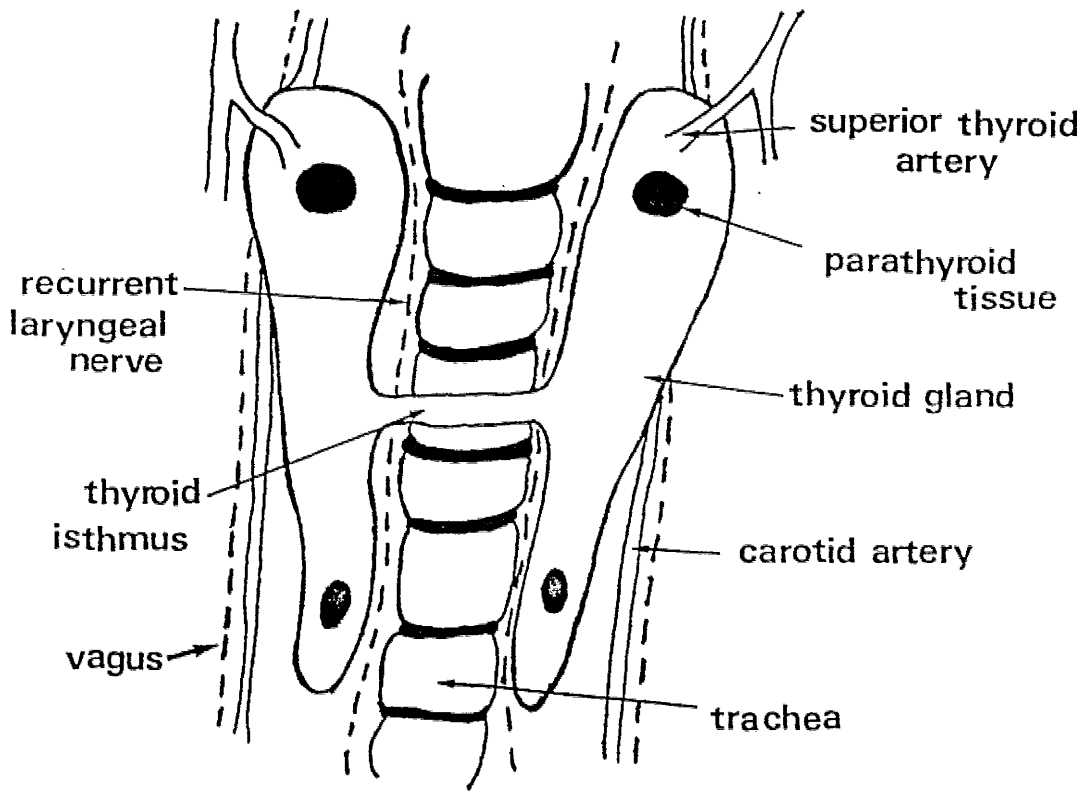
1) Animal Model - Maternal thyroparathyroidectomy (TPTX)

Female Sprague Dawley rats were maintained on a high calcium, low vitamin D diet for two weeks prior to mating. This so-called 'pink dough' diet was made from highly purified flour, which was essentially vitamin D-free. It was supplied by the Department of Medicine (Manchester University), and consisted of

4250g white unfortified flour, 500g egg albumin, 100g sodium chloride, 10g ferric citrate, 112g Na_2HPO_4, 20g vitamin B mix, 40ml fat soluble vitamin drops, 3.8% calcium carbonate and 161mmol/kg phosphate (in 5kg). Ashed mineral content being 370mmol/kg calcium and 10.5mmol/kg magnesium.

On day 13 of gestation, rats were anaesthetized with ether and the neck region was cleaned with 0.5% chlorhexidine gluconate antiseptic. A ventral line incision was made in the neck and the salivary glands were freed by blunt dissection to reveal the muscle overlying the trachea. This sternohyoid muscle was split into two strap muscles, along the midline, using scissors and these were then retracted to reveal the trachea. The two lower muscle layers running down either side of the trachea, were dissected out and retracted. The thyroid and parathyroid glands were then visible under a light microscope (x20 magnification; see Figure 4.1).

Figure 4.1 The neck region in the rat, showing the thyroid and parathyroid glands



The parathyroids appeared as slightly paler than the surrounding thyroid tissue, but to ensure complete parathyroidectomy, the thyroids had to be removed as well. The parathyroids were dissected out from the thyroid tissue and carefully discarded. The thyroid isthmus, connecting the two lobes of the thyroid gland, was severed and each lobe was gently pulled upward and laterally, freeing it from the trachea with a dissecting needle. Care was taken not to damage the recurrent laryngeal nerve running between the thyroid and the trachea. All pieces of thyroid and parathyroid tissue were carefully disposed of and the instruments regularly cleaned, as reimplantation of tissue is possible. The muscle layers were then pulled back over the trachea, the skin was sewn up with 3/0 braided silk suture (Ethicon Ltd.) and the animal was allowed to recover.

2) Protocol

For the next 7 days, these maternally TPTX rats were injected daily (s.c.) with either : a) 3μg L-thyroxine (T_4; Sigma Chemical Company Ltd.) in 0.1ml (1:100) ethanol/propylene glycol solution (1,2 propanediol); or b) 3μg L-thyroxine and 30ng $1,25(OH)_2D_3$, in 0.1ml (1:100) ethanol/propylene glycol solution ($1,25(OH)_2D_3$ kindly supplied by Dr.Davies, Roche Products, Welwyn Garden City). The animals were maintained on the 'pink dough' diet throughout the experiment and 3% calcium gluconate (BDH Chemicals Ltd.) was added to their drinking water. Our control group of pregnant rats, were those maintained on the 'pink dough' diet only (without any surgical intervention), i.e. they were vitamin D deficient, but not TPTX. In retrospect, a

control group of animals maintained on a normal rat pellet diet might have been included. However, it is possible to compare the data in this chapter with that in Chapter 3, where animals were maintained on the normal rat pellet diet.

On day 21 of gestation, the mothers were anaesthetized with 110mgkg^{-1} sodium thiobutabarbital (Inactin, BYK Gulden) and a placenta was perfused through the fetal circulation, as described previously (Chapter 2). Two tracers, ^{45}Ca and ^{3}H-sucrose (used as an extracellular marker) were injected at time zero. Three maternal carotid blood samples (0.5ml) were taken at 3, 13 and 23 minutes, and four 4-minute perfusate collections were made (i.e. 4-8, 8-12, 12-16 and 16-20 minutes). The animals were kept warm throughout the experiment using a dissection lamp and maternal body temperatures ranged from 34-37°C. Blood gases were measured on maternal blood and on the Krebs Ringer perfusate, umbilical artery 'inflow' and umbilical vein 'outflow', using the Corning blood gas analyser. Both ionized (ICA1 Radiometer, Copenhagen) and total (Perkin Elmer atomic absorption spectrophotometer 2380) calcium concentrations, were measured in maternal blood (see Chapter 3). Ionized calcium concentrations were also measured in Krebs 'inflow' and 'outflow' samples.

Four weighed fetuses were collected (if possible) from each experiment and they were frozen prior to total calcium analysis, by the fetal ashing method described in Chapter 3. The results from the four fetuses were pooled to give a mean figure for each rat and the data was expressed as mg calcium per fetus or per gram wet weight of fetus. Seven animals in each experimental

group were used for collection of maternal and fetal blood only. These plasma samples were taken for thyroxine (Dr.M.Addison, Pendlebury Biochemistry Dept.) and $1,25(OH)_2D_3$ measurements (Dr.B.Mawer, Dept.of Medicine, Manchester University). Thyroxine (T_4) and its metabolically active form, triiodothyronine (T_3), were measured using a solid phase radioimmunoassay and $1,25(OH)_2D_3$ was measured by the method of Mawer et al (1985). Blood for these measurements could not be taken from the animals whose placentas were actually perfused, as that blood was required for total and ionized calcium concentration analysis. The same criteria for rejection of experiments was used as before (see Chapter 2).

3) Calculations and Statistics

The first two perfusate samples were used to give a mean K_{mf} for each experiment. K_{mf} ^{3}H-sucrose and K_{mf} ^{45}Ca were calculated as described in Chapter 2 (equation 6), results being expressed as mean ± s.e.m., in μlmin^{-1}g^{-1} placenta (wet weight), with 'n' being the number of placentas. J_{mf} calcium was also calculated, using equation 9, expressed as nmolmin^{-1}g^{-1} placenta. The Student's 't' test was used for statistical comparisons, paired or unpaired as appropriate.

chap3

SECTION C : RESULTS

1) Viability Measurements

Table 4.1 shows the results for maternal blood pressure, perfusion pressure, maternal blood gases at the end of the

experiment (i.e. 23 minutes after isotope injection) and Krebs perfusate umbilical artery 'inflow' and umbilical artery 'outflow' ionized calcium concentrations.

Table 4.1 Viability measurements in the three experimental groups (mean ± s.e.m.; n= number of animals; * $p<0.01$, Krebs 'inflow' versus 'outflow')

Parameter	'Pink dough' controls	Maternal TPTX	Maternal TPTX + $1,25(OH)_2D_3$
Maternal blood pressure (kPa)	11.2 ± 0.96 n=9	9.2 ± 0.39 n=5	9.2 ± 0.67 n=5
Perfusion pressure (kPa)	2.41 ± 0.19 n=9	1.88 ± 0.33 n=7	2.20 ± 0.60 n=3
Maternal blood gases at the end : pH	7.30 ± 0.02	7.36 ± 0.03	7.39 ± 0.01
pCO_2 (kPa)	6.20 ± 0.30	4.30 ± 0.30	5.10 ± 0.60
pO_2 (kPa)	18.7 ± 2.60 n=8	16.5 ± 0.98 n=6	13.2 ± 0.97 n=5
Krebs ionized calcium concentration (mM) : 'inflow'	1.18 ± 0.01	1.21 ± 0.02	1.22 ± 0.02
'outflow'	1.12 ± 0.01* n=9	1.19 ± 0.04 n=7	1.19 ± 0.01 n=6

All of the above parameters suggest that the perfusions were viable and the animals were healthy. Blood pressure in the two experimental groups was lower (but not significantly) than in the control group, this is possibly due to the effects of surgery and hypocalcaemia (in the 'maternal TPTX only' group). Perfusion pressure and maternal blood gases were comparable in the three groups and both the Krebs 'inflow' and 'outflow'

samples appeared to be adequately oxygenated. Ionized calcium concentrations of Krebs 'inflow' were not significantly different to Krebs 'outflow', except for in the 'pink dough' control group. In this group, the ionized calcium concentration fell significantly ($p<0.01$; paired Student's 't' test) after passage through the placenta, which may reflect binding to high molecular weight substances or complexes.

2) Plasma Thyroxine measurements

A review of the literature indicated that an adequate replacement dose of L-thyroxine (T_4) for TPTX adult rats, was 1-2 μg $day^{-1}100g^{-1}$ body weight (Connors and Hedge,1981; Hefti et al,1983). In our rats, who showed an average body weight of 350g, the dose of 3μg per day, seemed to more than adequately replace plasma thyroxine levels.

Table 4.2 Maternal and fetal plasma T_4 and T_3 concentrations on day 21 of gestation (mean ± s.e.m.; n=7 rats)
* $p<0.05$, ** $p<0.01$, versus 'pink dough only'

Protocol	T_4 (nmol l^{-1})		T_3 (nmol l^{-1})	
	Fetal	Maternal	Fetal	Maternal
'Pink dough' controls	13.1 ± 1.8	17.9 ± 2.4	< 0.3	3.94 ± 0.73
Maternal TPTX only	9.3 ± 0.92	66.6 ± 16.4*	< 0.3	6.08 ± 1.10
Maternal TPTX + $1,25(OH)_2D_3$	7.3 ± 1.5*	133.6 ± 27.3**	< 0.3	4.03 ± 0.45

Table 4.2 shows the values for T_4 and T_3 in maternal and fetal plasma. Maternal T_4 levels were significantly ($p<0.05$) higher in the two groups with thyroxine replacement, whilst, fetal T_4

levels in the 'TPTX + $1,25(OH)_2D_3$' group were significantly ($p<0.05$) lower than in the 'pink dough only' group and were generally lower than maternal levels. Maternal plasma T_3 levels were comparable in the three groups, whilst fetal plasma T_3 levels were barely detectable.

3) Plasma $1,25(OH)_2D_3$ measurements

The dose of $1,25(OH)_2D_3$ used in these experiments, was similar to that used by other research groups (Lester et al, 1978; Gilbert et al,1980; Garel et al,1981C). Table 4.3 shows the concentrations of $1,25(OH)_2D_3$ ($pgml^{-1}$) and ionized calcium (mM) in maternal and fetal blood, measured in the same group of animals (i.e. those not used for perfusion). Unfortunately, the amount of maternal and fetal (i.e. pooled from many fetuses) plasma collected from one rat was often insufficient for accurate analysis and consequently, plasma from several rats had to be pooled (ideally, several millilitres were required). Also, $1,25(OH)_2D_3$ measurements in pregnant Sprague Dawley rats maintained on the normal rat pellet diet, are not available for comparison.

Table 4.3 Maternal and fetal plasma $1,25(OH)_2D_3$ and ionized calcium concentrations on day 21 of gestation
* $p<0.01$, 'TPTX only' versus 'pink dough only'
** $p<0.001$, 'TPTX only' versus other two groups
(mean ± s.e.m., n= number of animals; individual rats numbered 1-7, ie:those not used for perfusion)

Protocol		$1,25(OH)_2D_3$ ($pgml^{-1}$)		Ionized calcium concentration (mM)	
		Maternal	Fetal	Maternal n=7	Fetal n=7
'Pink dough' controls	1	15	29		
	2	10	0		
	3/4	--	0	1.11	1.37
	5	0	0	± 0.06	± 0.02
	6	33	26		
	7	--	18		
Maternal TPTX only	1	0	0		
	2/3/4/5/6	--	0	0.71**	1.21*
	4/5/6	5	--	± 0.04	± 0.04
	7	6	0		
Maternal TPTX + $1,25(OH)_2D_3$'					
	1/2/3/4/5/6	--	61		
	1/2/3/4	511	--	1.34	1.26
	5/6	188	--	± 0.09	± 0.05
	7	--	55		

Maternal blood ionized calcium concentration in the 'TPTX only' group, was significantly lower ($p<0.001$) than in the other two groups (who were not significantly different from each other). However, the maternal ionized calcium concentration in the 'TPTX + $1,25(OH)_2D_3$' group was significantly higher than that measured in rats on a normal rat pellet diet (see Chapter 3; 1.06 ± 0.02mM, n=42), whereas the 'pink dough only' group showed a comparable value. It is noteworthy, that in the actual perfusion experiment animals, the maternal blood ionized calcium

concentrations were comparable in the 'pink dough only' and the 'TPTX + $1,25(OH)_2D_3$' groups. This possibly reflects a difference in the time at which blood samples were taken for ionized calcium concentration analysis, with respect to the time of the last $1,25(OH)_2D_3$ injection. Fetal blood ionized calcium concentration in the 'TPTX only' group, was significantly ($p<0.01$) lower than in the 'pink dough only' group (this latter figure being comparable to that measured in fetuses from rats on a normal rat pellet diet; see Chapter 3; 1.42 ± 0.02mM, n=22). However, the 'TPTX + $1,25(OH)_2D_3$' group had a significantly ($p<0.05$) lower fetal ionized calcium concentration, when compared to animals on a normal rat pellet diet, but not when compared to the 'pink dough only' group.

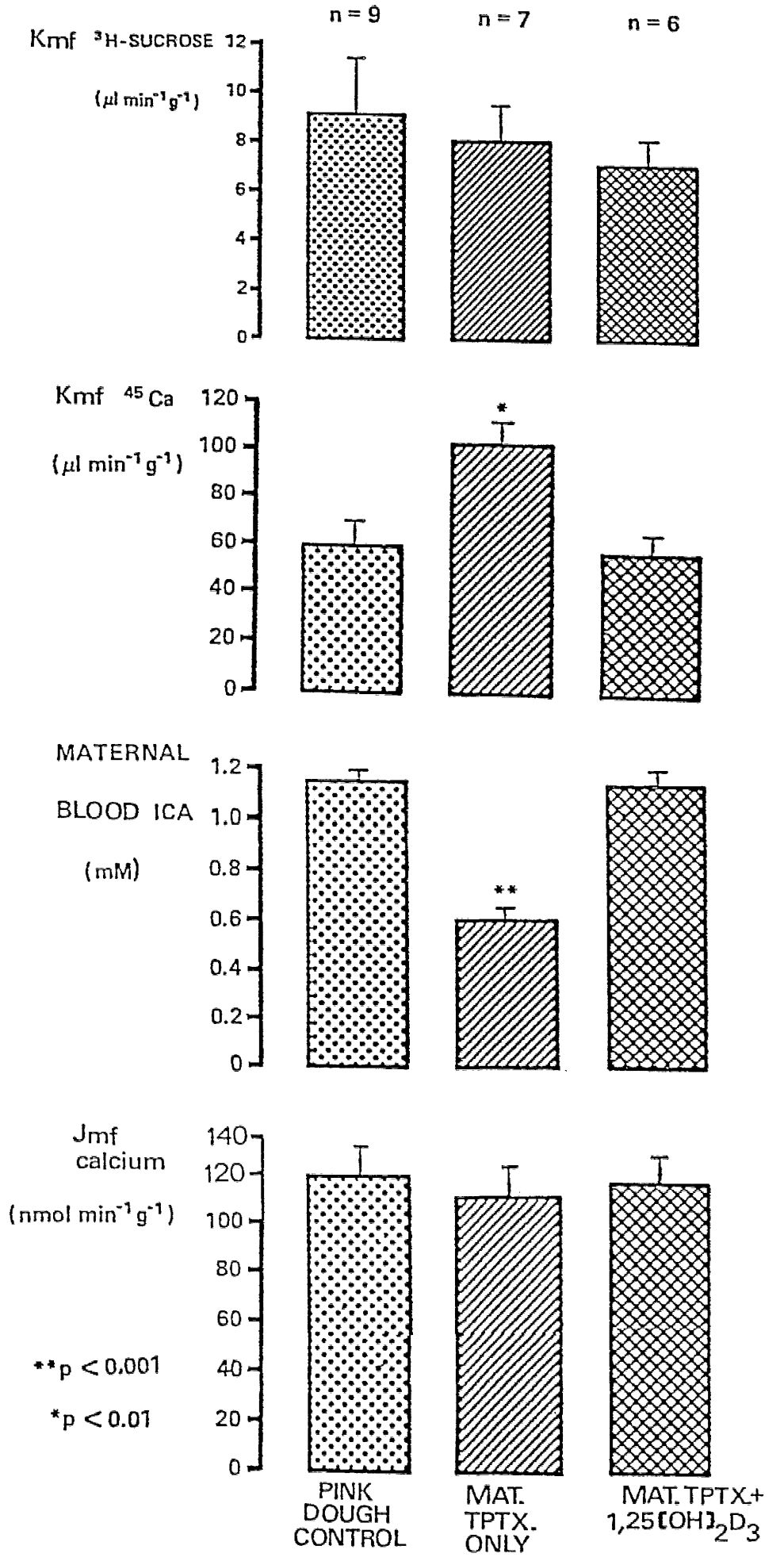
Unfortunately, the measurements of $1,25(OH)_2D_3$, do not express the degree of vitamin D deficiency in the three experimental groups, very clearly. The results being confused by the large scatter and the presence of 'pooled' samples, which meant that paired maternal/fetal data from the same rat was infrequent. However, looking at individual data points, the trend was for an increase in $1,25(OH)_2D_3$ concentration in both maternal and fetal plasma, rising from the 'TPTX only' group, to the 'pink dough only' controls, up to the 'TPTX + $1,25(OH)_2D_3$' group. This suggests a degree of maternal $1,25(OH)_2D_3$ deficiency, possibly also reflected as fetal $1,25(OH)_2D_3$ deficiency, which could be overcome by maternal injections of $1,25(OH)_2D_3$. Ideally, this assessment of vitamin D status would have been made in the same group of animals that were used for perfusion. This was not possible, but hopefully this data gives an approximate

representation of the vitamin D status of our experimental animals. Unfortunately, it is difficult to compare these plasma measurements of vitamin D metabolites with similar data in the literature, because of the variability in the sensitivity of the assays used, the differing amounts of vitamin D metabolites in the so-called 'normal' rat pellet diets and the varying amounts of ultraviolet light induced vitamin D_3 formation in the animals used.

4) Unidirectional maternofetal clearance and flux of calcium

Figure 4.2 shows the data for K_{mf} ^{3}H-sucrose, K_{mf} ^{45}Ca, maternal blood ionized calcium concentration (iCa) and J_{mf} calcium, in the three groups of animals. The clearance of the extracellular marker, ^{3}H-sucrose, was constant throughout, whilst the K_{mf} ^{45}Ca was significantly ($p<0.01$) higher in the 'TPTX only' group. However, this higher clearance simply reflects a difference in the specific activity of calcium, due to a significant ($p<0.001$) fall in maternal blood ionized calcium concentration in this group. When J_{mf} calcium was calculated (using equation 9), there was no significant difference between the three groups.

