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Vitamin D and Placental Calcium

William G. Schmitz
Loyola University Chicago

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By

William G. Schmitz

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Life

William G Schmitz was born on March 11, 1940 in San Francisco, California. After being graduated from Saint Aemydius grammer school in June, 1953, he entered Saint Joseph's College, the archdiocesan seminary for San Francisco, which he attended for five years. After leaving the seminary in June, 1958, he entered Saint Mary's College, California from where he was graduated with a Bachelor of Science degree in June, 1961.

He entered the Anatomy department, Stritch School of Medicine in September, 1961 and the Medical School in September, 1962. He was a graduate assistant in Anatomy in 1962 and 1963. He completed the work for both the Master of Science and Doctor of Medicine degrees in June, 1966.

Abstract

The histochemistry of the developing albino rat placenta has been studied with particular reference to the distribution of calcium and alkaline phosphatase with and without the influence of hypervitaminosis D; and with and without a living fetus (via umbilical cord ligation). Umbilical cord ligation performed before the 16th day of gestation was followed by hemorrhagic necrosis with subsequent rapid placental resorption. Umbilical cord ligation after the 16th day of gestation was followed by complete collapse of all of the fetal vessels and swelling of the tissue separating the maternal and fetal circulation, with consequent necrosis of the areas where the swelling was severe enough to obliterate the maternal sinuses.

Calcium became demonstrable in the altered stroma and possibly in the trophoblast of the labyrinth following cord ligation and appeared to accumulate more rapidly the nearer to term that ligation occured. Vitamin D administered in a dosage of 100,000 units for three days before sacrifice did not modify the deposition of calcium in the placental labyrinth. Alkaline phosphatase activity was demonstrated in the trophoblast lining maternal blood channels. Its distribution was not altered after fetal

death as long as maternal blood flow was normal. Calcium and alkaline phosphatase activity was reduced or completely absent in ischemic areas which were frankly necrotic.

The placental alteration following fetal death cannot be distinguished from the lesion ascribed as being unique to hypervitaminosis D by Potvliege(1962). It is concluded that both may have their origin in an interference with fetal blood flow through the placenta.

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Introduction

The placenta has evolved to transfer, between mother and fetus, the substances required for survival and growth during intrauterine development. This organ transports materials from the maternal blood stream to the fetus for the synthesis of fetal tissue, while it also transports in the opposite direction a number of waste products of metabolism. It is customary to divide transport processes into two major classes: simple physical diffusion and active transport. Simple physical diffusion across membranes involves no metabolic activity and is adequately described in quantitative terms by the Fick equation. Active transport, on the other hand, is defined as the net transfer of a substance against a chemical potential gradient, or work done on the substance transported.

The hemochorial placenta, which represents the maximum expression of the invasive activity of the chorion into maternal tissue, is the principal placental element in all higher mammals. Fetal blood vessels vascularize the chorion which has invaded the uterine mucosa to form a complex placental membrane which is bathed by maternal blood on one side and fetal capillaries on the other. Bridgeman (1948) traced the histology of the

developing placenta of the albino rat. She observed that from the thirteenth day of gestation until parturition, the placenta is a button-like disc whose convex surface joins with the uterine mucosa. Separating the decidua basalis and the labyrinth--the area of physiologic exchange--is the spongy zone, which extends to the lateral margin of the disc. This zone is approximately fifteen cell layers thick. Morphologically these cells are large, usually round in appearance but sometimes triangular. Their nuclei are large and oval and sometimes contain prominent nucleoli. Large tortuous maternal channels separate the cells, but the fetal capillaries do not invade this area. Another type of cell present in this zone are glycogen cells which have dark nuclei and clear cytoplasm when stained with hemotoxylin and eosin B- orange G.

The labyrinth is composed of maternal sinuses and fetal capillaries separated from one another by capillary endothelium, a variable layer of connective tissue elements, including the basement membrane, and trophoblast, which lines the irregularly shaped maternal blood channels. With the light microscope, the trophoblast appears to be syncytial. It is across these layers that metabolites and excretory products must be transported in order to be exchanged. Studies by Amoroso (1961)

with the electron microscope during late gestation revealed that the separating membrane is composed entirely of fetal tissue, typically consisting of two or three sheets of cellular trophoblast, with the innermost cells resting upon a basement membrane which separates the chorionic epithelium and the fetal capillaries. The walls of the fetal capillaries adjacent to the trophoblast possess a basement membrane of their own and the two contiguous basement membranes are everywhere separated by thin strands of collagen.

Jollie (1963) used the electron microscope to study the placental labyrinth from the twelfth day of gestation until term, and described the presence of four discrete cytoplasmic elements present between fetal and maternal blood streams. From the lumen of the allantoic vessel outward to the maternal sinus, they are as follows: (a) endothelium of the capillary with its underlying basement membrane; (b) a syncytial lipid rich cytoplasmic layer (element III) with widely scattered nuclei; (c) a relatively thick cellular layer (trophoblast II) presumed to be trophoblast; and finally, (d) a layer of cellular, usually relatively thin trophoblast (trophoblast I) which often extends microvilli and or pseudopod-like projections into the maternal blood sinus.