Figure 4.2 The effect of maternal parathyroid/vitamin D status, on unidirectional maternofetal calcium transfer across the rat placenta on day 21 of gestation. Unpaired Student's 't' test compares 'TPTX only' data with the other two groups. (mean ± s.e.m.; n= number of placentas)



5) Fetal Total Calcium Content

Table 4.4 shows the fetal total calcium content in the three groups. The data has been expressed as mg calcium fetus^{-1} or mg calcium g^{-1} fetal wet weight. By whatever method used, there were no significant differences between the three groups. Similarly, there were no differences in mean fetal wet weight, fetal dry ash weight or placental wet weight.

Table 4.4 Fetal total calcium content on day 21 of gestation (mean ± s.e.m.; n= number of animals, i.e. the mean of four fetuses)

Protocol	Fetal Total Calcium	
	mg calcium fetus^{-1}	mg calcium g^{-1} fetus
'Pink dough' control (n=7)	5.473 ± 0.29	1.570 ± 0.04
Maternal TPTX only (n=7)	4.979 ± 0.64	1.518 ± 0.08
Maternal TPTX + $1,25(OH)_2D_3$ (n=6)	4.893 ± 0.27	1.565 ± 0.05

SECTION D : DISCUSSION

1) Assesment of maternal and fetal parathyroid and vitamin D status

The problem of the existence of accessory parathyroid tissue is important in the interpretation of experiments using the chronically TPTX animal (Kenny,1962). It was possible to check for this in the 'maternal TPTX only' group, as all the animals were very hypocalcaemic and prone to tetanic spasms. However, in the 'maternal TPTX + $1,25(OH)_2D_3$' group, maternal plasma calcium concentration was normal, consequently it was impossible to assess whether a successful parathyroidectomy had been performed. Animals from both these groups seemed to have lower maternal blood pressures (but not significantly), than was seen in the control group. However, overall the animals chosen were healthy and only viable perfusion experiments were analysed.

A lack of parathyroid hormone (PTH) leads to a decrease in intestinal calcium absorption, plasma calcium concentration, bone formation and resorption, and in kidney tubular reabsorption of calcium (Sammon et al,1970). It also reduces the production of $1,25(OH)_2D_3$ and maintenance on a low vitamin D/high calcium diet, as in these experiments, may further reduce the stimulus for $1,25(OH)_2D_3$ production. Administration of $1,25(OH)_2D_3$ can overcome the decrease in intestinal calcium absorption and enhance the deposition into and the release of calcium from bone (Rizzoli et al,1977).

Measurement of $1,25(OH)_2D_3$ levels in these experiments, suggested that the 'pink dough only' and the 'maternal TPTX

only' groups had lower $1,25(OH)_2D_3$ levels, as compared to levels in normal rats. Unfortunately, we do not have any measurements of $1,25(OH)_2D_3$ in our pregnant Sprague Dawley rats fed on a normal rat pellet diet. However, Halloran et al (1979) have measured maternal blood vitamin metabolites in pregnant rats, on day 20 of gestation; $25OHD_3$ $7.9ngml^{-1}$, $24,25(OH)_2D_3$ $1.8ngml^{-1}$ and $1,25(OH)_2D_3$ $87.5pgml^{-1}$. Such measurements will obviously depend on the sensitivity of the assay, the amount of $1,25(OH)_2D_3$ in the animal's diet and the degree of ultraviolet light induced formation of vitamin D_3 in the skin. Daily maternal injections of 30ng $1,25(OH)_2D_3$ certainly seemed to overcome any deficiency of this vitamin D metabolite in our animals. Also, trends in maternal $1,25(OH)_2D_3$ plasma levels seemed to be mirrored by fetal $1,25(OH)_2D_3$ levels. However, fetal levels of $1,25(OH)_2D_3$ in the rat have previously been reported to be undetectable, $24,25(OH)_2D_3$ being the main circulating fetal vitamin D metabolite (Weisman et al,1976; Noff and Edelstein, 1978). However, our data suggests that this may not always be the case, as even the supposedly vitamin D deficient 'pink dough' animals, had measurable fetal $1,25(OH)_2D_3$ levels. Measurements have been made in the sheep (Abbas et al,1987B; Paulson et al,1987), which showed that $1,25(OH)_2D_3$ is lower in fetal than maternal plasma. However, it is really the concentration of 'free' $1,25(OH)_2D_3$ that is important, as this appears to be the physiologically active fraction (see Chapter 1, Section E2). In man, total $1,25(OH)_2D_3$ concentration in cord blood is lower than maternal, but 'free' $1,25(OH)_2D_3$ is higher (Bouillon,1983).

Significant correlations have been found between fetal and maternal plasma levels of both $25OHD_3$ and $24,25(OH)_2D_3$, but not $1,25(OH)_2D_3$, in the rat (Nguyen et al,1988). This suggests that fetal $25OHD_3$ and $24,25(OH)_2D_3$ are mainly derived from the mother by placental transfer, whereas fetal $1,25(OH)_2D_3$ is probably a mixture of placental $1,25(OH)_2D_3$, maternal $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$ produced by the fetal kidney. A similar situation is found in man (Hillman and Haddad,1974; Weisman et al,1978A), but again caution is required when interpreting data, if only total and not 'free' levels of the vitamin D metabolites are known.

Nguyen et al (1988) highlight the problem of assuming vitamin D deficiency without actually obtaining a measure of vitamin D status. They showed that neither maternal nor fetal plasma (total) $25OHD_3$, $24,25(OH)_2D_3$ or $1,25(OH)_2D_3$, were significantly affected by maternal TPTX. Fetal $1,25(OH)_2D_3$ certainly could be maintained by the fetal kidney (Weisman et al,1976) and possibly by the placenta itself (Tanaka et al,1979). Indeed, in the sheep, maternal TPTX caused a significant fall in maternal $1,25(OH)_2D_3$, accompanied by only a small fall in fetal $1,25(OH)_2D_3$ (Ross et al,1980B). Thus, the fetus seems to be able to maintain its vitamin D status, in the face of maternal vitamin D deficiency. Therefore, it is obviously very important to assess both maternal and fetal vitamin D status, in experiments where the role of vitmin D metabolites in pregnancy is being studied. However, our study highlights the problems of obtaining accurate paired maternal/fetal plasma measurements.

2) The role of the maternal thyroid status

Replacement of thyroid function by injecting thyroxine (T_4), was performed in order to minimize the changes caused by maternal TPTX. Indeed, it must be remembered that calcitonin was not replaced.

Our data suggests that T_4 does not cross the rat placenta (fetal levels being similar, irrespective of maternal levels) and does not alter maternal or fetal T_3 levels. It has previously been suggested that the rat placenta is permeable to T_4, but how much crosses to the fetus is unclear (Gray and Galton,1974). Devaskar et al (1986) recently reported placental transfer of T_4 and T_3 in the pregnant rabbit. Indeed, administration of T_3 to the doe seemed to stimulate fetal lung maturation, both functionally and morphologically (Church et al, 1987). There is much controversy over placental transfer of thyroid hormones in man (Dussault et al,1969; Wu et al,1973), but it is generally believed that transfer is negligable (Ekins, 1985). However, thyrotrophin releasing hormone readily crosses the human placenta and stimulates the fetal pituitary to release T_3 (Moya et al,1986).

The fetal rat T_4 and T_3 levels in these experiments were lower than maternal levels, which is slightly different to findings in the near term pregnant guinea-pig (Castro et al,1986). They found higher fetal plasma T_4 and lower fetal plasma T_3 levels, when compared to maternal plasma. Also, in the rat fetus T_3 levels were hardly measurable, whilst in the fetal guinea-pig T_3 was clearly detectable. Again, it is better to quote levels of 'free' T_4/T_3, when thinking in terms of cellular transport and

activity (Castro et al,1986). T_4 and T_3 being physiologically active in the 'free' form, circulating in the blood bound to thyroxine binding globulin. Clearly thyroid hormone profiles in the fetus and the adult differ markedly, coupled with large inter species variations.

The placenta itself has been implicated as a probable site for fetal T_3 metabolism (Castro and Braverman,1985). This is based on the fact that inner-ring deiodination of T_4 and T_3 can occur in the in situ perfused guinea-pig placenta. Similar studies have been performed in the rat (Roti et al,1982) and human (Roti et al,1981) placenta and this may be one of the causes of the apparent placental impermeability to thyroid hormone. The placental inner-ring deiodination activity was not affected by maternal or fetal hypo- or hyperthyroidism (Suzuki,1986). These effects of thyroid status on placental deiodinating activity seemed to be placental specific, as there were changes in T_4 to T_3 deiodination in the liver and kidney in these pregnant rats. Clearly, the fetal rat thyroid is active from as early as day 15 (Kawoai,1987), when thyroglobulin can be detected, with T_4 and T_3 following two days later.

Overall, we therefore had two groups of rats ('pink dough only' and 'pink dough + TPTX') that were vitamin D deficient, one group ('pink dough + TPTX') that was also PTH deficient and one group ('pink dough + TPTX + $1,25(OH)_2D_3$') that was PTH deficient, but with supranormal levels of $1,25(OH)_2D_3$. All three groups had adequate T_4 and T_3 levels.

3) Maternal control of placental calcium transfer?

In our experiments, the combination of chronic maternal vitamin D deficiency produced by diet and maternal TPTX, led to a much greater fall in maternal, than fetal blood ionized calcium concentration. This resulted in a larger maternofetal ionized calcium concentration gradient than was seen in the other two groups, and agrees with the work of Chalon and Garel (1985A). In the 'maternal TPTX + $1,25(OH)_2D_3$' group, the low maternal blood ionized calcium concentration produced by maternal TPTX, was overcome by $1,25(OH)_2D_3$ injection, although there was still a small (but not significant) fall in fetal blood ionized calcium concentration - in fact a reversal of the maternofetal ionized calcium gradient was seen. It must be remembered, that these paired measurements of maternal and fetal ionized calcium concentration were unfortunately made in a group of animals not used for placental perfusion. However, it can be assumed that similar changes in ionized calcium levels will have occurred in the placental perfusion group also. Despite these changes, both the maternofetal calcium flux across the perfused rat placenta and the net calcium flux (as measured by calcium accretion), were comparable in the three groups. The changes seen for $K_{mf}{}^{45}Ca$ simply reflected the prevailing maternal blood ionized calcium concentration and therefore a change in the specific activity of calcium. The clearance of the extracellular marker ^{3}H-sucrose, was not altered by maternal vitamin D/PTH deficiency, suggesting that passive placental permeability was also unaffected.

A similar study was carried out by Mughal et al (1985; using

the in situ perfused rat placental model), maternal vitamin D deficiency being obtained by maintaining a strain of Wistar rats on a vitamin D deficient diet over several generations and throughout pregnancy. Once again, neither the clearance of the extracellular marker or J_{mf} calcium were affected by this vitamin D deficiency or by replacement $1,25(OH)_2D_3$ injections. Their control vitamin D deficient group had a mean maternal plasma $1,25(OH)_2D_3$ concentration of 29 ± 6pg/ml, whilst the $1,25(OH)_2D_3$ supplemented group had a concentration of 172 ± 29pg/ml (fetal blood levels were not measured). In the study of Mughal et al (1985), total calcium content of the fetuses was also not affected by maternal vitamin D deficiency or replacement, which suggests that the net flux of calcium to the fetus was constant. This agrees well with our data and suggests that the maternal PTH/$1,25(OH)_2D_3$ status does not influence maternofetal or net calcium flux across the perfused rat placenta. As described previously (Chapter 1, Section E2), similar conclusions have been reached by other workers (Halloran and DeLuca,1981; Brommage and DeLuca,1984).

In Chapter 3, the acute effect of maternal hypo- and hypercalcaemia on maternofetal calcium flux was investigated, whilst in this study, maternal calcaemia was altered by a more chronic method being controlled by maternal $1,25(OH)_2D_3$ and PTH. It is thus possible to plot graphs similar to those shown in Chapter 3 (Figures 3.5-3.8), to express how a change in maternal blood ionized calcium concentration (produced by diet and TPTX) affects the other parameters measured. The data (i.e. the mean K_{mf} of the first two perfusate collections) from the three

Figure 4.3 Regression plot (R) for $K_{mf}{}^{45}Ca$ with a variable maternal blood ionized calcium concentration, brought about by surgical and dietary interventions. (each point is the mean K_{mf} of the first two perfusate collections)

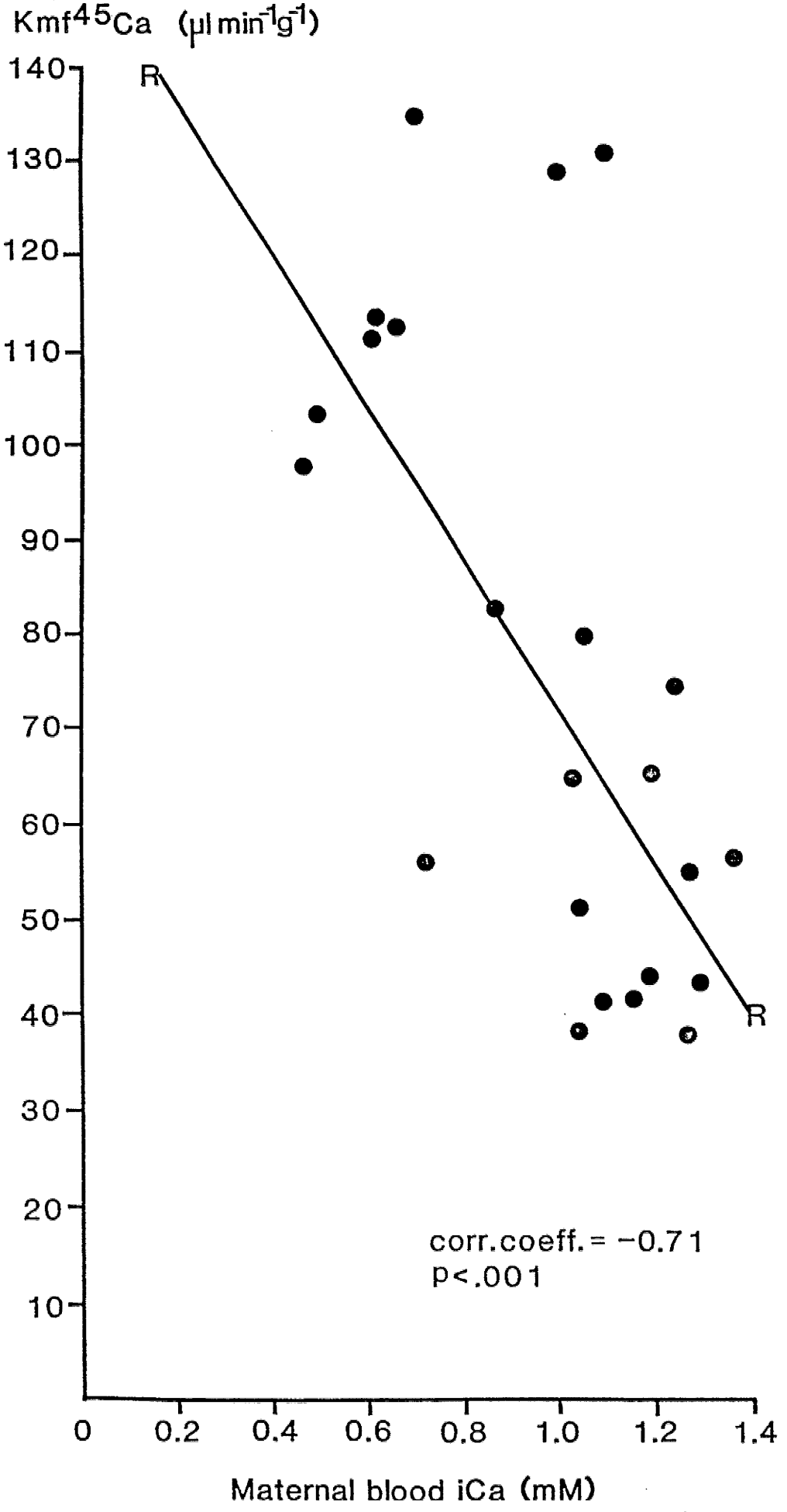


Figure 4.4 Regression plot (R) for J_{mf} calcium with a variable maternal blood ionized calcium concentration, brought about by surgical and dietary interventions (each point is the mean J_{mf} of the first two perfusate collections)

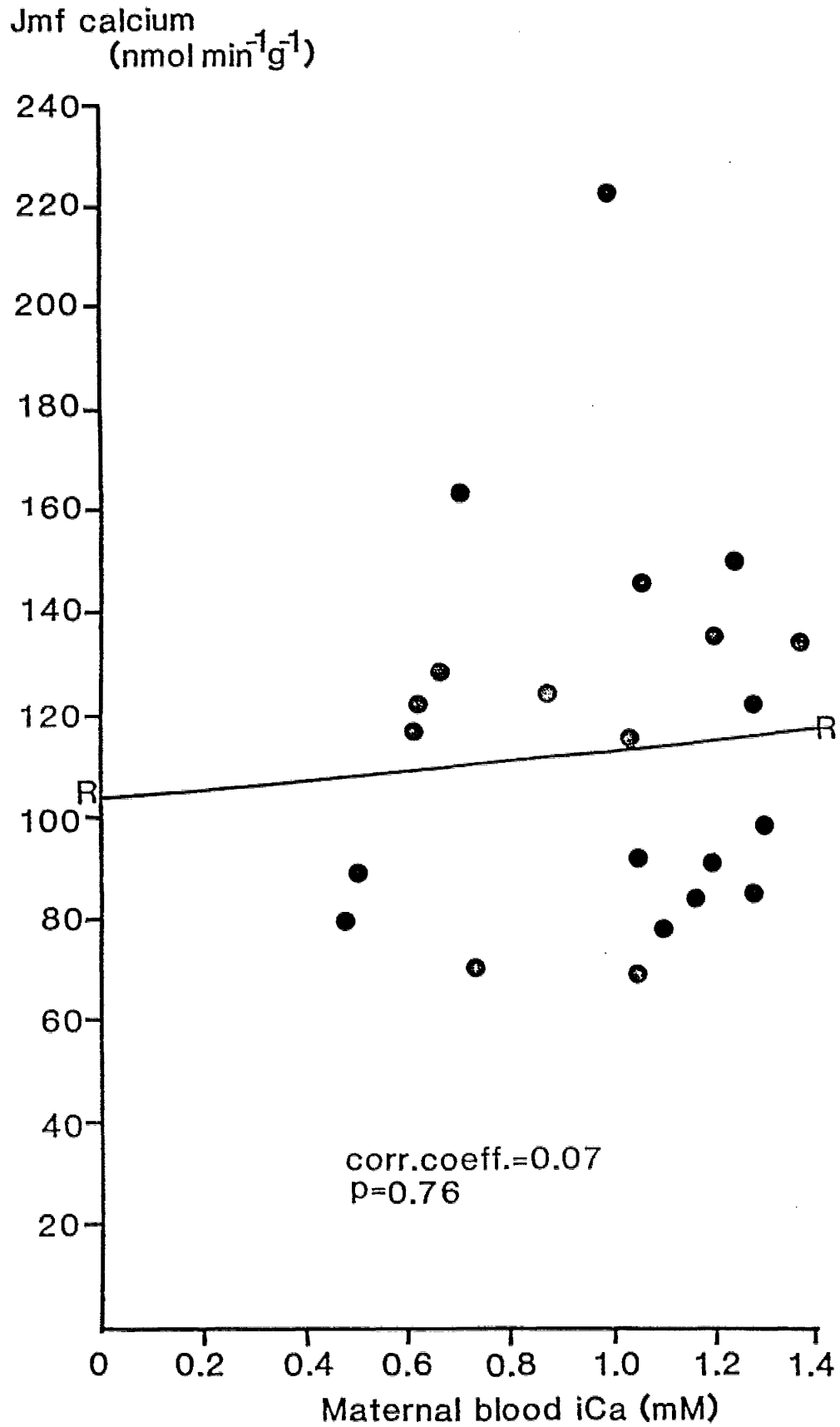
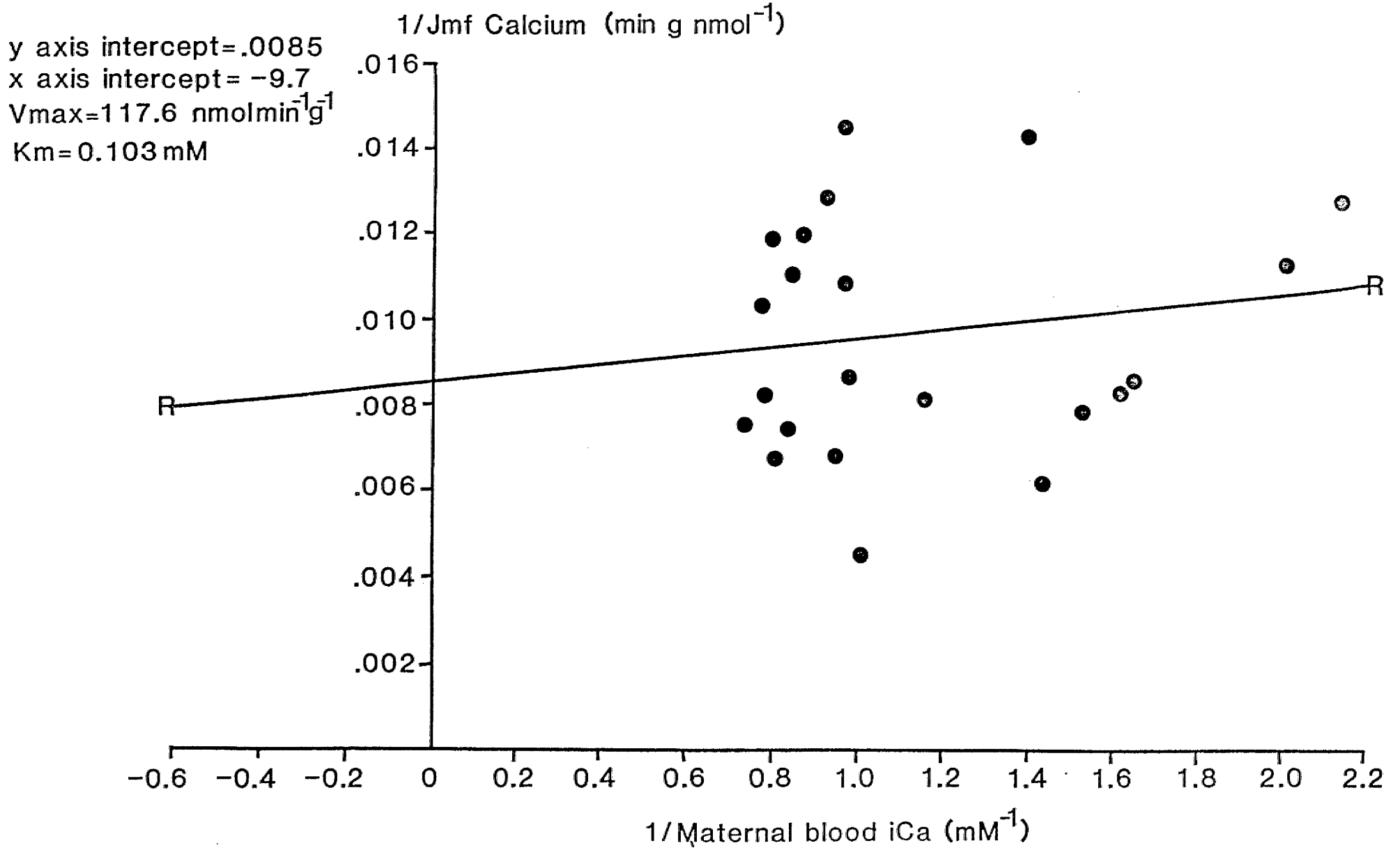


Figure 4.5 Lineweaver Burk plot showing the regression plot (R) of $1/J_{mf}$ calcium versus 1/maternal blood ionized calcium concentration. (see Chapter 3 for calculation of V_{max} and K_m)



groups were pooled and Figure 4.3 shows the expected significant ($p<0.001$) correlation between maternal blood ionized calcium concentration (iCa) and $K_{mf}{}^{45}Ca$. However (as in Chapter 3), there was no correlation between J_{mf} calcium (or $K_{mf}{}^{3}H$-sucrose) and maternal blood ionized calcium concentration (iCa; Figure 4.4). From this data, a Lineweaver Burk plot gave a V_{max} of 117.6 nmolmin^{-1} g^{-1} and a K_m of 0.103mM (Fig.4.5). However, as before, the regression line was not significant and therefore cannot provide reliable data. Nevertheless, comparison to the results in Chapter 3 show that the apparent K_m's are very similar. This suggests that the placental transport system for calcium from mother to fetus, is saturated at low maternal blood ionized calcium concentrations.

There is some discrepancy between our data and other reports in the literature. For example, Chalon and Garel (1985A) showed that maternal TPTX in rats caused a significant fall in fetal total calcium content, fetal blood total calcium and fetal growth; whereas we only observed a fall in fetal ionized calcium concentration. Gilbert et al (1980) showed that the fall in fetal plasma ionized calcium concentration, could be overcome by the grafting of one parathyroid gland into the TPTX mother. Garel et al (1981C) overcame all the fetal changes mentioned above, by maternal $1,25(OH)_2D_3$ injections, whilst Ibrahim et al (1984; rats) and Weatherley et al (1983; sheep), did not even see a fall in fetal blood ionized calcium concentration, after maternal TPTX. Injection of $1,25(OH)_2D_3$ into normal vitamin D replete guinea-pigs or ewes, significantly increased fetal total calcium content, but only slightly (not significant) stimulated

maternofetal calcium transfer in the third trimester and not at all, near term (Durand et al,1983A;1983B). So in some species it may be possible to stimulate placental calcium transfer with maternal $1,25(OH)_2D_3$, perhaps because the placental calcium transporter is working submaximally. Placental calcium transfer to the fetus may therefore be protected from maternal vitamin D deficiency in some species, but not in others. However, the different results also possibly reflect various degrees of vitamin D deficiency, incomplete TPTX and possible toxic effects of supra-physiological levels of $1,25(OH)_2D_3$.

It may be assumed that calcitonin was deficient in the TPTX, but not in the non-operated animals, consequently our data could also suggest that maternal calcitonin deficiency does not influence maternofetal calcium transport. This agrees with the work of Barlet (1985A) in 77 day gestation fetal lambs, but not in 140 day gestation fetal lambs or goats, where maternal calcitonin deficiency caused an increase in fetal bone calcium and maternofetal calcium transfer (Barlet and Garel,1975 -goat; Barlet,1985A -sheep). It is possible however, that maternal TPTX in our experiments did not produce maternal calcitonin deficiency, as the placenta has been shown to be capable of secreting calcitonin into the maternal circulation (Balabanova et al,1987B). It has been suggested that the role of maternal calcitonin during pregnancy is simply to protect the maternal skeleton from excessive resorption (Stevenson et al,1979). Maternal calcitonin should not affect the fetus, as it does not appear to cross the placenta (Garel et al,1969).

Garel et al (1980) have reported increased fetal basal bone

resorption in fetuses from TPTX mothers. This was normalized by maternal injections of $1,25(OH)_2D_3$ (suggesting some maternal $1,25(OH)_2D_3$ control), but also by maternal thyroxine injection. The thyroxine effect itself may be mediated through vitamin D metabolites, as thyroxine can increase the in vitro production of $24,25(OH)_2D_3$ in the rat (Kano and Jones,1983). Thyroxine does not seem to cross the placenta in our rat model, but it is possible that this hormone has a controlling influence over placental calcium transfer, possibly via vitamin D metabolites. It would be interesting to repeat our experiments without T_4 replacement. Similarly, the use of Ketoconazole (Glass and Eil, 1986), which significantly lowers maternal $1,25(OH)_2D_3$ levels in man, would provide another model in which to study the maternal control of placental calcium transport. Ketoconazole is a cytochrome P_{450} inhibitor and inhibits kidney ω-hydroxylation of $25OHD_3$; the problem with this being that it probably has other effects, as well as reducing $1,25(OH)_2D_3$ levels.

In summary, maternal control (via PTH and/or $1,25(OH)_2D_3$) of maternofetal and net placental calcium flux across the rat placenta does not seem to be important. This seems to contrast with the evidence (albeit indirect) in man, where vitamin D supplements during pregnancy seem to be beneficial in preventing fetal rickets and neonatal hypocalcaemia (Delvin et al,1986; Mallet et al,1986), possibly reflecting species differences.

CHAPTER 5

FETAL CONTROL OF PLACENTAL CALCIUM TRANSPORT IN THE RAT

SECTION A : INTRODUCTION

It has been suggested that the fetus is autonomous when it comes to calcium homeostasis (Care and Ross,1984). Many fetal endocrine organs are certainly active *in utero*, including the parathyroids (Thomas et al,1981), thyroid 'C' cells (Garel et al,1981A; Jarzab et al,1984) and the 25-α-hydroxylase activity of the kidney (Weisman et al,1976). Parathyroid hormone (PTH) and calcitonin do not seem to cross the placenta (Garel et al, 1969; Garel and Dumont,1972), hence all fetal PTH and calcitonin is presumably fetally derived. Removal of fetal PTH and calcitonin by thyroparathyroidectomy (TPTX) certainly seems to reduce fetal blood ionized calcium concentration, which can be overcome by PTH replacement (Pic,1965; Garel et al,1971). It has been suggested that the placenta may also be a site of PTH and calcitonin production (Balabanova et al,1987A;1987B). Fetal $1,25(OH)_2D_3$ can be derived from the mother across the placenta (Haddad et al,1971), be produced by the placenta itself (Tanaka et al,1972) or by the fetal kidney (Weisman et al,1976), which means that it is difficult to define the origin of any fetal $1,25(OH)_2D_3$. It is also difficult to pinpoint the site of action of any of these three hormones, they may be affecting fetal bone or kidney, or having a direct effect on the putative calcium pump in the placenta.

The control of fetal blood ionized calcium concentration and maternofetal calcium transfer across the *in situ* perfused rat placenta, has been studied in this chapter by investigating the effect of fetally administered hormones. Placental calcium

transfer in placentas from intact fetuses, was compared to that in placentas from decapitated fetuses. Fetuses were decapitated using the method of Jost (1947), which provides a means of grossly thyroparathyroidectomizing (TPTX) fetuses, but obviously may also remove other active endocrine glands. The hormones PTH and $1,25(OH)_2D_3$ were administered to the fetus either by subcutaneous (s.c.) injection or they were introduced into the 'fetal' perfusate.

Similarly, the action of the diterpene, forskolin, was investigated by addition to the 'fetal' perfusate. Forskolin (Seamon and Daly,1981A;1986) is a potent stimulator of adenylate cyclase, acting rapidly and reversibly in membrane and solubilized preparations, as well as in intact tissues and cells (Seamon et al,1981). Forskolin activates hormone-sensitive adenylate cyclase, even in the absence of a functional stimulatory guanine nucleotide regulatory protein (N_s protein; Seamon and Daly,1981B), thus suggesting that forskolin acts directly on the catalytic subunit of adenylate cyclase. This in turn causes the release of the second messenger cyclic adenosine monophosphate (cAMP). The activity of PTH involves the activation of adenylate cyclase and generation of cAMP (see MacIntyre,1986), as does the release of calcitonin (Care et al,1970A; Care et al,1971). Forskolin could therefore mimic the actions of both PTH and calcitonin, but not $1,25(OH)_2D_3$.

Thus, the aim of our experiments was to investigate the role of fetal PTH and $1,25(OH)_2D_3$ in the control of fetal blood ionized calcium concentration and maternofetal placental calcium transfer in the rat near term. The use of forskolin would

hopefully mimic the actions of fetal PTH and/or suggest the involvement of other molecules which act via cAMP stimulation (e.g. calcitonin), in the control of fetal calcium homeostasis. Some preliminary investigations into the effect of calcitonin on fetal blood ionized calcium concentration were also carried out.

SECTION B : MATERIALS AND METHODS

1) Animal Model - Fetal decapitation (TPTX)

Two groups of female pregnant Sprague Dawley rats maintained on a normal rat pellet diet, were used in all the experiments described in this chapter. One group was left to develop as normal until day 21 of gestation, whilst in the other group fetal decapitation (TPTX) was carried out on day 19 of gestation by the following method.

The mothers were anaesthetized with ether and the abdomen was shaved and cleaned with 0.5% chlorhexidine gluconate antiseptic. A ventral midline incision was made through the skin and peritoneum, revealing the two uterine horns. Starting with the fetus next but one to the vagina, every third fetus was operated on. Fetal decapitation (TPTX) was performed using the method of Jost (1947). Thus, the selected fetus was gently positioned in the uterus, with its nose away from the placenta and umbilical cord. Using a 5/0 Mersilk braided silk suture with a round-bodied needle (20mm; Ethicon Ltd.), a 'purse-string' suture was threaded through the uterus around the head region. Using micro-scissors, a small incision was made in the uterine wall at the centre of the 'purse-string' suture. The head of the fetus was

then carefully eased out, ensuring that both the upper and lower jaws were freed. The suture was then quickly tied off, severing the head which falls free. The head was then viewed under a light microscope to check that sufficient tissue had been removed to ensure complete removal of the thyroids and parathyroids. Several fetal decapitations had to be performed in each horn, as the survival rate was poor. The uterine horns were then gently pushed back into the peritoneal cavity. The peritoneum was sutured using a continuous stitch of plain sterile catgut B.P. (3/0; Ethicon Ltd.), with a round-bodied needle (20mm). It was important to include the facia in the stitch and not just the muscle layer, to ensure adequate healing. The skin was then closed with 3/0 Mersilk braided silk suture with a cutting needle (25mm; Ethicon Ltd.), using interrupted stitching. The abdominal surface was cleaned with antiseptic and the mother was allowed to recover and develop as normal until day 21 of gestation.

On day 21 of gestation, placentas from both groups of rats (i.e. control and operated) were perfused via the fetal circulation by the usual method (see Chapter 2). Only placentas from live decapitated fetuses were perfused, i.e. those which moved on touching and had a patent umbilical circulation.

Before the umbilical vessels were cannulated, fetal blood was taken from either an intact or decapitated fetus for measurement of ionized calcium concentration (ICA1, Radiometer, Copenhagen). For this, an axial incision (under the arm) was made in the fetus and blood was collected into a glass Pasteur pipette, which had been previously aspirated in a recommended heparin

solution (see Chapter 3). Fetal blood ionized calcium concentrations are given in mM and were measured in all experiments described in this chapter. Similarly, maternal blood ionized and total calcium concentrations were also always measured, being used for the estimation of the mean ionized to total plasma calcium concentration ratio, required for the calculation of J_{mf} calcium (see Chapter 3). The criteria for rejection of perfusion experiments were the same as those described previously (see Chapter 2 and 3). The animals were kept warm throughout the experiments with a dissection lamp and/or a hot water plate, giving maternal body temperatures ranging from 34-37°C or maintained exactly at 37°C (see each section).

2) Experiments

a) Placental perfusion with 10^{-5}M forskolin

Once the fetal circulation of a placenta from either an intact or decapitated fetus was perfusing, ^{45}Ca (10μCi) and ^{51}Cr-EDTA (40μCi) were injected into the maternal jugular vein (at time zero). Thereafter, usually 5 maternal arterial blood samples (0.5ml) were taken during the experiment for analysis of radioisotope concentration. Two 4-minute control perfusate collections were made initially, at 4-8 and 8-12 minutes after radioisotope injection (i.e. collections A and B). Then, either : a) 10^{-4}M forskolin (Calbiochem, Behring Diagnostics) in a 1:10 95% alcohol/0.9% saline solution, or b) 1:10 95% alcohol/0.9% saline solution alone, were introduced into the Krebs Ringer perfusate via a side-arm in the perfusion tubing, using an

infusion pump at a rate of 0.052mlmin^{-1}, for 28 minutes. The final concentration of forskolin in the Krebs perfusate as it entered the umbilical artery was 10^{-5}M. Because of the lag time between switching on the infusion pump to drug or diluent actually entering the umbilical artery (approx. 5 minutes), the next two perfusate collections were not analysed (12-16 and 16-20 minutes; collections C and D). The following five 4-minute collections were then taken for radioisotope analysis (collections E-I). Clearances (K_{mf} ^{51}Cr-EDTA and K_{mf} ^{45}Ca) were calculated as described previously (see Chapter 2, equation 6) and were expressed as μlmin^{-1}g^{-1} placenta. Data was also calculated as the percentage change in K_{mf} (with 10^{-5}M forskolin or diluent) from the mean K_{mf} of the two control (A and B) collection periods. In the first set of experiments, maternal body temperatures ranged from 34-37°C for both the intact and decapitated groups. In a second set of experiments, placentas from intact fetuses only were perfused, whilst accurately maintaining maternal body temperatures at 37°C.

b) Forskolin Dose Response Curve

Further experiments were carried out in which placentas from intact fetuses (maternal body temperatures at exactly 37°C), were perfused with 10^{-7}M, 10^{-6}M and 10^{-4}M forskolin. Together with the intact 10^{-5}M forskolin and 'diluent only' data obtained above (Section 2a; measured at 37°C), a dose response relationship could be demonstrated. The data was expressed as the percentage change in K_{mf} (with forskolin) from the control collections (i.e. A and B). To produce a dose response curve

showing the effect of forskolin on K_{mf} ^{51}Cr-EDTA and K_{mf} ^{45}Ca, the maximal percentage change (either positive or negative) in K_{mf} (from control values) seen in the five experimental collection periods (E-I), was used. This maximal percentage change was then plotted against the increasing concentrations of forskolin used.

c) The effect of bPTH(1-84) injected subcutaneously (s.c.) into intact and decapitated fetuses

Animals with either intact or decapitated fetuses were taken on day 21 of gestation. The mothers were anaesthetized with 110mgkg^{-1} Inactin (BYK. Gulden) and the uterus was exposed. Intact and decapitated fetuses were injected s.c. into the back of the fetal body through the uterine wall, with either : 1) 1.0IU bPTH(1-84) in 10μl diluent, or 2) 10μl diluent alone. The bovine PTH was obtained from the National Institute for Biological Standards (London, U.K.) and the diluent consisted of 1.0% sodium acetate and 0.1% bovine albumin (protease free; Sigma Ltd. 7906), made up to pH 4.0 with glacial acetic acid. The dose of bPTH(1-84) chosen for these experiments (1.0IU) was similar to that used by Garel et al (1971). They showed a significant increase in fetal blood ionized calcium concentration in intact and decapitated fetuses with 0.25 and 0.5 IU/g fetus bPTH(1-84), four hours after the injection. The bPTH(1-84) diluent was important, as bPTH(1-84) works better at an acidic pH and the bovine albumin (protease free, to stop the peptide being broken down) acts as a protein-carrier preventing bPTH(1-84) from sticking to any plastic, etc.

The abdomen was then closed and the animals were maintained

anaesthetized with maternal body temperatures ranging from 34-37°C, for the next two hours. Then the fetal circulation of a placenta from one of these intact or decapitated injected fetuses was perfused as described previously (see Chapter 2), after measurement of fetal blood ionized calcium concentration. ^{45}Ca and ^{51}Cr-EDTA were injected at time zero and three maternal arterial blood samples were taken at 3, 13 and 23 minutes, while four 4-minute perfusate collections were made (4-8, 8-12, 12-16 and 16-20). Maternal blood ionized calcium concentrations were measured (mM) and K_{mf} ^{51}Cr-EDTA , K_{mf} ^{45}Ca and J_{mf} calcium were calculated (using equation 6 and 9 respectively). Data is expressed as the mean K_{mf} or J_{mf} of the first two perfusate collections.

d) The effect of $1,25(OH)_2D_3$ injected s.c. into intact and decapitated fetuses

The protocol used in these experiments was exactly the same as that explained in Section 2c, except that both intact and decapitated fetuses were injected through the uterine wall with either :1) 20ng $1,25(OH)_2D_3$ (Roche products) in 10μl 1:100 95% alcohol/0.9% saline solution, or 2) 10μl 1:100 95% alcohol/0.9% saline solution only. Here the choice of a dose was difficult, as $1,25(OH)_2D_3$ did not seem to have any hypercalcaemic effects on fetal blood ionized calcium concentration in our rats. A large dose of $1,25(OH)_2D_3$ (20ng) was chosen (as compared to circulating fetal rat plasma $1,25(OH)_2D_3$ levels of 50pgml^{-1}; Verhaeghe et al,1986), being unsure how much would reach the fetal circulation after random s.c. injection into the fetal

back. The abdomen was closed and the animals were maintained anaesthetized at a maternal body temperature of exactly 37°C, for the next two hours (cf.Section 2c; animals between 34-37°C). The placentas were than perfused as normal and results were expressed as the mean K_{mf} or J_{mf} of the first two perfusate collections.

e) Placental perfusion with rPTH(1-34) in intact and decapitated fetuses

Placentas from intact and decapitated fetuses were perfused in the normal way on day 21 of gestation (maternal body temperatures maintained exactly at 37°C). The Krebs Ringer perfusate was modified for these experiments by the addition of 0.1% bovine albumin (protease free; Sigma Ltd. A4378) and the perfusion tubing was primed with this Krebs solution for 10-15 minutes before the start of placental perfusion at the normal rate of 0.5mlmin^{-1}. ^{45}Ca and ^{51}Cr-EDTA were injected into the mother at time zero and five maternal arterial blood samples were taken during the experiment. Two control perfusate collections were made initially at 4-8 and 8-12 minutes (i.e. A and B). Then, either :1) 4ngml^{-1} synthetic rat [Nle^{8,21},Tyr^{34}] PTH (1-34) amide, i.e. rPTH(1-34) (Peninsula Laboratories Inc., California) in 1:10 diluent/Krebs, or 2) 1:10 diluent/Krebs alone, were introduced into the Krebs perfusate via a side-arm in the perfusion tubing, at a rate of 0.04mlmin^{-1}, for 40 minutes. The diluent consisted of 1.0% sodium acetate and 0.1% bovine albumin (protease free; Sigma Ltd. A4378), made up to pH 4.0 with glacial acetic acid. rPTH(1-34) is a synthetic peptide

derived from rat PTH, which only has the amino acids numbered 1-34 in the polypeptide chain (these are the only ones necessary for complete bioactivity of the PTH molecule). This molecule was used rather than bPTH(1-84), as it is not so easily degraded and consequently will retain more of its bioactivity during perfusion. The dose of rPTH(1-34) used for placental perfusion ($4ngml^{-1}$) was similar to that used by Galceran et al (1987), who perfused isolated bones from young dogs with bPTH(1-34) and stimulated cAMP release.