Review of Literature

An interesting and as yet not understood problem is the transfer of calcium from the mother to the fetus. An anticipated explanation for the passage of calcium would be that the calcium level of the fetal plasma is lowered by the drain of fetal bone formation with calcium moving from the maternal to the fetal blood in response to a concentration gradient. However, this mechanism may be ruled out for Bogart and Plass (1923) analyzed human maternal and fetal samples drawn simultaneously from the brachial and umbilical veins and found that the calcium content of the blood serum is consistently higher in the fetus at the time of birth than in the mother, in fact, higher in the fetus than that which is considered to be the upper limit for an adult. Hallman and Salmi (1953) also found calcium to exist at a higher concentration in fetal than in maternal blood. Plumlee et alii (1952) discovered that "immediately after Ca-^{45} ions are introduced into the blood, they are removed by several competing processes such as: (a) mixing with the body fluids and deposition in the soft tissue of the mother; (b) deposition in the bones of the mother; (c) transfer across the placenta to reach the fetal blood with subsequent

deposition in the fetal soft tissues and bones." Feaster et alii (1956) and Wilkinson (1953) found that in the rat, radioactive calcium is concentrated in the fetus. Orally administered Ca^{-45} was found to cross the placenta and be deposited in the feti at all stages of gestation studied, from the 14th to the 22nd day. The amount of labelled calcium transferred from mother to fetus, in a given length of time after administration, increased with increasing fetal age. Comar (1956) has shown that in the first two-thirds of gestation only a negligible amount of calcium can be found in the fetus, but in the last trimester a fantastic amount of calcium is transferred. In fact, each hour the rat fetuses drain almost 100% of the maternal blood calcium, and also that within one hour after administration of radioactive calcium, the fetuses had already incorporated the maximal quantity of the radioisotope.

In reference to histochemically demonstrable calcium deposits in the placenta itself, Wislocki and Dempsey (1946) studying the human placenta, reported that in the last quarter of gestation little calcium was normally demonstrable in the chorionic stroma. They believed that these deposits were physiological and distinguished them from other deposits of calcium which arose in the aging placenta to reach their

maximum at term. In this category they placed interstitial infiltrations of calcium which occurred irregularly in regressing villous infarcts and in necrotic foci of the spongy zone. Wislocki, Deane and Dempsey (1946) studying the rat placenta, observed calcium deposited in decidual and spongy zones of the aging placenta. Emmert (1957) found that in the cotton rat placenta during the last week of a 28-day gestation period, the labyrinthine (region of transfer) fetal stroma became infiltrated with a basophilic, metachromatic, PAS positive and ribonuclease resistant material with which calcium deposits were often associated.

In the albino rat, Emmert (1956) observed that while calcium deposition could not be demonstrated histochemically in the placental labyrinth during normal gestation, deposits developed quickly following the cessation of fetal blood flow. Tomaszewski (1962) found that calcium was deposited in the aging placental labyrinth of intact conceptuses during prolonged gestation.

The hypercalcemic effect of Vitamin D is promoted mainly by an increased intestinal absorption of ingested calcium. Eisenstein and Graff (1957) pointed out that an additional factor may reside in bone calcium mobilisation which takes

place at toxic dosage levels. Scarpelli, Tremblay and Pearse (1960) found that hypercalcemia causes cell injury at a mitochondrial level, and also that cell damage precedes the deposition of calcium in tissue. Potvliege (1962) studying the effect of hypervitaminosis D on gravid rats stated that the kidney, heart and the aorta are the most reliable indicators of hypervitaminosis and that each organ is affected in a characteristic pattern. In the placenta he describes the following alteration. "The characteristic lieson consisted of the deposition around the fetal capillaries of allantoic villi of a strongly PAS-staining substance which was separated from the maternal blood by only a thin and perhaps discontinuous membrane of trophoblastic cytoplasm. Calcification was a late event and was manifested only when trophoblastic impregnation with mucoprotein was considerable. Calcium was found mainly incrusting the infiltrated and degenerated villi (an inappropriate term, in a labyrinthine placenta), but was also deposited on less altered structures, notably in the walls of large fetal vessels and in the mesenchyme surrounding them, at their point of entrance into the placenta." However, Potvliege (1962) noted that the maximum amount of this material was found in the placentae of macerated fetuses which came from his maximally treated group

of animals. This raises the question as to what exactly is the etiology of this material, hypervitaminosis D, fetal death or something else.

Materials and Methods

Albino rats of the Sprague-Dawley strain were obtained from the Hormone Assay Laboratory, Chicago and bred in this laboratory. Dated matings were obtained by placing males and females together overnight and checking vaginal smears for sperm the next morning. The presence of residual sperm was taken to indicate the beginning of day zero of the gestation period. Food, in the form of Purina rat biscuits, and water were available ad libitum.

Preliminary work revealed that accumulation of calcium could best be demonstrated in normal placentae if the umbilical vessels were ligated after the fifteenth day of the gestation period. Calcium could be demonstrated histochemically when the animals were sacrificed in as short a time as six hours after ligation of the vessels, but to elicit a near maximal histochemical response, a period of twenty-four hours between the surgical procedure and the harvesting of the placentae was needed.

The animals receiving vitamin D were given 100,000 or 250,000 units dissolved in three ml of water for the two days preceding, as well as the day of sacrifice. The controls were injected with three ml of water for the same period of time. Placental samples were taken on the following days of gestation:

a) normal-- 13 to 21 days

b) experimental-- subjected to 100,000 units for three days
13 to 15 days
subjected to 250,000 units for three days
14 to 17 days

On the second day of injections, the following surgical procedure was performed on all the animals. After induction of anesthesia with ether, the abdomen was sterilized by swabbing with cotton saturated in 70% alcohol. A midline incision was made through the abdominal wall and then through the peritoneum. Strips of cotton soaked in warm saline (37 C) were placed on either side of the incision, and the uterine horns--one at a time--were exposed and laid on the cotton. To ligate the umbilical vessels, a small needle with surgical silk was passed through the uterine wall, under the vessels, which were generally easy to locate between the button-like placenta and the fetus, and then back through the musculature so that the

two ends could be drawn together and tied to completely occlude the vessels. One-half to two-thirds of the fetuses present in each female had their umbilical vessels ligated in this manner. The periotoneal and abdominal incisions were then sutured separately. The day following this operation the animals were sacrificed by the administration of an overdose of ether. Immediately after administration of the ether, five ml of blood, for determination of calcium blood levels, was withdrawn by cardiac puncture and placed in a water-bath at 37 C for 30 minutes. The blood was then centrifuged at 2500 RPM for ten minutes, the sera pipetted off and stored for analysis by the method of Kavocs and Tarnoky (1960). After the blood had been placed in a water-bath, the two uterine horns were cut out, the placentae separated from the uterine musculature and cut in half to aid penetration of the various types of fixatives.

Some placentae were placed in calcium acetate formol for 48 hours with a fixative change at 24 hours. After which they were embedded in tissuemat (M.P. 52.5 C) and sectioned at five to eight micra. The other placental tissue was frozen in a solution of 2- methyl butane which was chilled in liquid nitrogen, then placed in a freeze-dry machine for two to three days at a temperature of -35°C. The absorbant for the water

was phosphorus pentoxide. After removal from the machine, the placentae were double-embedded in cellodin and tissuemat and sectioned at five to six micra.

Several staining techniques were used: the general histology was demonstrated by hemtoxylin and eosin B-orange G; the distribution of calcium by the iron substitution technique of Hurst et alii (1951) and by the technique of Kashiwa and Atkinson (1963); the localization of alkaline phosphatase by the method of Burstone (1960) and the location of mucopolysaccharides by the method of Mowery (1963) using alcian blue and PAS.

Results

The following results are based on observations derived from studying the 225 ligated and 158 non-ligated placentae obtained from 46 pregnant rats, of these 92 are not reported on because of technical difficulties.

Morphology

Grossly, the chorio-allantoic placentae of the white rat form large white masses distributed evenly in the bicornuate uterus. They are at first cup-like in appearance, with a convex surface, which is the side of attachment to the uterus,

and a concave surface, into the center of which (hilum) the umbilical vessels penetrate. As gestation progresses, the placentae grow in size, reaching their greatest diameter between the sixteenth and seventeenth days of gestation. There is also a thickening from hilum to uterine insertion which changes the appearance of the placenta to a button-like shape.

Microscopically, the chorio-allantoic placentae of the white rat in the last trimester of gestation is subdivided into two major zones--the spongy zone and the labyrinth. The spongy zone is located between the decidua basalis and the labyrinth, and extends almost to the lateral margin of the placenta. The decidua basalis at this stage of gestation is a dense band of fusiform cells interlaced with connective tissue fibers which hinders mesometrial placental growth, forcing it to occur laterally. The spongy zone is approximately fourteen to eighteen cell layers wide and consists mainly of large irregularly shaped cells. A special type of cell located in the spongy zone is the glycogen storage cell, the majority of which are compressed into islands by the surrounding cells. On the fifteenth day of gestation, these cells are full of glycogen. The number of glycogen cells and

the intensity of the staining reaction gradually decreases as gestation progresses until at term very few can be found.

The placental labyrinth, late in the twelfth day of gestation, has been formed by trabecular cords of fetal tissue delimiting maternal sinuses in which maternal blood circulates. The trabeculae are surfaced with trophoblast and contain vascular cores that collectively constitute a peripheral allantoic circulation. The thickness of this trophoblastic layer decreases as gestation progresses. Due to the anastomosing and branching of the labyrinthine plates, any given section of the labyrinth (Fig. 1) will reveal a cross-section in relation to one area, a longitudinal section in relation to another area and an oblique section through many other areas. The maternal blood channels were generally about 120 micra, while the fetal capillaries are usually 20-25 micra in diameter and consequently can contain at the most three or four red blood cells at a time. At term some of the nuclei of the trophoblastic layer appear abnormally large and actually bulge into the maternal sinuses. Between the trophoblastic layers and supporting the fetal capillaries occasionally is found a delicate layer of fibrocytes with their intertwined arrangement of fine collagenous and reticular fibers.

Ligation of Umbilical Cord

Ligation of the umbilical vessels during the last trimester of gestation produced the following developmental changes. The placentae did not appear to increase in diameter beyond what it was in a normal seventeen day animal--it did, however, increase in thickness from hilum to insertion, but this may be accompanied by a decrease in diameter. Ligation of the umbilical vessels on days twelve through fourteen, when the labyrinth is already well-vascularized from the fetal side, revealed the presence of areas of hemorrhegic necrosis in portions of the spongy zone and marked pyknosis of many of the nuclei in the spongy zone. The trophoblastic fetal capillaries are collapsed within 24 hours by the swollen edematous labyrinthine plates and contain no red blood cells. The maternal sinuses are smaller in diameter, contain fewer red blood cells and the labyrinthine plates are wider than in the thirteen to fifteen day control animals. Except for the areas of hemorrhagic necrosis the glycogen cells of the junctional zone were not affected by the ligation of the vessels, their number and location comparing favorably with the control series.

Ligation of the umbilical vessels on days fifteen through twenty-one produced the following changes. There were no areas

of hemorrhagic necrosis in the junctional zone and the glycogen cells underwent normal development and disintegration. The major area of change was found in the labyrinth (Fig. 2). All of the fetal vessels were collapsed, their endothelium merging with, and usually indistinguishable from, the surrounding trophoblast. The trophoblastic layer was edematous, severe enough in some areas to cause obliteration of the maternal sinuses and consequent necrosis, but in most areas, only partial obliteration. The maternal sinuses were engorged with red blood cells. Many macrophages were present in the necrotic areas where the maternal sinuses were obliterated.