As with the forskolin experiments, because of the lag time before rPTH(1-34) or diluent reached the umbilical artery, the next two perfusate collections were not analysed (12-16 and 16-20 minutes; collections C and D). The following eight 4-minute collections were then taken for radioisotope analysis (i.e. E-L). Clearances were calculated as described previously (equation 6) and maternal blood ionized calcium concentrations were measured (mM). Data was expressed as the percentage change in K_{mf} (with rPTH(1-34) or its diluent) from the mean K_{mf} of the two control (A and B) collection periods.

Unfortunately, we had no measure of the bioactivity of rPTH (1-34) once it had passed through the perfusion tubing. Consequently, several precautions had to be taken to ensure that maximum bioactivity of rPTH(1-34) was retained after passage through the perfusion tubing. As with bPTH(1-84) injection, the peptide diluent was important. A protease-free protein carrier, i.e. bovine albumin, had to be added to the Krebs Ringer perfusate and to the diluent, to solubilize the peptide. An acid pH was required for maximal bioactivity and in a few preliminary

experiments it was found that the pH of the perfusate coming out of the umbilical vein cannula did not rise above pH 7.24, even after an hour of perfusion. Unfortunately, a more concentrated solution of the diluent with a more acidic pH, caused clearances across the placenta to fall and the placenta often went white. It was also important to prime the perfusion tubing with the Krebs plus bovine albumin solution, otherwise the rPTH(1-34) would stick to the walls of the tubing and non-specifically to tissue proteins in the placenta.

f) Placental perfusion with $1,25(OH)_2D_3$ in intact and decapitated fetuses

The protocol used in these experiments was exactly the same as that described in Section 2e, except that $1,25(OH)_2D_3$ was added to the fetal perfusate instead of rPTH(1-34). The perfusion tubing was again primed with the Krebs/bovine albumin solution before placental perfusion, but this time 0.3% bovine albumin (protease free; Sigma Ltd. A4378) was added (cf.Section 2e; a higher concentration of bovine albumin was used, to act as a source of vitamin D binding protein). Therefore, after two control collections, either :1) $50pgml^{-1}$ $1,25(OH)_2D_3$ (Roche Products) in a 1:100 95% alcohol/0.9% saline solution plus 0.2% bovine albumin (Sigma Ltd. A4378), or 2) 1:100 95% alcohol/0.9% saline solution plus 0.2% bovine albumin only, was added to the perfusate. For perfusion with $1,25(OH)_2D_3$ a dose of $50pgml^{-1}$ was chosen, as this is close to the fetal circulating levels of $1,25(OH)_2D_3$ in the rat (Verghaeghe et al,1986). Ron et al (1984) suggested the addition of bovine albumin (Sigma Ltd. A7638,

protease-free) to the $1,25(OH)_2D_3$ diluent and to the Krebs perfusate, to act as a source of vitamin D binding protein. Also, the perfusion tubing had to be primed with Krebs plus bovine albumin, to prevent $1,25(OH)_2D_3$ binding to the tubing. Data is again expressed as the percentage change in K_{mf} (with $1,25(OH)_2D_3$ or diluent) from the mean K_{mf} of the two control collections (A and B). These animals were maintained with maternal body temperatures of exactly 37°C throughout the experiment.

g) The effect of calcitonin injected s.c. into intact fetuses, on fetal blood ionized calcium concentration

Three female Sprague Dawley rats were taken on day 21 of gestation and were anaesthetized with 110mgkg^{-1} Inactin (BYK. Gulden). After laparotomy, the two uterine horns were exposed and several fetuses in both horns were injected. Those in the right horn were injected with 0.20μg synthetic salmon thyro-calcitonin (Sigma Ltd.) in 20μl diluent, whilst those in the left horn were injected with 20μl diluent only. The diluent consisted of 1.0% sodium acetate and 1.0% bovine albumin, made up to pH 4.8 with acetic acid. The abdomen was closed and the animals were maintained with maternal body temperatures of exactly 37°C. Fetal blood ionized calcium concentration was then measured in pairs of fetuses (one from each horn) 30 minutes after the injection and thereafter, every 15 minutes. The results shown are those for paired fetal blood ionized calcium concentrations, 90 minutes after injection of calcitonin. Placental perfusions were not carried out in this study.

3) Statistics

All data is given as a mean ± s.e.m., expressed per gram wet weight of placenta and 'n' is the number of placentas. Statistical comparisons were made using the Student's 't' test, paired or unpaired as appropriate.

SECTION C : RESULTS

1) The effect of forskolin on K_{mf} ^{51}Cr-EDTA and K_{mf} ^{45}Ca, in the perfused rat placenta on day 21 of gestation

Figures 5.1 and 5.2 show the percentage change from control values for K_{mf} ^{51}Cr-EDTA and K_{mf} ^{45}Ca respectively, comparing saline diluent to 10^{-5}M forskolin perfusion in placentas from intact 21 day gestation fetuses. This data was obtained in animals maintained with maternal body temperatures of exactly 37°C. The trends seen were not significantly different from those experiments with maternal body temperatures ranging from 34-37°C, although the absolute values of K_{mf} ^{45}Ca were higher. Figures 5.1 and 5.2 also show the absolute values for K_{mf} ^{51}Cr-EDTA and K_{mf} ^{45}Ca in the two control periods (collections A and B). There were no significant differences in K_{mf} for either tracer between the first and second control period, or between the two groups, i.e. those to receive 10^{-5}M forskolin and those to receive saline diluent. The percentage change in K_{mf} ^{51}Cr-EDTA from control values with 10^{-5}M forskolin, was comparable to that seen with saline diluent only (Fig.5.1). However, the K_{mf} ^{45}Ca was significantly increased with the addition of 10^{-5}M forskolin, both above the control period values and when

Figure 5.1 The effect of perfusion with 10^{-5}M forskolin or its diluent on K_{mf} ^{51}Cr-EDTA, in placentas from intact fetuses on day 21 of gestation. (mean ± s.e.m., n=number of placentas)

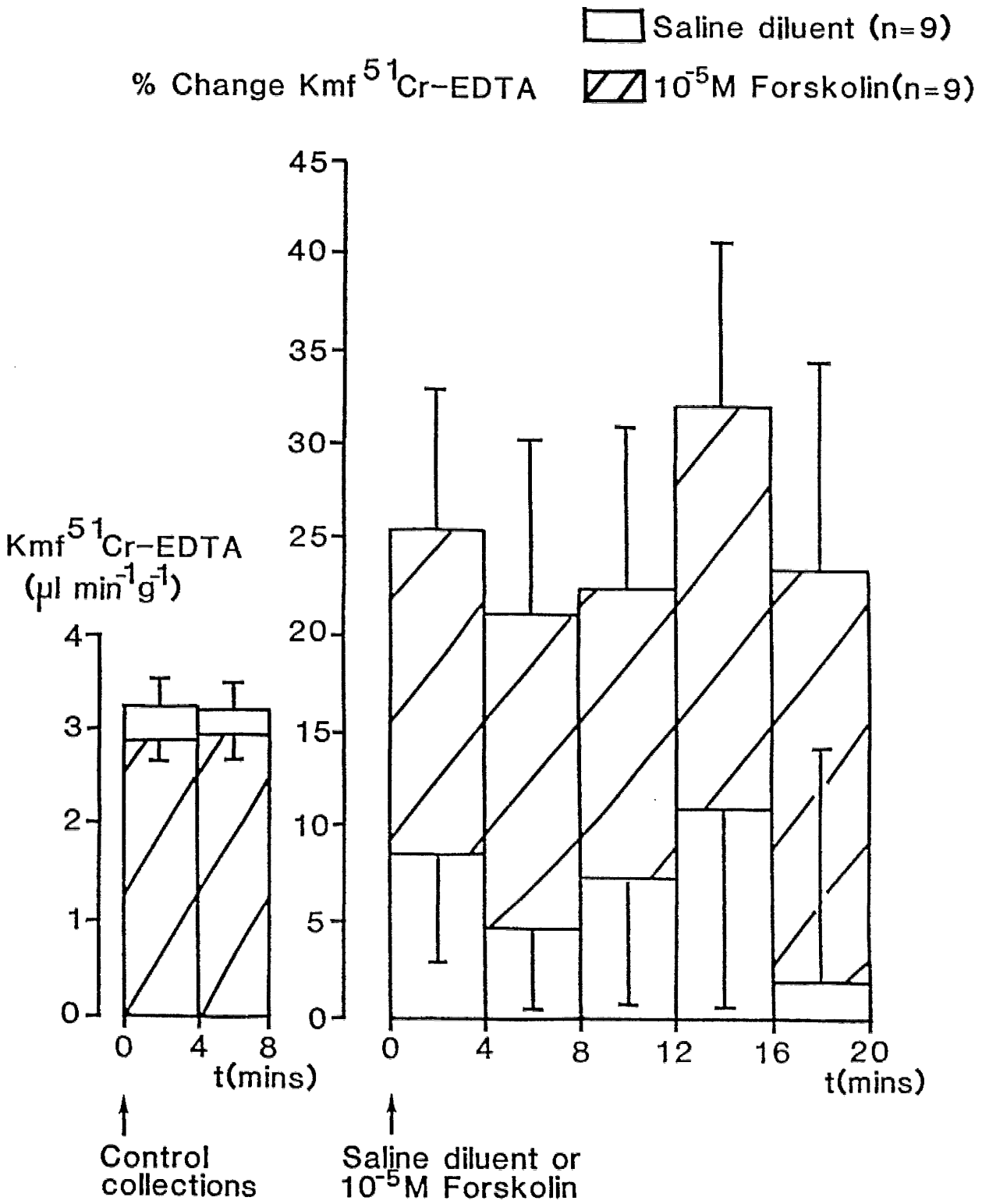
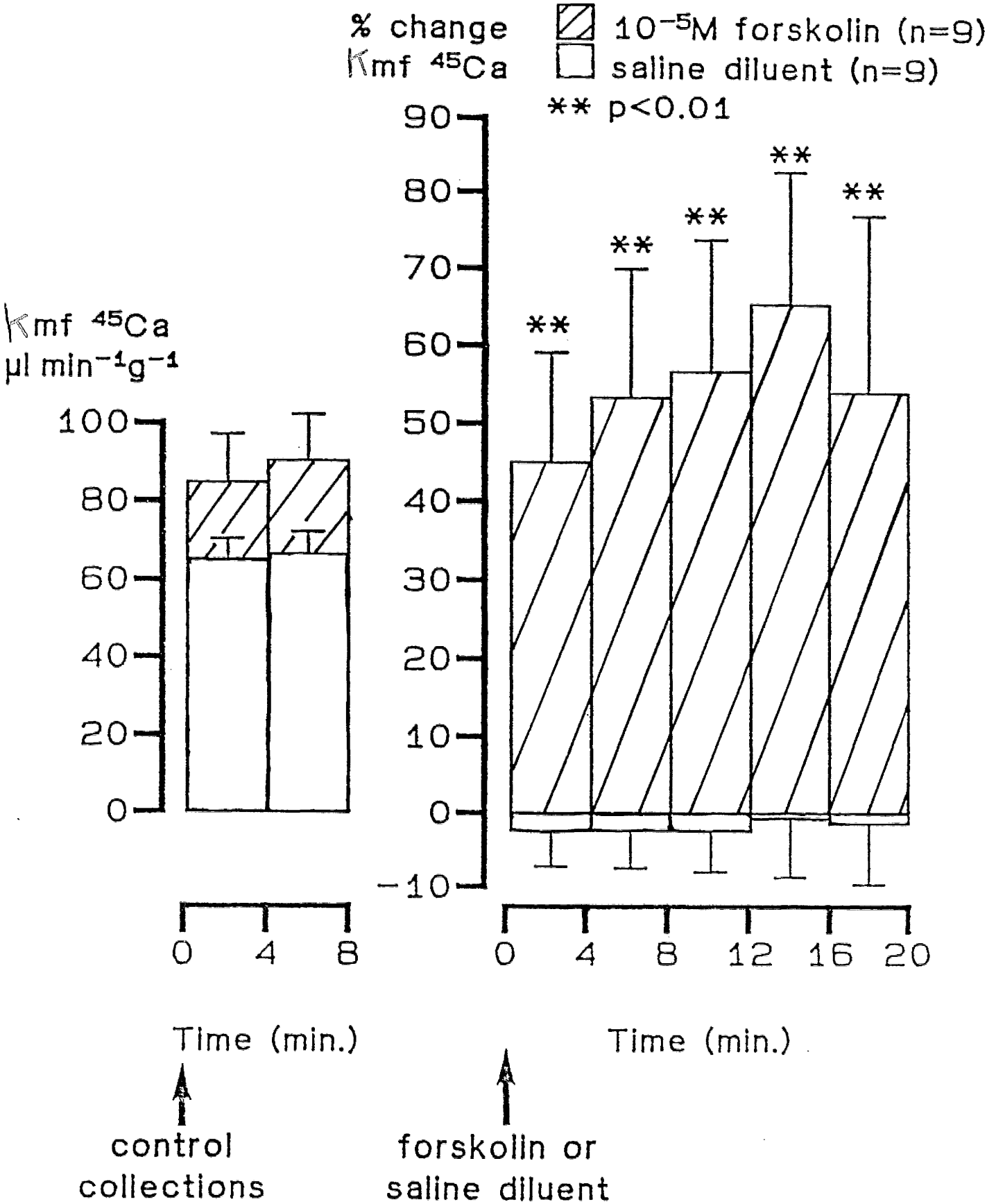


Figure 5.2 The effect of perfusion with 10^{-5}M forskolin or its diluent on $K_{mf}{}^{45}Ca$, in placentas from intact fetuses on day 21 of gestation. Unpaired Student's 't' test compared the percentage change in $K_{mf}{}^{45}Ca$ with 10^{-5}M forskolin versus saline diluent, for each experimental period (mean ± s.e.m., n=number of placentas)



compared to the percentage change with saline diluent (Fig.5.2).

The data from placentas of 21 day gestation decapitated fetuses has been expressed in a similar way, in Figures 5.3 and 5.4. These animals had maternal body temperatures ranging from 34-37°C and this set of experiments was not repeated at exactly 37°C. Comparison of data from the bPTH(1-84) injected group, with that from the $1,25(OH)_2D_3$ injected group, showed that a rise in maternal body temperature did not significantly increase $K_{mf}{}^{45}Ca$ in placentas from decapitated fetuses (see Section C2). In placentas from decapitated fetuses, 10^{-5}M forskolin significantly ($p<0.05$) increased $K_{mf}{}^{51}Cr$-EDTA (when compared to saline diluent), but only in two experimental collection periods. Perfusion with saline diluent significantly ($p<0.05$) increased $K_{mf}{}^{45}Ca$ above control values (in the first three experimental collection periods), but the increase seen with forskolin was still significantly ($p<0.05$) greater than that with saline diluent (at least in the three middle experimental collection periods). Unfortunately, in the group which were to receive saline diluent, the $K_{mf}{}^{45}Ca$ showed a significant ($p<0.01$; paired Student's 't' test) increase between the first and the second control period, suggesting that a steady state had not been reached. However, the mean of these two collection periods was still used for comparison to the experimental collection periods as we had no other baseline to work from. By using the mean of the two control collection periods in this unsteady state situation, we are probably actually over-estimating the percentage change in $K_{mf}{}^{45}Ca$ seen with saline diluent perfusion. Hence, comparison of the percentage change in

Figure 5.3 The effect of perfusion with 10^{-5}M forskolin or its diluent on K_{mf} ^{51}Cr-EDTA, in placentas from decapitated fetuses on day 21 of gestation. Unpaired Student's 't' test compared the percentage change in K_{mf} ^{51}Cr-EDTA with 10^{-5}M forskolin versus saline diluent, for each experimental period (mean ± s.e.m., n=number of placentas)