Vitamin D Treatment

The development of the placentae of the rats administered 100,000 units of vitamin D for three days was similar to that observed in the control animals with the exception of some labyrinthine alterations which will be discussed later. Most of the placentae whose umbilical vessels were ligated on days twelve or thirteen quickly underwent hemorrhagic necrosis and reabsorption. The placentae of the animals whose umbilical vessels were ligated at or after fourteen days gestation attained a normal size. The thickness of the spongy zone, the number and location of the glycogen cells and the

differentiation of the labyrinth were all normal in this latter group. In some of the placentae of the rats administered vitamin D, especially those sacrificed on the thirteenth, fourteenth or fifteenth day of gestation, a hyaline appearing substance is evident surrounding the allantoic vessels as they branch and penetrate deep into the labyrinth. This substance is not present in the similar control group. In areas where it is minimally present, it is found between the fetal blood vessels and the outer layer of trophoblast. As the deposits increase in size, they gradually force the lumen shut and push the trophoblast into the maternal blood sinus. The small patchy areas of this hyaline material eventually coalesce, to replace even larger areas of trophoblast. The initial lesions are present in the region of the insertion of the umbilical vessels, and as they increase in size they extend deeper into the labyrinth, reaching toward the reticular zone.

Alkaline Phosphatase

Alkaline phosphatase is discretely localized in the trophoblast serving as the lining for all of the maternal sinuses located in the labyrinth (Figs. 8 and 9). The reaction is strongly positive and limited to the outer portion of the cytoplasm--possibly the trophoblastic layer I described by

Jollie in his electron miscroscope study. In the junctional zone, most of the maternal sinuses are lined by positive reacting cells, strong in intensity, but not as strong as those found in the labyrinth, which are also localised to the most peripheral part of the cell. The distal surface of the epithelium of the yolk-sac also reveals strong and well-localized reacting sites. No positive reacting sites were observed in relation to glycogen cells, fetal blood vessels or trophoblastic nuclei. The intensity of the staining reaction increases in the places listed above, from the thirteenth day of gestation--the first of our study--until term, although there is a minimal detectable difference histochemically between the strength of the reaction on the nineteenth, twentieth and twenty-first days. As the age of the placenta increases, positive reacting leukocytes are seen more frequently in the decidua basalis, in the sinuses of the junctional zone and sometimes in the area immediately adjacent to the clumped degenerating glycogen cells. In the placentae whose umbilical vessels were ligated, no deposits were detectable in the areas of hemorrhagic necrosis, but they were present, although of lesser intensity, in the edematous cytoplasm encircling the maternal sinuses in the labyrinth and

junctional zone. In the placentae of the animals treated with vitamin D, there was no change in the location or intensity of alkaline phosphatase activity in the non-ligated animals. There was no correlation between the intensity of alkaline phosphatase activity and the hyaline like substance found in some of the placentae in which the umbilical cords were ligated. There is decreased intensity of alkaline phosphatase activity in ischemic or frankly necrotic areas.

Calcium

Calcium is histochemically demonstrable in many areas of the chorio-allantoic placenta, but we are primarily interested in the deposits found in the labyrinthine stroma, because of the close proximity of the maternal and fetal circulations in this area. Normally on the thirteenth day of gestation small deposits are demonstrable by the method of Kashiwa (1963) in the trophoblast of the allantoic vascularized chorionic villi. The intensity of the staining reaction of these deposits gradually decreases until the middle of the fifteenth day of gestation when they are no longer histochemically demonstrable.

After ligation of the umbilical vessels on the thirteenth, fourteenth or fifteenth day of gestation, there is a minimal

increase of calcium deposits by the method of Hurst et alii (1951) or of Kashiwa (1963) in the stroma and trophoblast in the following twenty-four hours. However, twenty-four hours post-ligation (Figs. 4-7) in an animal that is sixteen or seventeen days pregnant, a positive staining reaction of faint to light intensity can be found in the labyrinthine trophoblast, located primarily on the trophoblast serving as the lining for the maternal sinuses. This reaction has increased to moderate intensity twenty-four hours after ligation on the eighteenth day of gestation. Twenty-four hours after ligation on the nineteenth day of gestation, twelve hours after ligation on the twentieth day of gestation, and six hours after ligation on the twenty-first day of gestation, the staining reaction has progressed to heavy intensity. In the areas of hemorrhagic necrosis, found in the ligated placentae, scattered areas of calcification are found where the degeneration is most severe.

The results of administering 100,000 units of vitamin D are found in table IV. The results of administering 250,000 units of vitamin D are not given because of the large fluctuations in maternal blood serum observed. In the placentae of the rats treated with vitamin D, calcium deposits are found

in the same areas, and are of the same intensity as those found in the placentae of the rats which were not administered vitamin D. Calcium deposits were not associated with the hyaline appearing substance which was observed in some of the placentae of the animals which had received vitamin D.

Calcium deposits which are not influenced by ligation of the umbilical vessels are scattered deposits which are found among the muscle fibers of the decidua basalis and deposits lining the sinuses of the junctional zone as well as being scattered throughout their cell volumns. These deposits gradually increase in amount as gestation progresses regardless if the umbilical vessels are ligated or not.

Discussion

Umbilical cord ligation which causes fetal death within six hours created the following changes in the placenta of the albino rat during the last trimester of gestation. Ligation if performed prior to the fifteenth day of gestation produced areas of hemorrhagic necrosis in the spongy zone with complete collapse of the trophoblastic fetal capillaries and edematous swelling of the trophoblastic layer in the labyrinth. If the ligation were performed after the fifteenth day of gestation,

the major area of change was found in the labyrinth in which (a) the fetal vessels were collapsed, (b) swelling of the trophoblast, the severity of which varied in different regions, was present, and (c) granularity of the trophoblast, which increased as the time after ligation of the umbilical vessels increased, developed. In some areas the swelling and granularity was severe enough to cause obliteration of the maternal sinuses with consequent necrosis in the areas where the maternal vascular supply was destroyed. In most areas, only partial obliteration resulted from the above factors.