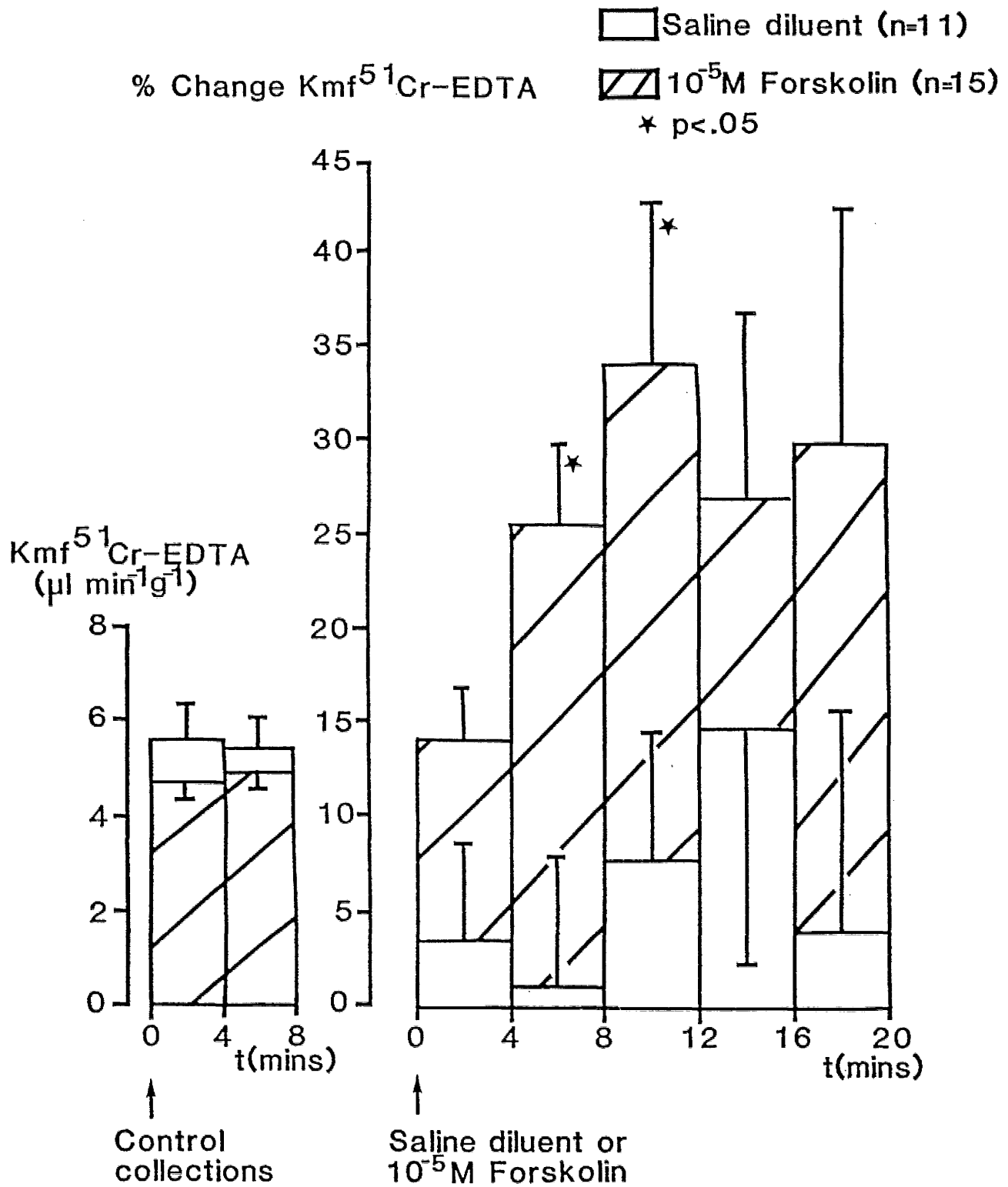
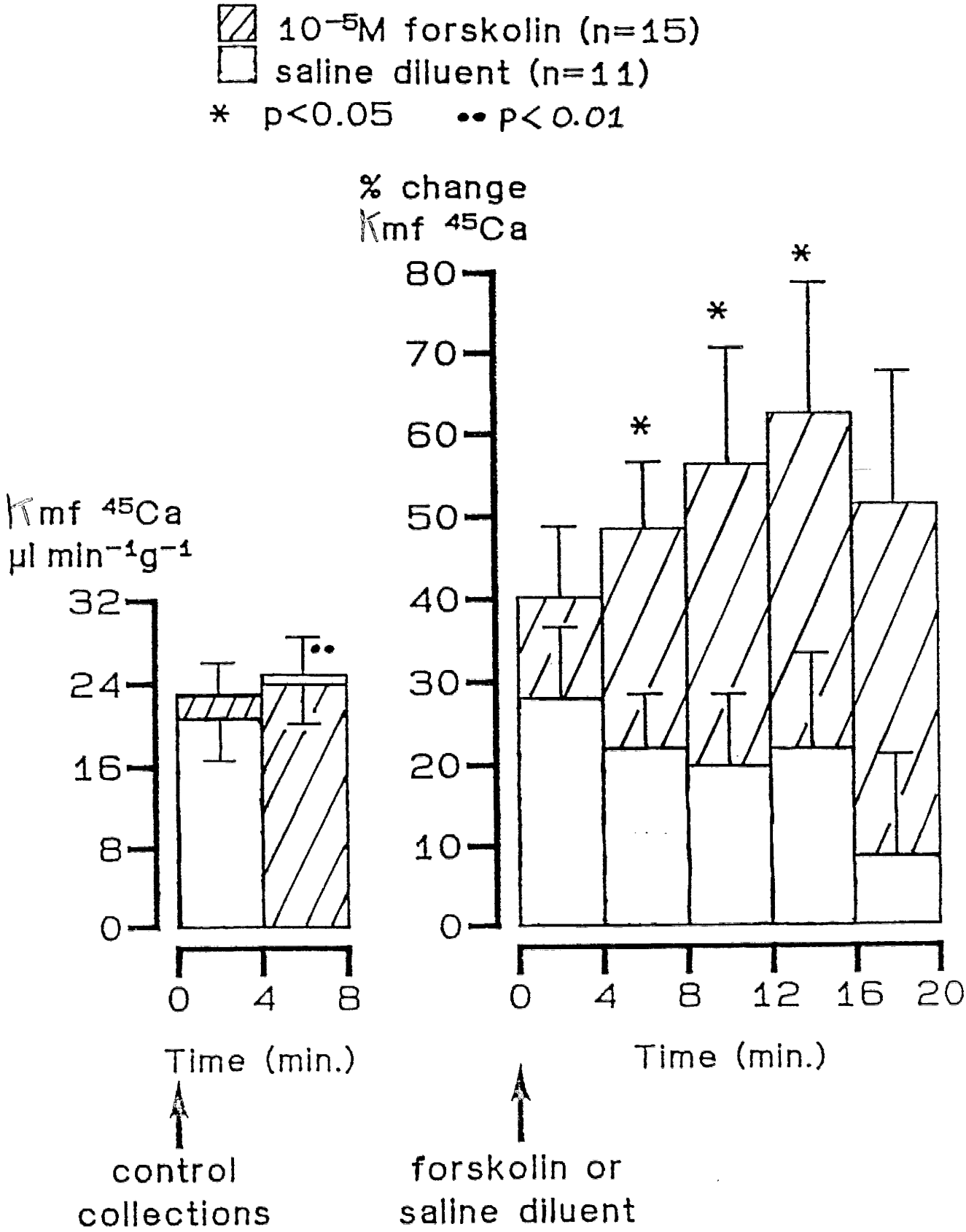


Figure 5.4 The effect of perfusion with 10^{-5}M forskolin or its diluent on $K_{mf}{}^{45}Ca$, in placentas from decapitated fetuses on day 21 of gestation. Unpaired Student's 't' test compared the percentage change in $K_{mf}{}^{45}Ca$ with 10^{-5}M forskolin versus saline diluent, for each experimental period (* $p<0.05$). Paired Student's 't' test compared the first control period versus the second control period (•• $p<0.01$). (mean ± s.e.m., n=number of placentas)



$K_{mf}{}^{45}Ca$ seen with forskolin perfusion, to that with saline diluent perfusion, may be even greater than that calculated here. The control period $K_{mf}{}^{51}Cr$-EDTA and $K_{mf}{}^{45}Ca$ values measured in these two groups, ie: those to receive saline diluent and those to receive forskolin, were not significantly different from each other.

Therefore, the data obtained with $10^{-5}M$ forskolin perfusion in placentas from intact and decapitated fetuses on day 21 of gestation was comparable, with the exception of the significant effect of forskolin on $K_{mf}{}^{51}Cr$-EDTA in the latter group. The major differences to note between data from intact and decapitated fetuses, were that both control measurements of $K_{mf}{}^{45}Ca$ and fetal blood ionized calcium concentrations were significantly ($p<0.001$) lower in the decapitated group. Fetal blood ionized calcium concentration measurements in intact and decapitated fetuses were 1.42 ± 0.02mM (n=22) and 1.22 ± 0.015mM (n=38) respectively.

The effect of forskolin perfusion was investigated further in placentas from 21 day gestation intact fetuses, using different concentrations of the drug (in animals with maternal body temperatures of exactly 37°C). The effect of $10^{-7}M$ forskolin perfusion was not significantly diffferent to that of saline diluent perfusion for either $K_{mf}{}^{51}Cr$-EDTA or $K_{mf}{}^{45}Ca$. Neither $10^{-6}M$ nor $10^{-4}M$ forskolin had any significant effect on $K_{mf}{}^{51}Cr$-EDTA (as with $10^{-5}M$), whilst $K_{mf}{}^{45}Ca$ was significantly ($p<0.05$) increased by $10^{-6}M$ forskolin, but only in the first three experimental collection periods. $10^{-4}M$ forskolin significantly ($p<0.01$) increased $K_{mf}{}^{45}Ca$ in all five of the experimental

Figure 5.5 Forskolin dose response curve, showing the maximal percentage change in K_{mf} ^{51}Cr-EDTA with increasing doses of forskolin, in placentas from intact fetuses on day 21 of gestation. (mean ± s.e.m., n=number of placentas)

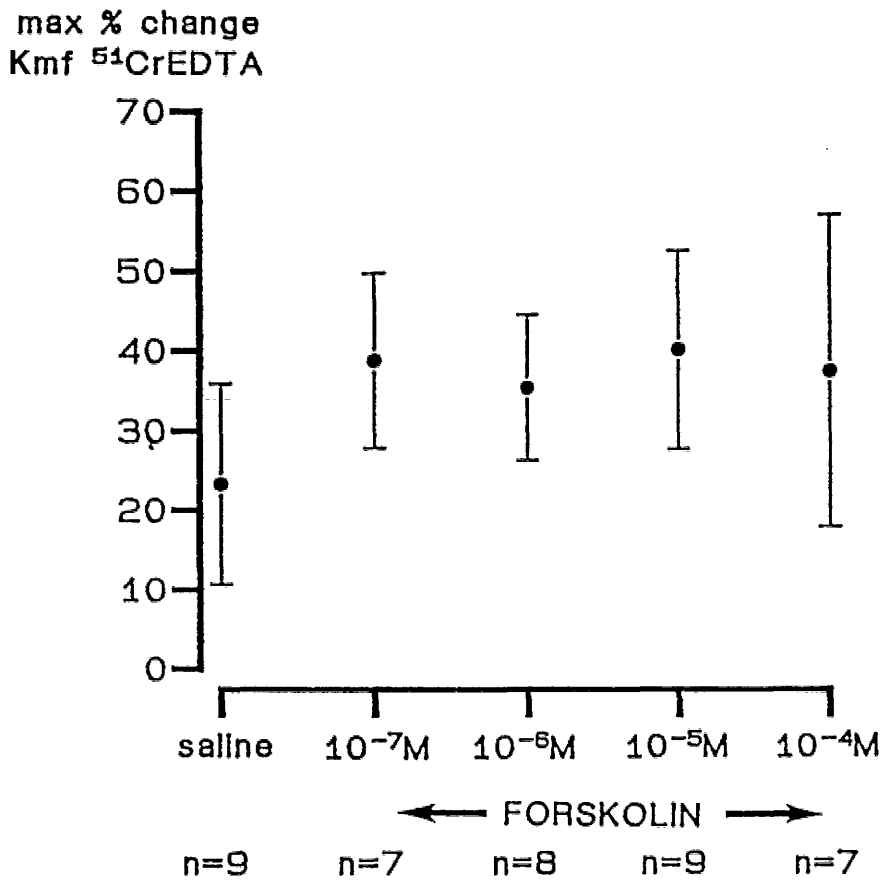
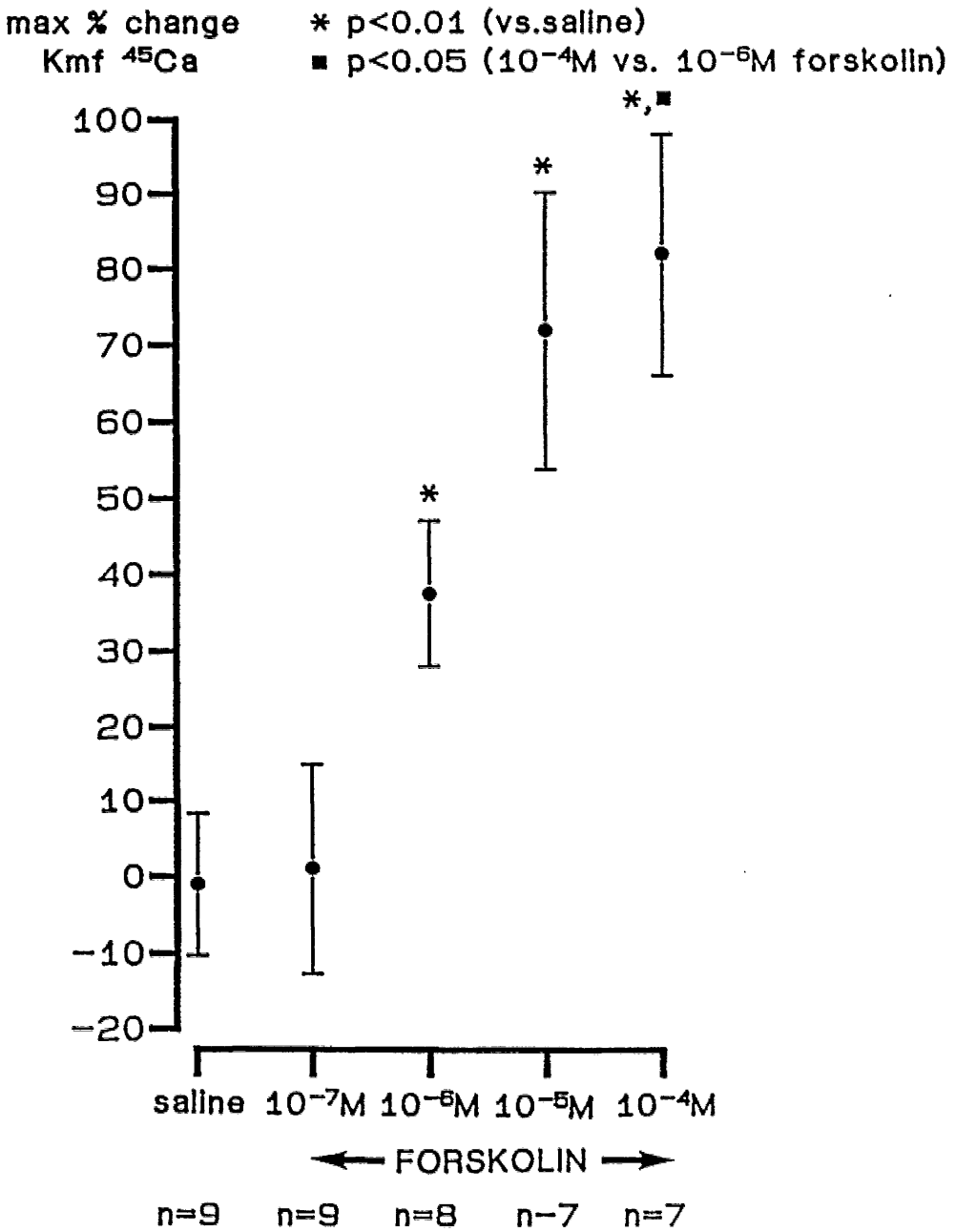


Figure 5.6 Forskolin dose response curve, showing the maximal percentage change in $K_{mf}{}^{45}Ca$ with increasing doses of forskolin, in placentas from intact fetuses on day 21 of gestation. Unpaired Student's 't' test compared the change with $10^{-4}M$, $10^{-5}M$ and $10^{-6}M$ versus saline diluent (* $p<0.01$). ■ $p<0.05$ compared the change with $10^{-4}M$ versus $10^{-6}M$. (mean ± s.e.m., n=number of placentas)



periods (as with 10^{-5}M). Figure 5.5 and 5.6 show the dose response curves for the effect of forskolin on $K_{mf}{}^{51}$Cr-EDTA and $K_{mf}{}^{45}$Ca, respectively. Here, the maximal percentage change in K_{mf} (from control values) has been plotted and for $K_{mf}{}^{51}$Cr-EDTA there were no significant differences between any of the concentrations of forskolin used and saline diluent. However, 10^{-6}M, 10^{-5}M and 10^{-4}M forskolin all showed a significant ($p<0.01$) stimulation of $K_{mf}{}^{45}$Ca above control levels, when compared to saline diluent. Also, 10^{-4}M forskolin showed a significantly ($p<0.05$) greater effect on $K_{mf}{}^{45}$Ca than was seen with 10^{-6}M forskolin.

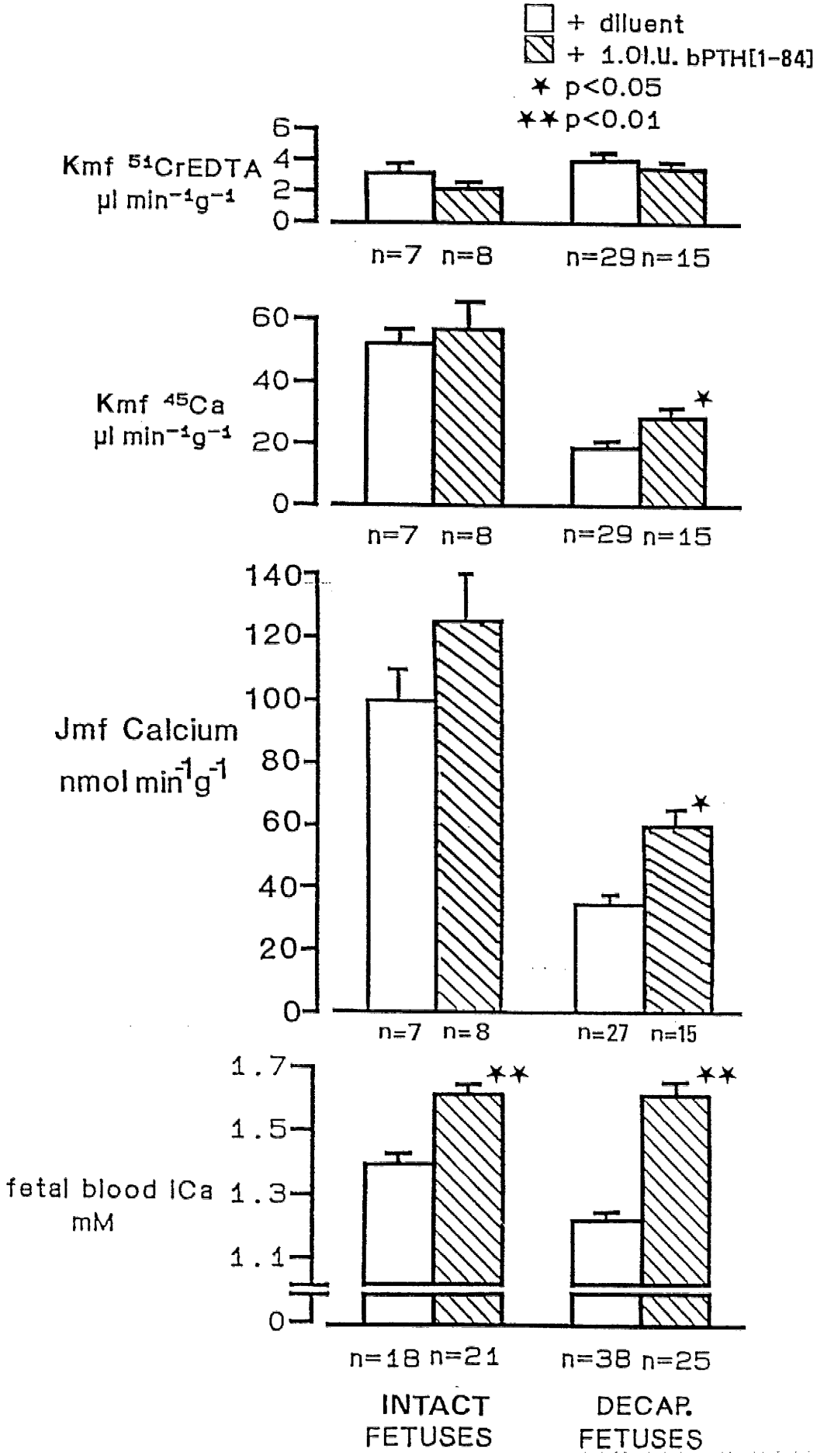
Maternal blood ionized calcium concentrations were not significantly different between any group in these forskolin studies and therefore the effects of forskolin on $K_{mf}{}^{45}$Ca as described, are identical for J_{mf} calcium.

2) The effect of bPTH(1-84) and 1,25(OH)$_2$D$_3$ injected s.c. into intact and decapitated fetuses

In this study, the bioactivity of our bPTH(1-84) was checked by injecting 0.58 IU s.c. into intact fetuses, leaving three hours and then measuring fetal blood ionized calcium concentrations. Comparison of fetuses in one horn injected with bPTH(1-84) to the other horn injected with diluent, showed a significant ($p<0.001$; paired Student's 't' test) increase in fetal blood ionized calcium concentration with bPTH(1-84) (from 1.39 ± 0.04mM, n=8, to 1.69 ± 0.04mM, n=8).