Emmert (1957), utilising Caesarian section with the placenta left in situ or umbilical cord ligation in the cotton rat reported that the labyrinthine stroma, at first in small areas and later throughout the entire placenta, became infiltrated with a granular basophilic material associated with deposits of calcium and that the trophoblast eventually developed areas of swelling and blistering which was sometimes severe enough to occlude maternal blood channels which caused ischemia of portions of the spongy zone and decidua basalis. Emmert (1958) reported that in the presence of an intact fetal blood flow, calcium was not histochemically demonstrable using alizarin red in the labyrinth; but after cessation of the

fetal blood flow, calcium quickly accumulated.

Tomaszewski (1962) found that in albino rats the first area of change after umbilical cord ligation in prolonged gestation was in the lateral margins where areas of retraction of the labyrinthine plates was evident. He divided the changes observed into two categories: (a) slight retraction of the labyrinthine plates with edematous tissue mainly limited to the lateral margins; (b) large areas of the labyrinthine margin were devoid of labyrinthine plates due to severe retraction with widespread edematous regions in the labyrinthine.

Pritchard and Huggett (1947) observed two kinds of degenerative changes in the albino rat placenta: (a) after destruction of fetuses on the tenth through the twelfth days of gestation, the peripheral portions of the placenta underwent hemorrhagic infarction and histological differentation proceeded along normal lines except in the labyrinth where the fetal vessels were obliterated and their endothelium merged with the trophoblast to form a composite syncytium; (b) after destruction of fetuses on the sixteenth through nineteenth days of gestation, gross edema of the labyrinth was evident. In many places the thin syncytial strands of trophoblast appeared to have broken down. Around the collapsed fetal

vessels, a thick layer of exudate, containing fibrin strands and isolated maternal red blood cells was seen. In areas where edema was severe, necrosis followed obliteration of the maternal circulation; elsewhere the trophoblast remained until term.

Some of the ligated placentae of the rats administered vitamin D developed hyaline appearing deposits which initially appear between the allantoic vessels and the surrounding trophoblast. These deposits increased in size, until in some of the placentae they extended from the area of insertion of the allantoic vessels into the placenta, penetrating deep toward the junctional zone.

Potvliege (1962) studied the effect of hypervitaminosis D in gravid rats and discovered clear cut histological differences only in the maximally treated mothers who had received 40,000 units of vitamin D daily for the last nine days of gestation. He described the initial deposits which were found particularly in placentae of macerated fetuses, as foamy PAS staining material which accumulated around the fetal capillaries beneath the syncytial trophoblast. He analysed this material histochemically and concluded that it was free of fat, fibrin, acid mucopolysaccaride or glycogen and that it was not transformed into

collagen even in advanced stages. This description is very similar to what Emmert (1956 and 1957) described as following fetal death or as the consequence of aging, neither procedure involving the administration of vitamin D. Calcification was a late event and was manifested only when villous impregnation with this mucoprotein was considerable. He also noted that the material first appeared in the region of the insertion of the fetal vessels into the placenta and progressed inward toward the maternal surface as gestation advanced. It must be pointed out, however, that the experimental procedure varied considerably between Potvliege's study and ours. He anesthetized his rats daily with ether, inserted a gastric tube and administered the vitamin D dissolved in oil, every day for the last nine days of gestation, varying the dosage from 0 units to 40,000 units in his maximally treated series. It certainly seems possible that these deposits may be the result of interruption of the fetal circulation, and not to the hypervitaminosis.

Calcium is histochemically demonstrable in many areas of the chorio-allantoic placenta. Calcium deposits which are found as part of the consequences of aging are found among the debris of the decidua basalis and are scattered throughout the cell columns of the spongy zone. Wislocki and Dempsey (1946)

observed in the human that variable but considerable quantities of calcium accumulate in the chorionic stroma during the second and third quarters of pregnancy, which diminish as pregnancy advances. They distinguish these from other calcium deposits which arise in the aging placenta and reach their maximum at term. These latter deposits occur irregularly in infarcted villi and in necrotic foci of the spongy zone. In the albino rat placenta, Wislocki and Dempsey (1946) observed that calcium was deposited in the decidua basalis and spongy zone of the aging placenta. However, it must be pointed out that they used the von Kossa technique for histochemical demonstration of calcium, which stains calcium only by inference since the reaction material is actually either carbonate or phosphate, and that they did not or could not reproduce all of their results with other direct staining techniques.

Hard (1946) stated that in the guinea pig slight scattering of preformed calcium occurred in the spongy zone as well as in the walls of the placental arteries and veins, which increase with advancing age. Emmert (1956) stated that in the cotton rat during the last week of a 28-day gestation period, the fetal stroma of the labyrinth became infiltrated with a granular, metachromatic PAS positive, ribonuclease resistant

material with which calcium deposition was associated. McCance and Widdowson (1961) stated that there is a rapid buildup of calcium in the labyrinth of the pig between the twentieth and forty-sixth day of gestation with subsequent decline to much lower concentration as the rate at which calcium deposition in the fetal bones goes up. Widdowson and Spray (1951) demonstrated that in the last trimester of gestation, the placenta contains negligible amounts of calcium, if not over mature or pathologically calcified.

After ligation of the umbilical vessels, the calcium deposits which accumulate in or on the fetal side of the trophoblast increase in quantity in shorter periods of time as the gestation period advances. Emmert (1958) found that after the 18th day of gestation in the albino rat, there is a rapid accumulation of calcium in the labyrinth after cessation of fetal blood flow. Tomaszewski (1962) discovered that in prolonged gestation after umbilical cord ligation, the increase in intensity of the loci of calcium deposits in the trophoblast of the labyrinth was dependent on the length of time that the fetus had been dead. Since there was no greater accumulation of calcium in comparable periods of time in the labyrinth of the placentae exposed to a higher concentration of calcium,

than in the control placentae, we conclude that a high maternal blood calcium level is not primarily responsible for the deposition of calcium in the labyrinth.