Figure 5.7 shows the effect of bPTH(1-84) or its diluent (2 hours after injection) on $K_{mf}{}^{51}$Cr-EDTA, $K_{mf}{}^{45}$Ca, J_{mf} calcium and

Figure 5.7 The effect of fetally injected 1.0IU bPTH(1-84) or its diluent on placental calcium transfer, in placentas from intact and decapitated fetuses on day 21 of gestation. Unpaired Student's 't' test compared bPTH(1-84) data versus diluent data. (mean ± s.e.m., n=number of placentas)

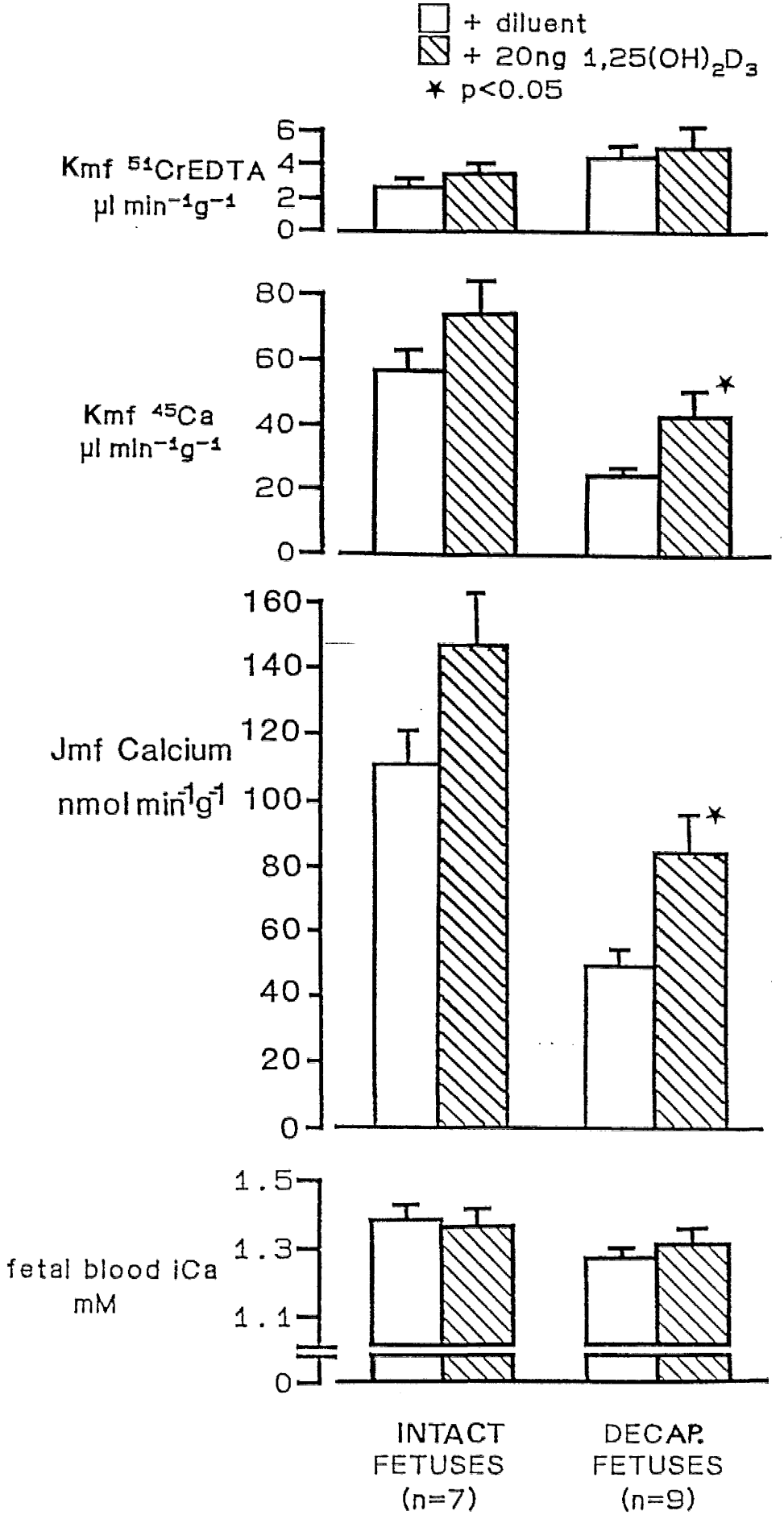


fetal blood ionized calcium concentration (iCa), in intact and decapitated fetuses. 1.0 IU bPTH(1-84) significantly ($p<0.01$) increased fetal blood ionized calcium concentration in both intact and decapitated fetuses after 2 hours, as compared to diluent injection. Fetal decapitation itself caused a significant ($p<0.001$) fall in fetal blood ionized calcium concentration when compared to control intact fetuses, this being increased up to and above control levels by bPTH(1-84). bPTH(1-84) seemed to have no effect on K_{mf} ^{51}Cr-EDTA in either group, nor on K_{mf} ^{45}Ca or J_{mf} calcium in placentas from intact fetuses. However, the significant ($p<0.001$) fall in K_{mf} ^{45}Ca caused by fetal decapitation (as compared to controls) was significantly ($p<0.05$) reversed by bPTH(1-84), but not back up to control (intact fetus) levels. A similar effect was seen for J_{mf} calcium in the decapitated group, but here the bPTH(1-84) stimulated increase was even greater, due to a significantly higher ($p<0.01$) maternal blood ionized calcium concentration in this group when compared to the decapitated group with diluent injection only. The maternal blood ionized calcium concentration in the 'intact + bPTH(1-84)' group, was also significantly ($p<0.05$) higher than the intact group with diluent injection only. However, this difference was not large enough to significantly affect J_{mf} calcium.

Figure 5.8 shows the effect of $1,25(OH)_2D_3$ or diluent injection on K_{mf} ^{51}Cr-EDTA, K_{mf} ^{45}Ca and fetal blood ionized calcium concentration (iCa). Once again, fetal decapitation caused a significant ($p<0.05$) fall in fetal blood ionized calcium concentration, but $1,25(OH)_2D_3$ had no effect on fetal

Jmf calcium

Figure 5.8 The effect of fetally injected 20ng $1,25(OH)_2D_3$ or its diluent on placental calcium transfer, in placentas from intact and decapitated fetuses on day 21 of gestation. Unpaired Student's 't' test compared $1,25(OH)_2D_3$ data versus diluent data. (mean ± s.e.m., n=number of placentas)



blood ionized calcium concentration in either intact or decapitated fetuses (cf. bPTH(1-84)). Similarly, $1,25(OH)_2D_3$ had no effect on K_{mf} ^{51}Cr-EDTA in both groups, nor on K_{mf} ^{45}Ca in placentas from intact fetuses. However, as with bPTH(1-84), the significant ($p<0.001$) fall in K_{mf} ^{45}Ca seen in placentas from decapitated fetuses was significantly ($p<0.05$) reversed by 20ng $1,25(OH)_2D_3$, but not back up to control (intact fetus) levels. Maternal blood ionized calcium concentration measurements in this $1,25(OH)_2D_3$ study were comparable in all four groups, hence similar trends were seen for J_{mf} calcium.

To summarize these results, $1,25(OH)_2D_3$ did not have the same effect as bPTH(1-84) on fetal blood ionized calcium concentration, but both hormones stimulated maternofetal calcium flux in placentas from decapitated fetuses where the flux was low to start with.

It is noteworthy, that although the animals used for the bPTH(1-84) study showed maternal body temperatures ranging from 34-37°C and those for the $1,25(OH)_2D_3$ study had maternal body temperatures of exactly 37°C, there seemed to be little difference in the absolute values of K_{mf} ^{45}Ca in placentas from either intact or decapitated fetuses in these experimental groups.

3) Placental perfusion with rPTH(1-34) and $1,25(OH)_2D_3$ in intact and decapitated fetuses

In a few preliminary experiments, it was checked that the dose of rPTH(1-34) used (4ngml^{-1}) would cause a rise in fetal blood ionized calcium concentration in intact fetuses. 4ng rPTH(1-34)

was injected into intact rat fetuses in one uterine horn, whilst fetuses in the other horn received diluent only. After two hours, rPTH(1-34) had caused a significant ($p<0.001$; paired Student's 't' test) increase in fetal blood ionized calcium concentration, from 1.32 ± 0.03mM (n=12) to 1.51 ± 0.03mM (n=12).

Figures 5.9 and 5.10 show the percentage change in $K_{mf}{}^{45}Ca$ (with rPTH(1-34) or diluent) from control values, in placentas from intact and decapitated fetuses respectively (all animals maintained exactly at 37°C). The absolute $K_{mf}{}^{45}Ca$ values were significantly ($p<0.001$) lower in placentas from decapitated fetuses (as usual), but perfusion with rPTH(1-34) had no significant effect on $K_{mf}{}^{45}Ca$ in placentas from either intact or decapitated fetuses. $K_{mf}{}^{51}Cr$-EDTA was also not significantly affected in either group (data not shown). In the placentas from intact fetuses the control $K_{mf}{}^{45}Ca$ values were slightly, but significantly higher ($p<0.05$) in the group to receive rPTH(1-34), than in the group to receive diluent.

The perfusion of placentas from intact fetuses with $1,25(OH)_2D_3$, had no significant effect on either the percentage change in $K_{mf}{}^{51}Cr$-EDTA (data not shown) or $K_{mf}{}^{45}Ca$ (Figure 5.11) from control values. Unfortunately, in the placentas from decapitated fetuses, the control values of $K_{mf}{}^{51}Cr$-EDTA were significantly higher ($p<0.05$) in the group to receive $1,25(OH)_2D_3$ than in the group to receive diluent. The opposite was found for $K_{mf}{}^{45}Ca$, with control values being significantly ($p<0.01$) higher in the group to receive diluent. Nevertheless, $K_{mf}{}^{51}Cr$-EDTA was unchanged by $1,25(OH)_2D_3$ perfusion in placentas

Figure 5.9 The effect of perfusion with 4ng/ml rPTH(1-34) or its diluent on $K_{mf}{}^{45}Ca$, in placentas from intact fetuses on day 21 of gestation. Unpaired Student's 't' test compared rPTH(1-34) control data versus diluent control data. (mean ± s.e.m, n=number of placentas)

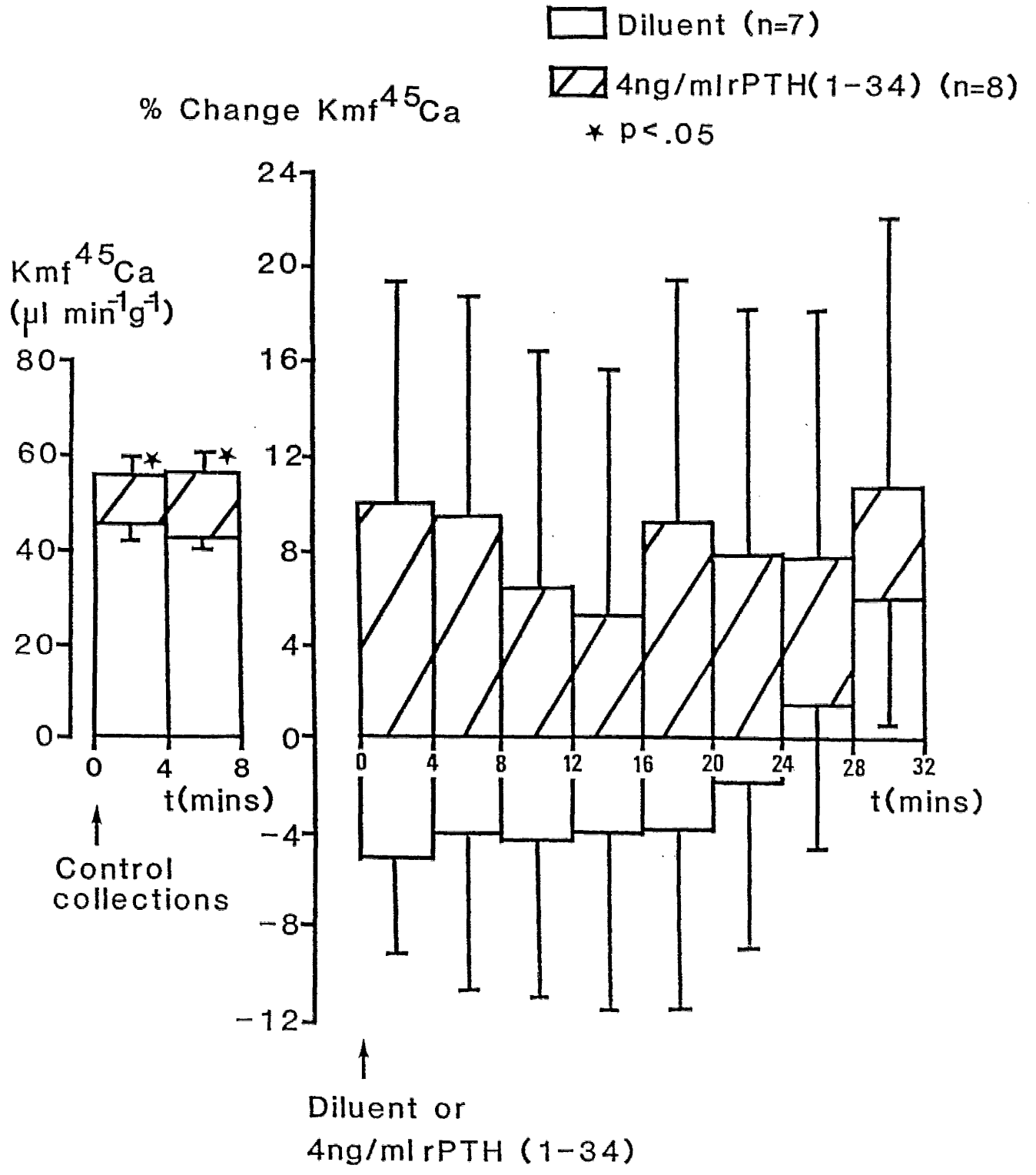
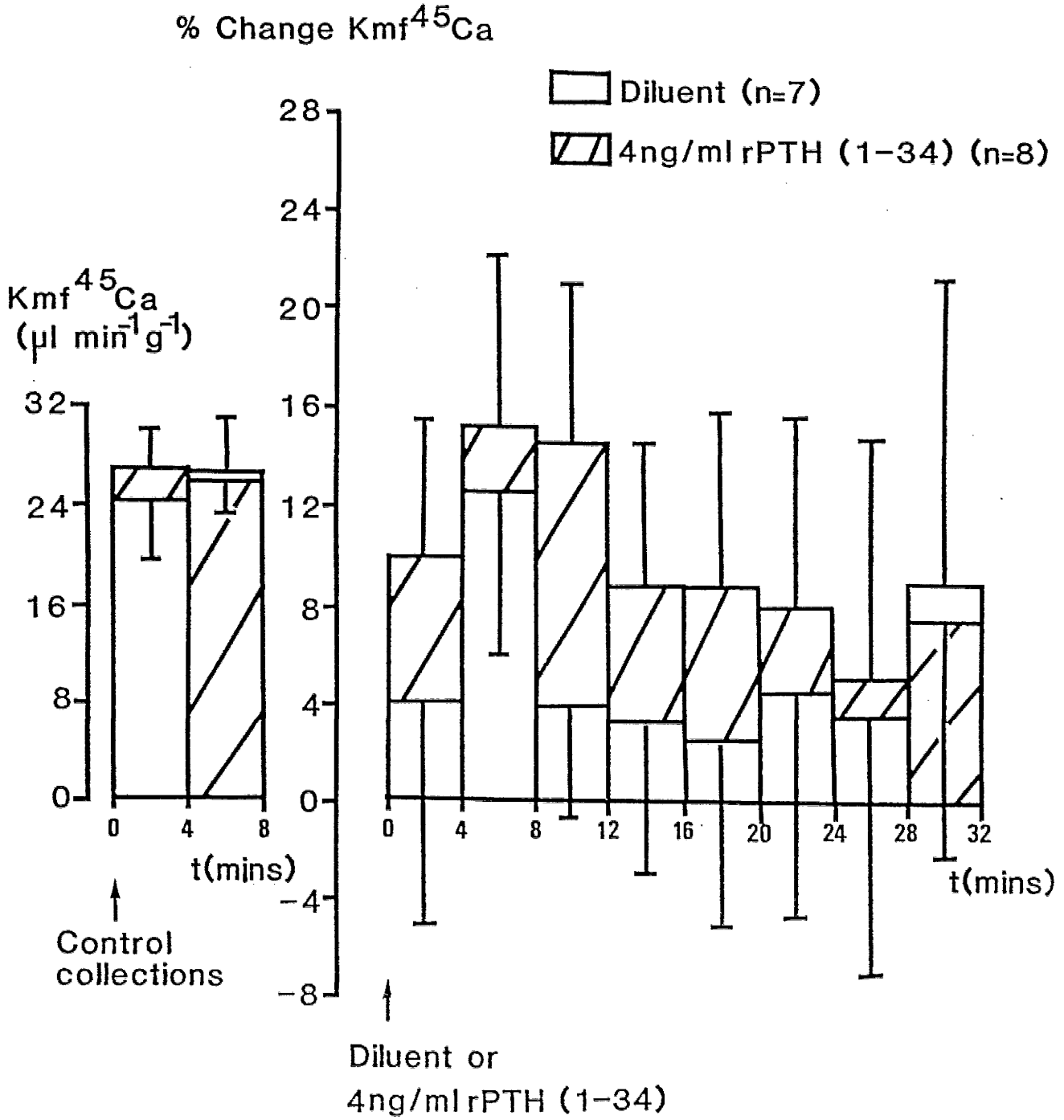


Figure 5.10 The effect of perfusion with 4ng/ml rPTH(1-34) or its diluent on $K_{mf}{}^{45}Ca$, in placentas from decapitated fetuses on day 21 of gestation. (mean ± s.e.m., n=number of placentas)



from decapitated fetuses (Fig.5.12), when compared to diluent perfusion. Whereas, the hormone significantly increased $K_{mf}{}^{45}Ca$ in 7 out of 8 experimental periods, as compared to diluent perfusion (Fig.5.13). There were no differences in maternal blood ionized calcium concentration between any of the groups in these perfusion studies, so J_{mf} calcium followed the same trends as $K_{mf}{}^{45}Ca$.

4) The effect of calcitonin injected s.c. into intact fetuses, on fetal blood ionized calcium concentration

Injection of 0.2μg calcitonin into normal intact 21 day gestation fetuses, caused a significant ($p<0.01$; paired Student's 't' test) fall in fetal blood ionized calcium concentration after 90 minutes (but not before). Fetal blood ionized calcium concentration falling from 1.40 ± 0.03mM (n=9), to 1.24 ± 0.04mM (n=9).

Figure 5.11 The effect of perfusion with 50pg/ml 1,25$(OH)_2D_3$ or its diluent on $K_{mf}{}^{45}Ca$, in placentas from intact fetuses on day 21 of gestation. (mean ± s.e.m, n=number of placentas)

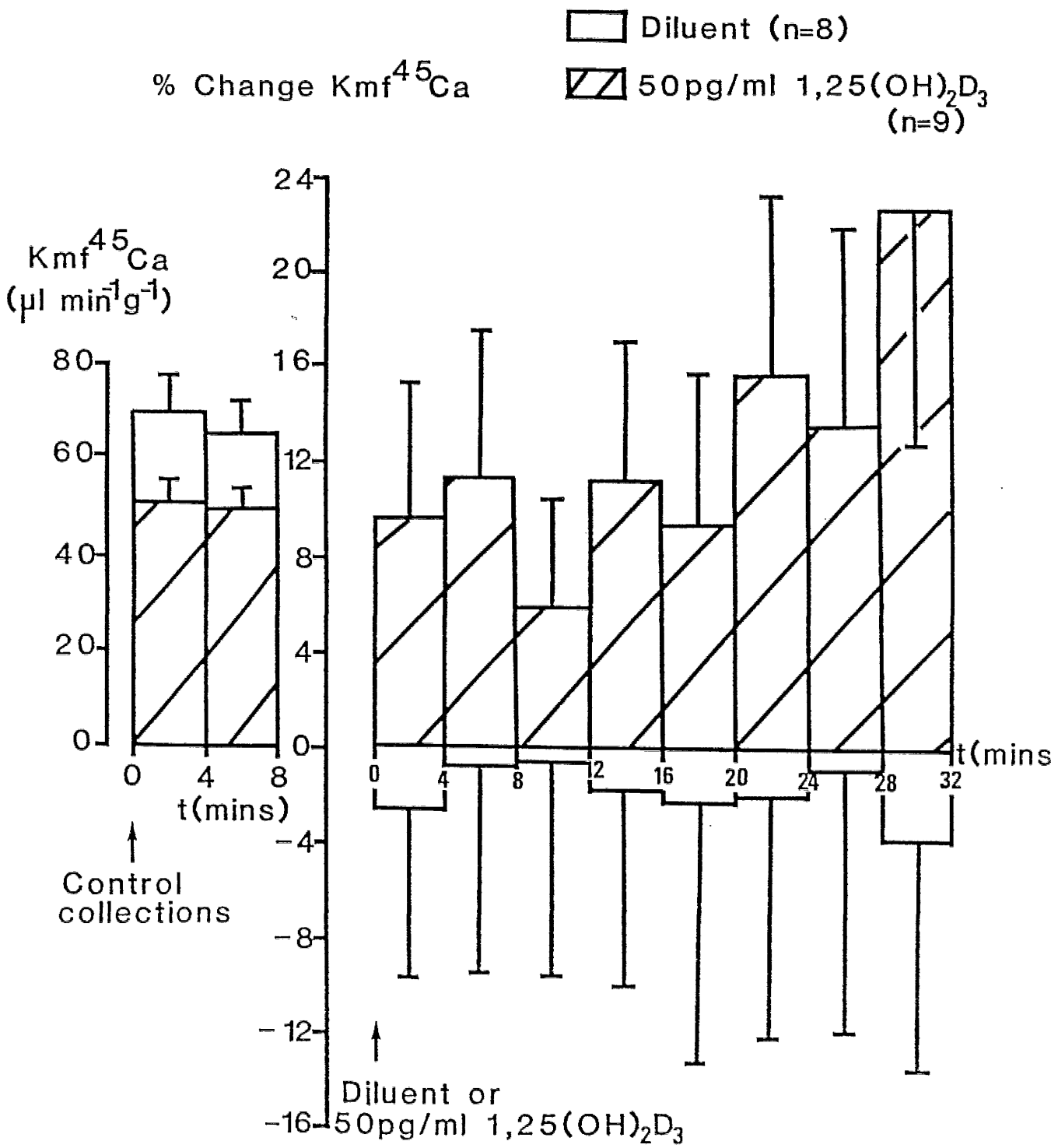


Figure 5.12 The effect of perfusion with 50pg/ml $1,25(OH)_2D_3$ or its diluent on $K_{mf}{}^{51}Cr$-EDTA, in placentas from decapitated fetuses on day 21 of gestation. Unpaired Student's 't' test compared $1,25(OH)_2D_3$ control data versus diluent control data. (mean ± s.e.m., n=number of placentas)

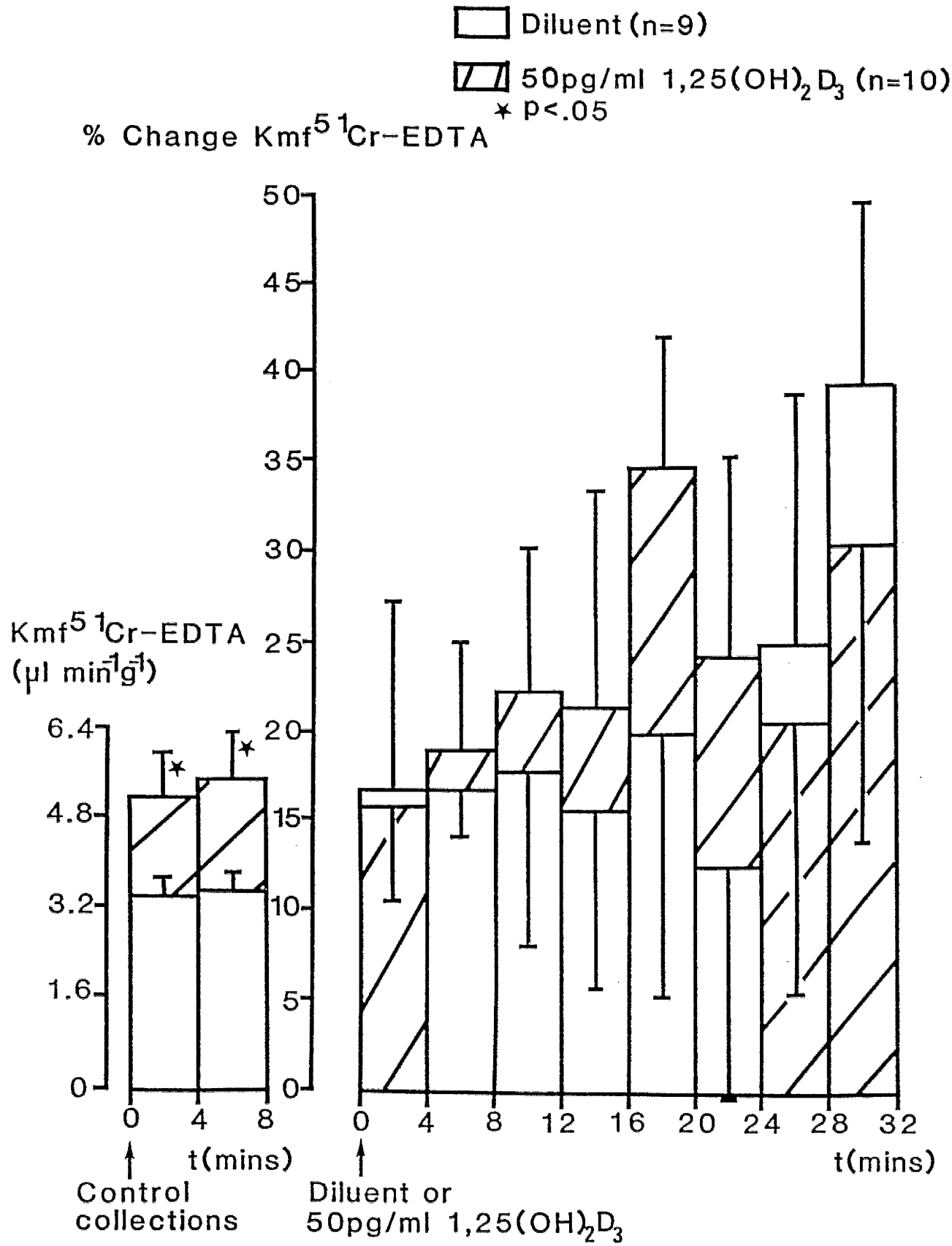
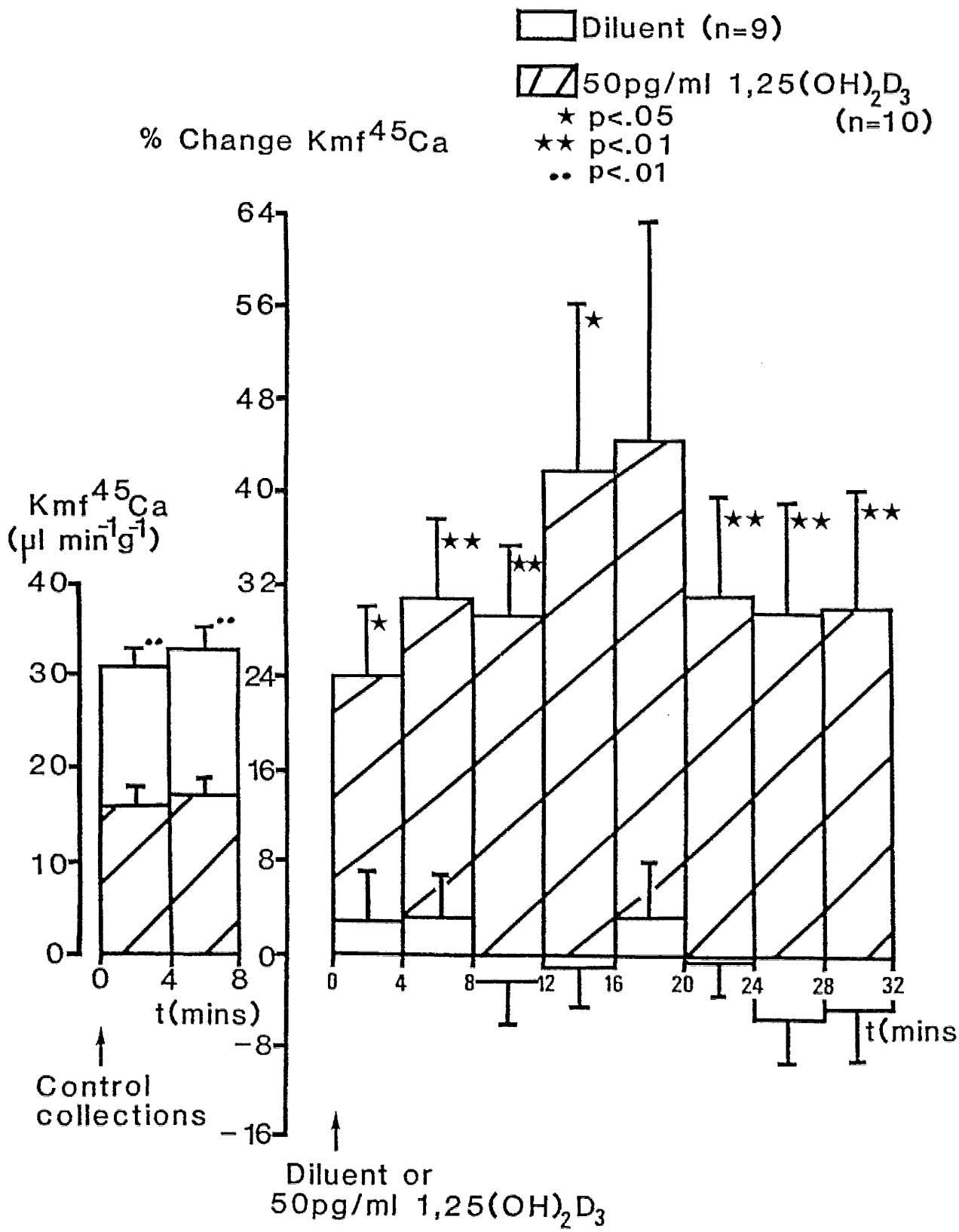


Figure 5.13 The effect of perfusion with 50pg/ml $1,25(OH)_2D_3$ or its diluent on $K_{mf}{}^{45}Ca$, in placentas from decapitated fetuses on day 21 of gestation. Unpaired Student's 't' test compared the percentage change in $K_{mf}{}^{45}Ca$ with $1,25(OH)_2D_3$ versus diluent, for each experimental period (* $p<0.05$, ** $p<0.01$). Unpaired Student's 't' test compared $1,25(OH)_2D_3$ control data versus diluent control data (•• $p<0.01$).
(mean ± s.e.m., n=number of placentas)



SECTION D : DISCUSSION

1) Viability of the fetal decapitation model

Fetal thyroparathyroidectomy by decapitation (Jost,1947) is a very gross preparation, because of the accompanying hypophysectomy and loss of brain tissue. However, the selective ablation of fetal thyroid and parathyroid tissue under the microscope (Chalon and Garel,1985B), is extremely difficult and still requires major surgery and handling of the uterine tissue, as well as leaving uncertainty as to whether all the tissue has been removed. It is not clear what role if any, the fetal pituitary and brain may have in the control of placental transfer. However, the fact that it is possible to overcome the fall in fetal rat blood ionized calcium concentration caused by fetal decapitation, by injection of PTH (Garel et al,1971; see Section C2), suggests that at least some fetal endocrine target tissues are still viable in the decapitated fetus. The main problem with the decapitation method was the poor success rate, losses resulting from maternal death caused by blood loss, as well as death of the decapitated fetuses *in utero*.

Growth may be affected by fetal decapitation, although it is difficult to assess due to the lack of the head tissue. The fetuses do however continue to grow until birth, after decapitation *in utero* and, in the rabbit, although there were large variations in fetal growth in animals which were submitted to intrauterine surgery, there was no statistical difference in body growth between decapitated and control fetuses (Jost,1954). Rat fetuses submitted to any kind of surgery in the uterus,

usually suffer from some reduction of the growth rate, although again this is not usually a significant effect. Therefore, Jost (1954) concluded that the fetal pituitary gland and brain have at the most a slight effect on fetal growth. Fetal decapitation seems to prolong gestation, delaying parturition of decapitates in pigs (Stryker and Dzuik,1975). This is probably due to the reduced activity of the adrenals in decapitates. The actual weight of decapitated pig fetuses being comparable to normals by birth. However, they contained 5-10% less protein than intact fetuses and had a higher water content (Stryker and Dziuk,1975). Furthermore, fetal decapitation did not alter the histology of the placenta in pigs, but altered lipid metabolism, elevating placental glycogen levels and increasing fatty acid synthesis (Ramsay et al,1985). This may support the concept of a fetal influence on placental metabolism.

From our observations, the fetuses decapitated in utero certainly seemed to be smaller (1.96 ± 0.05g; n=107; fetal weight minus head tissue), but there was a large size range, consequently it was difficult to assess how growth retarded they were. However, it was clear that placental weights of decapitated fetuses were not significantly different from normal (0.43 ± 0.008g; n=107; cf. Chapter 2). Our fetal decapitations on day 19 of gestation certainly caused a significant ($p<0.001$) fall in fetal blood ionized calcium concentration, which agrees with Garel et al (1971; on day 18.5). Fetal blood ionized calcium concentration (mM) fell to a mean of 1.22 ± 0.015mM (n=38), however, this was still higher than the mean maternal blood ionized calcium concentration of 1.15 ± 0.02mM (n=27; as

measured in animals which had undergone fetal TPTX surgery). Therefore although the maternofetal calcium concentration gradient had been reduced, it was not totally abolished. However, Chalon and Garel (1985B) performed both selective fetal TPTX and decapitations on day 19.5 of gestation. They showed a significant fall in fetal blood ionized calcium concentration after fetal TPTX, but not after decapitation. This suggests that the timing of fetal decapitation is crucial.

The surgery itself may have affected the intact fetuses, therefore, ideally only one group of operated animals should have been used, studying either an intact or a decapitated fetus from each animal. However, because of the low success rate encountered, it was decided that two separate groups of animals should be used. It was also difficult to assess whether complete TPTX had been achieved by decapitation. Although a significant fall in fetal blood ionized calcium concentration was taken as a successful TPTX by decapitation, these measurements were variable and it was often difficult to eliminate experiments on the basis of fetal blood ionized calcium concentration alone (i.e. it was difficult to set a 'cut-off' point).

Due to the great differences in the handling of control (unoperated) and operated animals, great significance cannot be placed on comparisons between the two groups as any effects may be due to surgery in general, rather than TPTX alone. In contrast, the effects of injection or perfusion with specific drugs or hormones within each group, seem worthy of greater consideration.

One of the unfortunate anomalies which appeared in two sets of

experiments (see Fig.5.10 and 5.13), was that the initial control $K_{mf}{}^{45}Ca$ values were significantly different in the control (i.e. diluent perfused) group when compared to the experimental group. However, these differences were not reflected in fetal or maternal blood ionized calcium concentrations and they may simply reflect the great inter-animal variations. It would have been useful to have measured fetal levels of vitamin D metabolites and PTH in all groups, to assess any differences. Also, it would be interesting to know whether fetal decapitation reduced fetal $1,25(OH)_2D_3$ and PTH levels. Care et al (1986) reported that fetal TPTX in sheep actually had no effect on fetal $1,25(OH)_2D_3$ levels.

2) The efffects of forskolin in the perfused rat placenta

The discovery that the diterpene forskolin activates hormone-regulated adenylate cyclase in membranes and intact cells, has provided a valuable tool for studying both the biochemistry and regulation of the enzyme and for studying the physiological functions of cAMP in a wide range of preparations. Forskolin was extracted from the roots of the plant Coleus forskohlii and its ability to stimulate adenylate cyclase was discovered in 1981 (Metzger,1981; Seamon et al,1981). It is a lipophilic molecule, so has limited solubility in water and is able to pass through cell membranes with relative ease and stimulate adenylate cyclase intracellularly. Activation of adenylate cyclase by forskolin occurs with an EC_{50} (half maximal stimulation) of approximately $10^{-5}M$. This potency varies according to the tissue, with a range of EC_{50}'s between $5x10^{-6}M$ and $4x10^{-5}M$

(Seamon and Daly,1981A). The final level of intracellular cAMP elicited by forskolin will obviously be determined by the rate of synthesis of adenylate cyclase and the rate of hydrolysis by cyclic nulceotide phosphodiesterases. Such steady state levels of cAMP frequently being attained after 5-10 minutes in the presence of forskolin. Therefore, forskolin acts rapidly and reversibly, and is highly selective by its activation of adenylate cyclase only (Seamon and Daly,1986).

The action of forskolin has been investigated in many tissues and these actions are varied. In smooth muscle forskolin causes relaxation and vasodilation. An intra-arterial injection will cause a rise in peripheral blood flow and general hypotension (Lindner et al,1978). In blowfly salivary glands forskolin increased cAMP, fluid secretion and calcium transport, with maximal stimulation at 10^{-5}M (Litosch et al,1982). It can also increase chloride and sodium transport across several epithelia (Cuthbert and Spayne,1982) and can stimulate ATP-dependent calcium transport in rat parotid basolateral membrane vesicles (Helman et al,1986). However, it inhibits glucose transport in adipocytes (Kashiwagi et al,1983). It remains to be determined if forskolin inhibition of glucose transport is occurring via interaction of forskolin at adenylate cyclase or due to an action of forskloin at a site other than adenylate cyclase, possibly the glucose transporter.

The placenta certainly exhibits adenylate cyclase activity (Milewich et al,1982), which can be stimulated via adrenergic receptors. These have been found throughout the placenta (Schocken,1982), with β-receptors being localized mainly in

fetally-facing placental plasma membranes (Whitsett et al,1980). By analogy with other tissues (e.g. bone; Brown,1983; Chambers et al,1985), PTH or calcitonin could also increase placental adenylate cyclase activity.

Perfusion of forskolin through the rat placenta of intact fetuses certainly increased calcium transfer in a dose dependent fashion. The EC50 was between 10^{-6}M and 10^{-5}M, which agrees well with the potency found for raising cAMP levels in other tissues (Seamon and Daly,1981A). Also, the effect of forskolin in these placentas seemed to be specific to calcium transfer, as the clearance of ^{51}Cr-EDTA was not altered. This specifcity was less clear in placentas from decapitated fetuses, where clearances of both ^{51}Cr-EDTA and ^{45}Ca were increased - the reasons for this are unclear. It could be that forskolin does have some non-specific effects in the placenta, e.g. increasing blood flow or passive permeability, which are of greater importance to these placentas from decapitated fetuses, however this requires further investigation. Also, forskolin does not increase K_{mf} ^{45}Ca back up to normal levels in placentas from decapitated fetuses, possibly suggesting that other hormones which do not act via cAMP, may be involved in the control of placental calcium transfer. It is clear however, that forskolin could be mimicing the effect of a variety of fetal peptide hormones, two of which could be PTH and calcitonin (Hakeda et al,1985; Van Der Plas et al,1985).

Finally, it is possible that forskolin is having other effects in the placenta, which may in turn secondarily influence maternofetal calcium transfer. For example, forskolin has been

shown to stimulate transfer of sodium and chloride in some epithelia (Cuthbert and Payne,1982) and to inhibit glucose transfer (Kashiwagi et al,1983). It would be interesting to investigate whether the effect seen here with forskolin is really specific to calcium transfer or whether it is a general effect on carrier-mediated types of transport.

3) The role of fetal PTH in the control of placental calcium transfer

It has been suggested in the literature that fetal PTH plays a role in the generation of fetal rat hypercalcaemia (Garel, 1970; Garel et al,1971). The fetal rat parathyroids are certainly active *in utero* (Thomas et al,1981), but it is not clear whether fetal PTH simply acts on fetal bone or via stimulation of $1,25(OH)_2D_3$ release, or whether it acts directly on the putative calcium transporter in the placenta.

In investigating the role of fetal PTH, the first problem was to decide which molecule of PTH to use and to choose a dose and time course for the experiments. Bovine PTH seems to have previously been used most frequently, both as the purified bPTH(1-84) molecule and the synthetic bPTH(1-34) (Dacke and Shaw,1987; Galceran et al,1987). In most systems, including fetal rats, rabbits and chicks, these two molecules show equivalent potencies in terms of cAMP production and calcium mobilization (Goltzman,1978; Freitag et al,1979; Silve et al, 1982). But in the perfused canine bone, bPTH(1-34) was more potent in terms of cAMP production (Galceran et al,1987). Bovine PTH is certainly active in non-related species, consequently the

species difference does not seem to be a problem. Also, the 1-34 peptide fragment of PTH is entirely adequate for the biological activity of PTH (Brown,1983). Both bPTH(1-84) and rPTH(1-34) were available, so bPTH(1-84) was used for injection into the fetuses, whilst rPTH(1-34) was used for placental perfusion, as this molecule is thought to be more stable and less prone to degradation during perfusion.

The dose of PTH used for both sets of experiments, was that which raised fetal blood ionized calcium concentration 2 hours after injection into the fetus. The dose of 4ngml^{-1} rPTH(1-34), was similar to that used by Galceran et al (1987), however, this dose is rather high when compared to immunoreactive PTH levels in fetal rat plasma (Thomas et al,1981), i.e. 0.4ngml^{-1} on day 21 of gestation. Also, in the perfused duodenal loop, calcium transfer was stimulated with 0.26-0.52 ngml^{-1} bPTH(1-34) (Nemere and Norman,1986). In preliminary experiments (not shown), a dose of 0.4ngml^{-1} did not increase fetal blood ionized calcium concentration two hours after fetal injection and also had no effect on maternofetal calcium flux. Therefore, the larger dose seemed a reasonable one to use, especially as we had no measure of the amount of rPTH(1-34) being lost during perfusion, due to binding to the tubing or the alkaline pH, etc.

For the other set of experiments where bPTH(1-84) was injected directly into the fetus, maternofetal calcium flux was measured two hours after the injection, as this is when the rise in fetal blood ionized calcium concentration was observed. The dose used here (1.0IU) was slightly higher than that quoted by Garel et al (1971; 0.25IU g^{-1} fetus), but again it was not known how much of

the injected bPTH(1-84) was actually getting into the fetal circulation. Also, the time course chosen for these experiments may have missed the maximal effect of bPTH(1-84) on maternofetal calcium flux.

It is clear from the results that fetal decapitation significantly ($p<0.001$) reduced fetal blood ionized calcium concentration and that this could be increased up to and above normal levels by injection of bPTH(1-84). bPTH(1-84) was also active in increasing fetal blood ionized calcium concentration in intact fetuses. However, bPTH(1-84) only had a stimulatory effect on maternofetal calcium flux in placentas from decapitated fetuses, i.e. where the flux was low to start with. This suggests a permissive role for PTH in controlling placental calcium transfer. In these experiments, bPTH(1-84) could be acting either directly on the placental calcium transporter or via the release of $1,25(OH)_2D_3$. The effect of bPTH(1-84) on fetal blood ionized calcium concentration in intact fetuses, was probably due to a direct action on fetal bone rather than any placental effect. However, in placentas from decapitated fetuses, bPTH(1-84) still did not stimulate maternofetal calcium flux back up to normal (cf. forskolin). This either reflects a response to the gross fetal surgery or suggests that other hormones are involved in the control of maternofetal calcium flux across the rat placenta.