Undoubtedly the fetal requirement for calcium is the governing factor in the transfer of calcium from mother to fetus. It is well known that serum calcium levels are lower in pregnant than in non-pregnant women, the difference probably being accounted for in lowered protein bound rather than the free calcium fraction. During the last portion of pregnancy, the concentration of fetal vascular calcium is higher than that found in the maternal circulation. Newman (1943) found that the calcium concentration began to fall in the pregnant human in the second or third month and reached its lowest value in the second or third month; this may be a reflection of the hypoalbuminemia of pregnancy. Mull and Bill (1934) reported that the maternal calcium concentration drops five to ten per cent from the normal average, until near term when it tends to rise. The experiments of McIsaac (1926) indicate that the excess of calcium in the fetal blood is a phenomenon peculiar to the last stages of pregnancy.

Since it is well known that the adult parathyroid gland regulates the vascular calcium concentration through the

effect of parathormone on the kidney, as well as through a direct effect on bone, the question may be asked if the fetal parathyroid may be responsible for the transfer of the calcium. Sato (1938) demonstrated that extract from the fetal calf parathyroid is effective in controlling calcium concentration in the rabbit. McIsaac (1928) found that at the time of ossification in the dog, the parathyroid glands are already morphologically differentiated and also that if parathormone was injected into the mother, it would raise the maternal blood calcium levels but not the fetal, while if it was injected into the fetus, it would raise the fetal but not the maternal blood calcium.

Kurhowski (1963) stated that the maintenance of physiological conditions of the conceptus in a maternal environment with excess circulating parathyroid hormone and an elevated serum calcium concentration suggests protection of the fetus either by a barrier impermeable to parathyroid hormone or by a particular resistance of fetal tissues to the deleterious effects of the hormone. He raised the question that the increase in serum calcium concentration may not have been produced by the passage of calcium across the placenta but by a rise in the carbon dioxide tension. Salmi (1954) was aware

of this danger of distortion of calcium values from low oxygen tension in work on calcium and showed that, although experimental hypoxia did raise the concentration of serum calcium, it was not enough to account for the observed maternal-fetal blood calcium differences.

Our work has demonstrated that calcium accumulates in the placenta after the influence of the fetus has been removed. This does not prove that the fetal parathyroid gland is not responsible for the transfer of calcium from the placenta to the fetal blood stream, but it does prove that the fetal parathyroid cannot be responsible for the initial accumulation of calcium in the placenta.

Alkaline phosphatase is localized in the labyrinth within the trophoblast which serves as the lining for the maternal blood sinuses, which is at least trophoblastic layer I described by Jollie in his electron microscope study. Wislocki and Dempsey (1946) reported that in humans, as well as in rats, alkaline phosphatase activity in the labyrinth is strongly present wherever the trophoblast bounds maternal blood channels, and as the placental laybrinth differentiates with increasing age, phosphatase accumulates in increasing amounts in the fine meshed syncytium bordering the maternal blood channels. In the

areas where degeneration is found either secondary to ligation or to the aging process, decreased alkaline phosphatase activity is found. Ahmed (1959) utilized differential centriguation to analyze full term placentae and reported that alkaline phosphatase was present in the following places: cytoplasm (37%), microsome (33%), mitochondria (13%), cell debris (8%) and nuclear fraction (7%). His histochemical observations were similar to those reported by Dempsey and Wislocki.

Hard (1946) studied the distribution of alkaline phosphatase in the guinea pig and reported that the only element in the embryonic portion of the placenta which exhibited a conspicuous alkaline phosphatase concentration was the trophoblast lining the maternal sinuses. Quantitatively he stated that phosphatase activity shows a sharp increase from day fifty to day fifty-five of the normal sixty-seven day gestation period and then gradually declines as term approaches.

The role of alkaline phosphatase has been the subject of much controversy. In the hypothetical scheme for the calcification process, alkaline phosphatase was thought by Robinson (1962) to split organic phosphate with the result that the local concentration of phosphate is increased and the solubility product of calcium phosphate is exceeded with deposition of

calcium phosphate resulting. But the presence of an organic phosphate serving as the substrate has never been demonstrated. It has been postulated by Neuman (1951) that phosphatase is related to preosseous cellular metabolism and to the elaboration of a calcifiable bone matrix. This latter point obviously cannot be applicable in the placental labyrinth because formation of bone would destroy the intimate association existing between maternal and fetal circulation.

Some authors feel that alkaline phosphatase results from the accumulation in the trophoblast of the alkaline phosphatase circulating in the maternal circulation, likening it to what Hupler (1945) stated in relation to the presence of the enzyme in the kidney, attributing its occurrence in the brush border as an attempt of the tubule cells to absorb the enzyme from the glomerular filtrate.

Hard (1946) felt that alkaline phosphatase is important in the phosphorylation of fats and lipids, stating that phosphatases have been implicated in the metabolism of phospholipids and sugars and in the absorption of sugars and fats through cellular membranes.

Summary and Conclusions

1) The histochemistry of the developing albino rat placenta has been studied with particular reference to the distribution of calcium and alkaline phosphatase with and without the influence of hypervitaminosis D; and with and without a living fetus (via umbilical cord ligation).

2) Umbilical cord ligation performed before the 16th day of gestation is followed by hemorrhagic necrosis with subsequent rapid placental resorption.

3) Umbilical cord ligation after the 16th day of gestation is followed by complete collapse of all of the fetal vessels, swelling of the tissue separating the maternal and fetal circulation, severe enough in regions to obliterate the maternal sinuses, and consequent necrosis of the involved areas.

4) Calcium became demonstrable in the altered stroma and possibly in the trophoblast of the labyrinth following cord ligation and appeared to accumulate more rapidly the nearer to term that legation occurred.

5) Alkaline phosphatase activity was demonstrated in the trophoblast lining maternal blood channels. Its distribution

was not altered by fetal death as long as maternal blood flow was normal.

6) Calcium and alkaline phosphatase activity was reduced or completely absent in ischemic areas which were frankly necrotic.

7) The placental alteration following fetal death cannot be distinguished from the lesion ascribed as being unique to hypervitaminosis D. It is concluded that both may have their origin in an interference with fetal blood flow through the placenta.

8) Vitamin D administered in a dosage of 100,000 units for three days before sacrificing did not modify the deposition of calcium in the placental labyrinth.

Bibliography

1) Ahmed, A. 1959 Placental Phosphatases. Biochem. Biophys. Acta, 34:313-325

2) Amoroso, E. 1961 Histology of the Placenta. Brit. Med. Bull. 17 No. 2:86

3) Bogert, J. and E. D. Plass 1923 Placental Transmission. J. of Biol. Chem. 56:297-307

4) Bridgeman, J. 1948 A morphological study of the development of the placenta of the rat. J. Morph. 83:61-68

5) _____ 1948 A Histological and Cytological Study of the Development of the Chorio-allantoic placenta of the white rat. J. Morph. 83:195-224

6) Burstone, M.S. Hydrolytic enzymes in dentinogensis and osteogensis
Calcification in Biological Systems, Edited by Reidar Sognnoes. pp. 217-243

7) Comar, C. L. 1956 Radioactive studies in pregnancy. Ann. N. Y. Acad. of Sci. 64:297

8) Eisenstein, G. 1957 Experimental Hypervitaminosis D. Proc. Soc. Exp. Biol. Med. 94:441-444

9) Emmert, L. A. 1956 Morphological and histochemical observations of placentation in cotton rat (Abstract).

Anat. Rec. 124:285

10) _____ 1957 Observations of the fetal membranes of the cotton rat (Abstract).
Anat. Rec. 127:289

11) Feaster, A. 1956 Placental transfer of Ca-^{45} in the rat.
J. Nutrit. 58:399

12) Hallman, N. and I. Salmi 1953 On Plasma calcium in cord blood and in the newborn.
Acta Peadiat. 42:126

13) Hard, W. L. 1946 Histochemical and quantitative study of phosphatase in the placenta and fetal membranes of the guinea pigs.
Am. J. Anat. 78:47-77

14) Hupler, A. 1945 Phosphatase in chemically injured kidneys.
Arch. Path. 39:133-141

15) Hurst, M., S. Vallerie, R. Hutton and W. Nuchalls 1951 A new histochemical reaction for calcium.
J. of Dent. Res. 4:489

16) Jollie, W. 1964 Age changes in rat placental labyrinth.
J. of Ultrastr. Res. 10:27-47

17) Kashiwa, J. and S. Atkinson 1963 The application of a new Schiff base, glyoxal-bis (2-hydroxy-anil) for the

cytochemical localization of ionic calcium.
J. Histochem. Cytochem. 11:258-264

18) Kovacs, G. S. and K. E. Tornoky 1960 A simple and rapid method for the simultaneous determination of calcium and magnesium for the same sample of blood serum.
J. Clin. Path. 13:160-162

19) Kwhowski, M. 1963 Parathyroid hormone and the placental barrier.
Arch. Int. Pharm. 146:245

20) McCance, R. and P. M. Widdowson 1961 Mineral metabolism of the fetus and newborn.
Brit. Med. Bull. 17:132

21) McIsaac, P. 1928 Studies on calcium metabolism III. Comparison in calcium metabolism of doe and litter in the rabbit.
Brit. J. Exp. Biol. 5:248-251

22) Mowry, R. W. 1963 The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins.
Ann. N. Y. Acad. of Sci. 106:402-423

23) Mull, J. W. and A. W. Bill 1934 Variations in serum calcium and phosphorus during pregnancy.

Am. J. Obst. and Gyn. 27:510

24) Neuman, W. F., V. Distefano and B. J. Mulryan 1951 The Surface Chemistry of bone III. Observations on the role of phosphatase.
J. Biol. Chem. 193:227

25) Newman, R. L. 1943 Blood calcium: A normal curve for pregnancy.
Am. J. Obst. and Gyn. 53:817

26) Plumlee, M. 1952 Placental transfer of Ca-^{45}.
Am. J. Physiol. 171:678.

27) Potvliege, P. 1962 Hypervitaminosis D_2 in gravid rats.
Arch. Path. (Chicago) 73:371-382

28) Pritchard, J. J. and A. Huggett 1947 Experimental fetal death in the rat; Histochemical changes in the membranes.
J. Anat. 81:212-222

29) Robinson, R. A. and M. L. Watson 1952 Collagen crystal relationships in bone as seen in the electron microscope.
Anat. Rec. 114:383

30) Salmi, I. 1954 On the influence of anoxia on plasma calcium.
Ann. Paediat. Fen. (Suppl. 2) 1:1954-1955

31) Sato, Y. 1936 Calcium metabolism of the fetus.
J. Chosen M. A. (Abstract) 28:49

32) Scarpelli, R., W. Tremblay, and T. Everson 1960 A comparative cytochemical and cytological study of vitamin D induced nephrocalcinosis.
Am. J. Path. 36:331

33) Tomaszewski, J. 1962 Observations on prolonged gestation.
Master's thesis Loyola University, Chicago, Illinois

34) Wasserman, R. H., C. L. Comar, M. M. Nold and F. W. Lengemann 1957 Placental transfer of calcium and strotium in the rat and rabbit.
Am. J. Physiol. 189:91-97

35) Widdowson, F. M. and A. Spray 1951 Chemical development in utero.
Arch. Dis. Childh. 26:205-214