One unexpected observation, was that maternal blood ionized calcium concentration was significantly ($p<0.05$) higher in those experiments where the fetuses had received bPTH(1-84) injection. Most reports in the literature suggest that PTH does not cross

the placenta, at least from mother to fetus (Garel and Dumont, 1972). However, our experiments may suggest that PTH can cross the placenta from mother to fetus or that PTH perhaps stimulates the release of $1,25(OH)_2D_3$ from the placenta, which crosses to the mother and causes a rise in maternal blood ionized calcium concentration.

Perfusion with rPTH(1-34) was carried out to investigate whether PTH was having a direct effect on the placenta. In fact, perfusion with rPTH(1-34) seemed to have no effect on maternofetal calcium flux in either preparation. Unfortunately, we had no measure of the rPTH(1-34) bioactivity in the Krebs coming out of the perfusion tubing after passage through the placenta, therefore it could be that the dose of rPTH(1-34) reaching the placenta was insufficient to have any affect on maternofetal calcium flux. However, it could also be explained if the effect of bPTH(1-84) injection in decapitated fetuses on placental calcium transfer, was due to the release of $1,25(OH)_2D_3$, which in turn acted on the putative placental calcium transporter.

4) The role of fetal $1,25(OH)_2D_3$ in the control of placental calcium transfer

Similar problems were encountered in designing these experiments as with the fetal PTH ones, i.e. the dose, bioactivity and likely time course for $1,25(OH)_2D_3$ were difficult to judge. Other workers have reported fetal blood hypercalcaemia in response to $1,25(OH)_2D_3$ (sheep- Barlet et al,1978; rats-Chalon and Garel,1983). Chalon and Garel (1983)

injected 2ng $1,25(OH)_2D_3$ into 19 day gestation rat fetuses and found an increase in fetal blood ionized calcium concentration by day 21, when compared to non-injected fetuses. However, the higher dose of 20ng $1,25(OH)_2D_3$ used here, had no effect on fetal blood ionized calcium concentration after two hours, but it could be that this was too short a time for any detectable response to have occurred. This suggests that the effect of $1,25(OH)_2D_3$ in this respect, is quite different to that of bPTH(1-84), which did increase fetal blood ionized calcium concentration within two hours. The dose of $1,25(OH)_2D_3$ used for perfusion experiments was close to the reported fetal plasma levels of $1,25(OH)_2D_3$ (Verghaeghe et al,1986; 54.7pgml^{-1}), although we had no measure of the $1,25(OH)_2D_3$ bioactivity in the Krebs after passage through the placenta.

The effect of fetally injected $1,25(OH)_2D_3$ on maternofetal calcium flux was simliar to that of bPTH(1-84), in that there was stimulation, but only in placentas from decapitated fetuses. However, unlike rPTH(1-34), $1,25(OH)_2D_3$ also stimulated maternofetal calcium flux when perfused through the placentas of these decapitated fetuses. This provides some evidence that the <u>in vivo</u> effect of PTH was via $1,25(OH)_2D_3$. These findings are supported by the presence of $1,25(OH)_2D_3$ receptors in the placenta (Pike et al,1980). However, the quick response of $1,25(OH)_2D_3$ in stimulating maternofetal calcium flux, suggests that a vitamin D dependent calcium binding protein is not involved in this increase in placental calcium transfer. $1,25(OH)_2D_3$ (like bPTH(1-84)) did not return calcium flux back to normal levels in placentas of decapitated fetuses and

together with the lack of effect on placentas of intact fetuses, again suggests that the hormone may have a permissive effect on placental transport. Also, bearing in mind the different effects of the two hormones on fetal blood ionized calcium concentration, it could be that fetal PTH has an acute effect on fetal calcium homeostasis, possibly via a bone effect, whereas $1,25(OH)_2D_3$ has a more long-term role maintaining the placental calcium transport. This is of course little more than speculation at present, but may provide a useful working hypothesis.

Comparing the effects of forskolin with those of PTH and $1,25(OH)_2D_3$, it is clear that they are quite different. In particular, the diterpene was able to stimulate maternofetal calcium transport across placentas from intact fetuses to supranormal levels. It is therefore quite likely that forskolin was mimicing an <u>in vivo</u> effect of a quite different hormone. This would be an interesting area for further investigation and one possible candidate might be the newly discovered PTH-related peptide (Rodda et al,1988). Finally, there has not been time to directly study the effects of fetal calcitonin on the placenta, but the reduction of fetal blood blood ionized calcium concentration (Garel et al,1968; Garel and Barlet,1978) that it caused, suggests that this would be worthwhile. Indeed, it could act directly on the placental calcium pump, as receptors for calcitonin have now been observed in human placental tissue (Nicholson et al,1988).

CHAPTER 6

FINAL DISCUSSION AND CONCLUSIONS

Final Discussion and Conclusions

1) The 'in situ' perfused rat placenta as a model for studies on the control of placental calcium transfer

The major aim of the investigation reported in this thesis was to obtain information on the control of placental calcium transfer by direct measurement of maternofetal clearances and fluxes of the cation. This involved the development of the technique of perfusing the fetal circulation of the rat placenta *in situ* and the subsequent validation of the model. It was clear from placental perfusion studies in other species that two particular questions concerning the model required investigation : a) was the passive permeability of the rat placenta altered by perfusion, and b) does active calcium transfer take place across the perfused rat placenta ?

a) Passive permeability of the perfused rat placenta

Comparison of clearance measurements made across the intact and the perfused rat placenta showed remarkable similarity (Chapter 2). Perfusion of the rat placenta does not therefore seem to increase permeability in the same way as was reported in the guinea-pig placenta (Hedley and Bradbury,1980). The permeability of the haemotrichorial rat placenta was also similar to that of the other haemochorial placentas, consequently, the extra two layers of trophoblast in the rat do not seem to greatly alter the rate of transfer of small molecules.

The K_{mf}/D_w ratio for ^{125}I-albumin (the largest molecule

studied) was very low suggesting restricted diffusion, whilst the clearances of all the other non-polar hydrophilic molecules were directly proportional to their diffusion coefficients in water. The other interesting observation was the high K_{mf}/D_w ratio for sodium. This could reflect a carrier-mediated transcelllular pathway for transfer (Sibley et al,1985), however it could also be explained by the influence of a transplacental potential difference (p.d.) on sodium transfer (Canning and Boyd,1984: Faber et al,1987) and/or the presence of heterogenous sized pores in the placenta (Štulc et al,1969). This is quite possible, but as yet it has been difficult to visualize any sort of transtrophoblastic channels across the haemochorial placentas, especially through the syncytial layers (Kaufmann et al,1987).

Finally, experiments investigating the role of the yolk sac suggested a pathway for sodium tansfer via this route, which agreed well with in vitro studies (Chan and Wong,1978; Gibson and Ellory,1984). It may be that the yolk sac is an important route for transport of molecules from the mother to the fetus in the rat, particularly in early gestation before the chorioallantoic placental development is complete.

b) Active calcium transfer across the perfused rat placenta

As discussed in Chapter 1, there is good evidence that placental calcium transport is an active carrier-mediated process. In fact, Štulc and Štulcová (1986) have previously provided evidence that this is the case in the perfused rat placenta and the study reported in Chapter 3 of this thesis

sought to confirm their observations. We found that the fetal plasma ionized calcium concentration was always higher than the maternal plasma calcium concentration and this 'uphill' concentration gradient appeared to be maintained in the perfused rat placenta even after removal of the fetus. The K_{mf}/D_w ratio for calcium measured in both intact and perfused placentas, was very high as compared to all the other molecules studied (including sodium), which can be explained by several alternatives as previously described for sodium. The possibility of an active transcellular component was thought to be most likely, especially as the maternofetal calcium flux in our experiments could be increased by a rise in maternal body temperature and decreased by the metabolic inhibitor, potassium cyanide. Investigations into the kinetics of the putative placental calcium transporter involved in maternofetal calcium transport across the perfused rat placenta, suggested that the carrier shows a very high affinity for calcium and seems to be saturated even at very low levels of maternal blood ionized calcium concentration. Therefore, active carrier-mediated transport of calcium does indeed appear to occur across both the intact and perfused rat placenta. However, despite the qualitative similarities between the two situations there did appear to be some quantitative differences in the data, as reported in Chapter 3. This requires further investigation, but may be partly explained by the variability in the data as discussed below.

No attempt was made to dissect the components of the placental calcium transfer mechanism in this study. However, by reference

to other studies on the placenta and the intestine (Chapter 1), the following model is postulated. Maternofetal calcium transfer may involve an initial passive saturable binding of calcium to the maternally-facing placental membranes, by attachment to high affinity binding sites (Shami et al,1974; Sweiry and Yudilievich,1984). This calcium may then be internalized by membrane pinocytosis (Terepka et al,1976) or through a facilitated diffusion mechanism (Bronner,1982), both possibly being regulated by CaBP. The calcium would then traverse the placenta transcellularly, possibly being sequestered on route by CaBP, endoplasmic reticulum, mitochondria, etc.; to be actively extruded on the fetal side, presumably by the action of a Ca^{2+}-ATPase (Fisher et al,1987).

It is clear that more specific experiments are needed, for example using antibodies specific for placental Ca^{2+}-ATPase and CaBP, in order to reveal their true role in placental calcium transport. Similarly, the maintenance of low intracellular calcium levels against a background of massive active maternofetal calcium flux has not been studied. Cultured rat and human placental cells (McArdle et al,1985; Kliman et al,1987) could be used to investigate this, making use of calcium sensitive dyes such as fura-2 (Cobbold and Rink,1987) to monitor changes in intracellular calcium during the stimulation of active maternofetal calcium transport, e.g. with forskolin (see below).

Two other interesting aspects of calcium transfer were touched on in Chapter 3. Firstly, on day 18 of gestation (term is day 23) the $K_{mf}{}^{45}Ca$ was much lower than on day 21, suggesting that

any calcium transfer at this stage was nearly all by simple diffusion. This agrees with the data of Chef (1969A), who showed an increase in net calcium flux to the rat fetus throughout gestation. Secondly, the yolk sac seemed to be capable of transporting calcium (as well as sodium) to the fetus on day 21 of gestation. There is also evidence for calcium transport across the yolk sac in the guinea-pig (Derewlany et al,1983) and therefore, it is possible that the yolk sac plays an important role in the transfer of calcium, particularly in early gestation.

Although both the permeability study and the investigation of the mechanisms of calcium transfer showed that the perfused rat placenta is a good model to study control of placental transfer, one problem has been the variability in the magnitude of calcium transfer from experiment to experiment. This variability may have been due to a number of causes :

i) Any study of placental transport is dependent on the accurate dating of time-mated animals. An inaccuracy of a couple of days is not too much of a problem for species where the gestation period is fairly long. However, for animals like the rat, even a difference of ± 0.5 day may be important. The female Sprague Dawley rats used for our experiments were placed with a male rat overnight and then were checked at about 10a.m. the following morning. If mating had occurred, vaginal plugs were visible in the bottom of the cage and this was numbered as day 1 of pregnancy (term being day 23). Consequently, the dating of these animals could be inaccurate by more than half a day, as the actual time of conception was unknown. This was probably one of

the main reasons for the large variation seen in K_{mf} measurements on day 21 of gestation and is also a problem when trying to make comparisons to other data reported in the literature. It is apparent that every research group seems to use a different system for time-mating animals and dates are often given as ± 0.5 day. It would therefore be useful if there could be an internationally recognised system for dating time-mated animals, with everyone being aware that these dates could be inaccurate by at least 0.5 day (Jost and Picon,1970).

ii) The experiments where the effect of maternal body temperature on $K_{mf}{}^{45}Ca$ was investigated, revealed a great temperature dependence of calcium transfer. Hence, the variability in measurements could have been affected by small changes in temperature. Other experimental effects may also have contributed, for example, surgery and length of anaesthesia, which are even more difficult to quantify.

iii) The rate of transport of molecules across the perfused placenta may also vary with time. The maximum time of perfusion used here was 40 minutes and $K_{mf}{}^{45}Ca$ did not seem to change during this time period. However, $K_{mf}{}^{51}Cr$-EDTA did seem to slowly increase with time in some experiments, suggesting that the diffusion permeability of the placenta was increasing. This effect on $K_{mf}{}^{51}Cr$-EDTA was not consistently found and varied with each placental preparation; placentas from decapitated fetuses did seem to be more susceptible to such changes.

iv) There was undoubtedly biological variability in the rate of transfer across placentas from different animals. This biological (as well as experimental) variability has also been

noted by authors investigating the permeability of the human placenta in vivo (Willis et al,1986; Bain et al,1988; Thornburg et al,1988).

In our investigations we have tried to overcome these problems by always carrying out control studies at the same time as experimental studies and where possible, by having control and experimental periods in the same animal.

2) Control of calcium transport across the near term rat placenta

It has previously been suggested that the fetus is relatively autonomous as regards calcium homeostasis (Care and Ross,1984). Our experiments using the pregnant rat near term certainly seem to agree with this. Alteration of the prevailing maternal blood ionized calcium concentration, achieved acutely by infusion or chronically by surgery and diet, had no effect on the maternofetal calcium flux. Similarly, maternal hypo-parathyroidism and chronic vitamin D deficiency, seemed to have no effect on either the maternofetal calcium concentration gradient or maternofetal and net calcium flux (Chapter 4). Therefore, the rat fetus seems to be largely protected from maternal hypo- and hypercalcaemia. Some explanation for this comes from the suggestion both in this study (Chapter 3) and that of others (Chalon and Garel,1985C; Štulc and Štulcová, 1986), that the calcium transporter in the rat placenta has a high affinity for the ion and transports at maximum rates even at very low ambient maternal plasma ionized calcium concentrations. However, in man the situation seems to be

slightly different in that chronic maternal vitamin D deficiency during pregnancy can cause fetal hypocalcaemia and neonatal rickets, with vitamin D supplements being able to rectify these problems (Brooke et al,1980; Mallet et al,1986). These experiments, of course, only indirectly suggest that maternal $1,25(OH)_2D_3$ can regulate the amount of calcium that the fetus receives in man. Unfortunately, no direct measurements of maternofetal calcium flux have been made to confirm this observation.

The fetus receives its $1,25(OH)_2D_3$ from the maternal circulation across the placenta (Haddad et al,1971), or it is synthesised by the placenta itself (Tanaka et al,1979; Whitsett et al,1981) or by the fetal kidneys (Weisman et al,1976), at least late in gestation. Therefore, one could perhaps explain the differences between rat and human, if the relative amounts of fetal $1,25(OH)_2D_3$ from these three sources were different. For example, in man most of the fetal $1,25(OH)_2D_3$ may come from the mother and hence, maternal vitamin D deficiency would greatly affect fetal $1,25(OH)_2D_3$ levels and calcaemia. In the rat, fetal $1,25(OH)_2D_3$ may come mainly from the placenta and fetal kidneys, and therefore, maternal vitamin D deficiency would not greatly affect fetal $1,25(OH)_2D_3$ levels and calcaemia. However, fetal nephrectomy on day 19.5 of gestation did not change fetal rat plasma calcium levels (Chalon and Garel,1985B), thus suggesting that the fetal kidneys are not involved in the control of fetal plasma calcium concentration; further investigation of the source of fetal $1,25(OH)_2D_3$ is therefore required.

It must be remembered that in the maternal TPTX experiments, the mothers were presumably also calcitonin deficient. Barlet (1985A) showed that calcitonin deficiency in late gestation in the sheep caused a rise in fetal calcaemia and increased maternofetal calcium flux. Therefore, any fall in maternofetal calcium flux which may have occured in our TPTX rats, may have been masked by a rise in maternofetal calcium flux caused by calcitonin deficiency. However, it seems unlikely that this could have masked a putative rise in maternofetal calcium transfer in $1,25(OH)_2D_3$ supplemented animals. Nevertheless, the role of maternal calcitonin requires further investigation.

Interestingly, Garel et al (1981B) showed that maternal TPTX in pregnant rats caused a significant reduction in placental CaBP. However, as we have shown that J_{mf} calcium does not alter in these conditions, this again questions the importance of placental CaBP in the transport of calcium across the placenta.

In direct contrast, our experiments which involved fetal decapitation (TPTX) caused a significant reduction in the maternofetal calcium concentration gradient and maternofetal calcium flux across the rat placenta. Replacement injections of both bPTH(1-84) and $1,25(OH)_2D_3$ significantly increased maternofetal calcium flux in placentas from decapitated fetuses, although not back up to normal levels. Fetal PTH has previously been shown to control fetal blood calcium levels in the rat (Garel et al,1971) and other workers have also shown a hypercalcaemic effect of fetal $1,25(OH)_2D_3$ in sheep (Barlet et al,1978) and rats (Chalon and Garel,1983), which was not observed in our experiments. However, fetal $1,25(OH)_2D_3$, both

injected directly into decapitated fetuses or added to the perfusate passing through a placenta of a decapitated fetus, seemed to be able to stimulate maternofetal calcium flux. The action of fetally injected PTH in these experiments may have been primarily due to PTH-stimulated release of fetal $1,25(OH)_2D_3$ (Fraser and Kodicek,1973), as perfusion with the peptide had no effect.

One possible working hypothesis arising from these different effects of PTH and $1,25(OH)_2D_3$ on fetal plasma ionized calcium concentration and maternofetal calcium transfer, is that whilst fetal PTH is particularly important for maintaining fetal plasma calcium concentration by mobilizing, for e.g. skeletal stores, fetal $1,25(OH)_2D_3$ (sources as yet unclear) is responsible for maintaining a constant supply of the cation by the placenta. The effect of $1,25(OH)_2D_3$ when administered by injection into the fetus or by perfusion (and indeed, injected PTH) was a permissive one, in that the hormone had no effect on placentas of normal intact fetuses. Thus, it could be that $1,25(OH)_2D_3$ is required for maintaining some component of the calcium transfer mechanism at an activity at which it is not rate limiting. Reduction of fetal $1,25(OH)_2D_3$ levels by decapitation might then reduce the activity of this component so that it does become rate limiting and subsequent administration of the hormone would then increase calcium transfer. An obvious candidate for this 'component' would be CaBP (despite reservations expressed earlier concerning its role in placental calcium transfer) and again this is a worthwhile area for future investigation.

It is again important to remember that fetal TPTX also removes

the influence of fetal calcitonin, especially as our preliminary experiments showed that fetal calcitonin had a significant hypocalcaemic effect when injected subcutaneously into a fetus (Chapter 5). This is another important area for further study.

There are many aspects of our research where more detailed information would have been useful. For example, in our perfusion experiments we had no measure of the rPTH(1-34) or $1,25(OH)_2D_3$ bioactivity after passage through the placenta. Similarly, we had no measure of the exact dose of fetally injected bPTH(1-84) or $1,25(OH)_2D_3$ which actually entered the fetal circulation. It would also be useful to assess different doses of these hormones, looking at the effects over different time periods, as the maximal effects may have been missed. Furthermore, it would be interesting to take measurements of PTH and $1,25(OH)_2D_3$ concentrations in fetal blood from both intact and decapitated fetuses, to see what endocrine effects fetal decapitation actually caused in the fetus. A different model of fetal TPTX would also have been useful, e.g. antibodies against PTH, and similarly, Ketoconazole could possibly have been used to produce maternal $1,25(OH)_2D_3$ deficiency (Glass and Eil,1986).

The experiments where forskolin was added to the fetal perfusate were carried out to investigate whether fetal hormones (like PTH and calcitonin) which act via cAMP, can stimulate maternofetal calcium flux. Indeed, in placentas from both intact and decapitated fetuses, forskolin was able to stimulate maternofetal calcium flux in a dose dependent manner. This could be mimicing the response to fetal PTH in placentas from decapitated fetuses. Howver, the effect of forskolin was quite

different to that of PTH, in that it worked when perfused through placentas of both normal intact and decapitated fetuses. The results therefore suggest the involvement of other hormones which act via cAMP. Consequently, it would be interesting to test such substances in the perfusion system, e.g. growth hormone, prolactin and β adrenergic agonists. A useful starting point might be to investigate which of these can raise placental cAMP levels.

In conclusion therefore, the rat fetus in utero may be capable of autonomously controlling maternofetal calcium transfer across the placenta. Although $1,25(OH)_2D_3$ and PTH seem important in this respect, other hormones may also be involved and calcitonin certainly requires more detailed investigation, as do prolactin (Barlet,1985B), growth hormone (Braithwaite,1975) and β adrenergic agonists (Brown,1983).

The origin of the hormones in the fetal circulation which may be important in the control of placental calcium transfer, is unclear. The relative amounts of fetal hormone derived from the three possible sources of mother, fetus or placenta, probably varies from species to species and may explain the differences in their control of placental calcium transfer.

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