36) Wilkinson, P. M. and F. E. Hoecker 1953 Selective placental transmission of radioactive alkaline earths and plutonium.
Trans. Kansas Acad. Sci. 56:341-363

37) Wislocki, G. B., H. W. Deane and E. W. Dempsey 1946 The histochemistry of the rabbit placenta.
Am. J. Anat. 78:281-318

38) Wislocki, G. B. and E. W. Dempsey 1946 Histochemical age changes in normal and pathological placental villi. Endocrinology 38:90-106

Table I

The Gestation Age Distribution of Albino Rat Placentae Obtained from Untreated Mothers Following Umbilical Cord Ligation of Some of the Conceptuses

Gestation Age At Ligation (Days)	Time Between Ligation And Sacrifice (Hours)	# Mothers	# Conceptuses Ligated	# Conceptuses Intact
13	24	1	4	3
14	24	1	5	5
15	6	1	7	4
	12	1	6	4
	24	2	14	10
	48	2	11	7
	72	2	8	4
16	6	1	5	3
	12	1	7	4
	24	2	7	1
	72	1	7	3
17	6	1	7	4
	12	1	6	3
	24	1	6	3
	48	1	3	4
	72	1	4	2

Gestation Age At Ligation (Days)	Time Between Ligation And Sacrifice (Hours)	# Mothers	# Conceptuses Ligated	# Conceptuses Intact
18	6	1	7	7
	12	1	4	3
	24	1	5	4
	48	1	7	4
	72	1	7	5
19	6	1	7	5
	12	1	6	6
	24	1	5	2
	48	1	6	5
20	6	1	5	4
	12	1	1	1
	24	1	5	3
21	6	1	5	5
	12	1	7	5

Table II

The Gestation Age Distribution of Albino Rat Placentae Obtained from Mothers Treated with 100,000 Units of Vitamin D Following Umbilical Cord Ligation of Some of the Conceptuses

Gestation Age At Ligation (Days)	Time Between Ligation And Sacrifice (Hours)	# Mothers	# Conceptuses Ligated	# Conceptuses Intact
13	24	3	13	6
14	24	3	16	12
15	24	6	21	17
16	24	4	13	25
17	24	2	8	9

Table III

Maternal Blood Calcium Levels from Sacrificed Pregnant Rats Administered Either 100,000 Units of Vitamin D in Three Ml of Water for Three Days Prior to Being Sacrificed or Three Ml of Water

Day	Control	Experimental
13	12.86	14.64
		14.91
		15.48
14	10.26	12.92
		14.64
		12.97
15	11.34	13.20
		12.92
		13.04
		12.86
		12.64
		12.88
16	11.56	13.92
		13.60
		13.90
		13.52
17	13.40	14.88
		14.40

Key for qualitative interpretation of calcium deposits in labyrinth

0- No Ca deposits

+- Traces of Ca deposits

++- Less than 1/2 of labyrinth shows evidence of Ca deposits

++- 1/2 to 3/4 of labyrinth shows evidence
+ of Ca deposits

++- Over 3/4 of labyrinth shows evidence
++ of Ca deposits

Table IV

Calcium deposition in the labyrinth of albino rat placenta obtained from untreated mothers following umbilical cord ligation of some of the conceptuses

Age	Hour Sacrificed At After Operation	Animal #	Ligated A	Ligated B	Ligated C	Non-ligated A	Non-ligated B
13	24	19	0	0	0	0	0
14	24	1	0	0		0	
15	6	33	0			0	0
	12	50	0	0		0	
	24	17	0			0	
	48	18	+			0	
		21	+	+		0	0
	72	34	++			0	
		35	+			0	
16	6	31	0	0		0	
		32	0			0	
	12	48	++	++	+	0	
	24	44	++				
	72	52	++ +	++ +		0	
17	6	43	+	+	+	0	0
	12	27	++			0	
	24	16	++	++		0	0

Age	Hour Sacrificed at After Operation	Animal #	Ligated A	Ligated B	Ligated C	Non-ligated A	Non-ligated B
17	48	53	++ ++			0	
	72	36	++ ++	++ ++		0	
18	6	37	+	0	+	0	
	12	46	++	++ +		0	
	24	28	++			0	
	48	23	++			0	
	72	39	++ ++	++ ++		0	
19	6	26	+	+		0	
	12	54	++			0	0
	24	40	++ ++			0	0
	48	41	++			0	0
20	6	38	++	++		0	
	12	56	++ +	++		0	
	24	20	++ ++	++ +		0	0
21	6	14	++	++		0	
	12	55	++ +	++ +	++	0	

Table V

Calcium deposition in the labyrinth of albino rat placenta obtained from mothers administered 100,000 units of vitamin D following umbilical cord ligation of some of the conceptuses.

Age	Hour Sacrificed At After Operation	Animal #	Ligated A	Ligated B	Ligated C	Non-ligated A	Non-ligated B
13	24	6	0	0		0	0
		9	0	0		0	0
		14	0	0	0	0	0
14	24	11	0	0	0	0	+
		15	0	0	0	0	0
15	24	13	0	0	0	0	0
		18	0	0		0	0
		2	0	0	0	0	0
		7	0	0		0	0
16	24	4	0	++ +	+	0	0
		5	++	++ +		0	0
		6A	++ +	++ +	0	0	0
		8	0	0		0	
17	24	9A	++ +	+	++	0	0
		10	++ +	++ +	++	0	0

Key for Photomicrographs

MS	maternal sinus
FC	fetal capillary
RBC	red blood cells
ST	swollen trophoblast
TN	trophoblastic nucleus
EN	endothelial nucleus
HD	hyaline appearing deposits
Ca	calcium deposition
AP	alkaline phosphatase activity

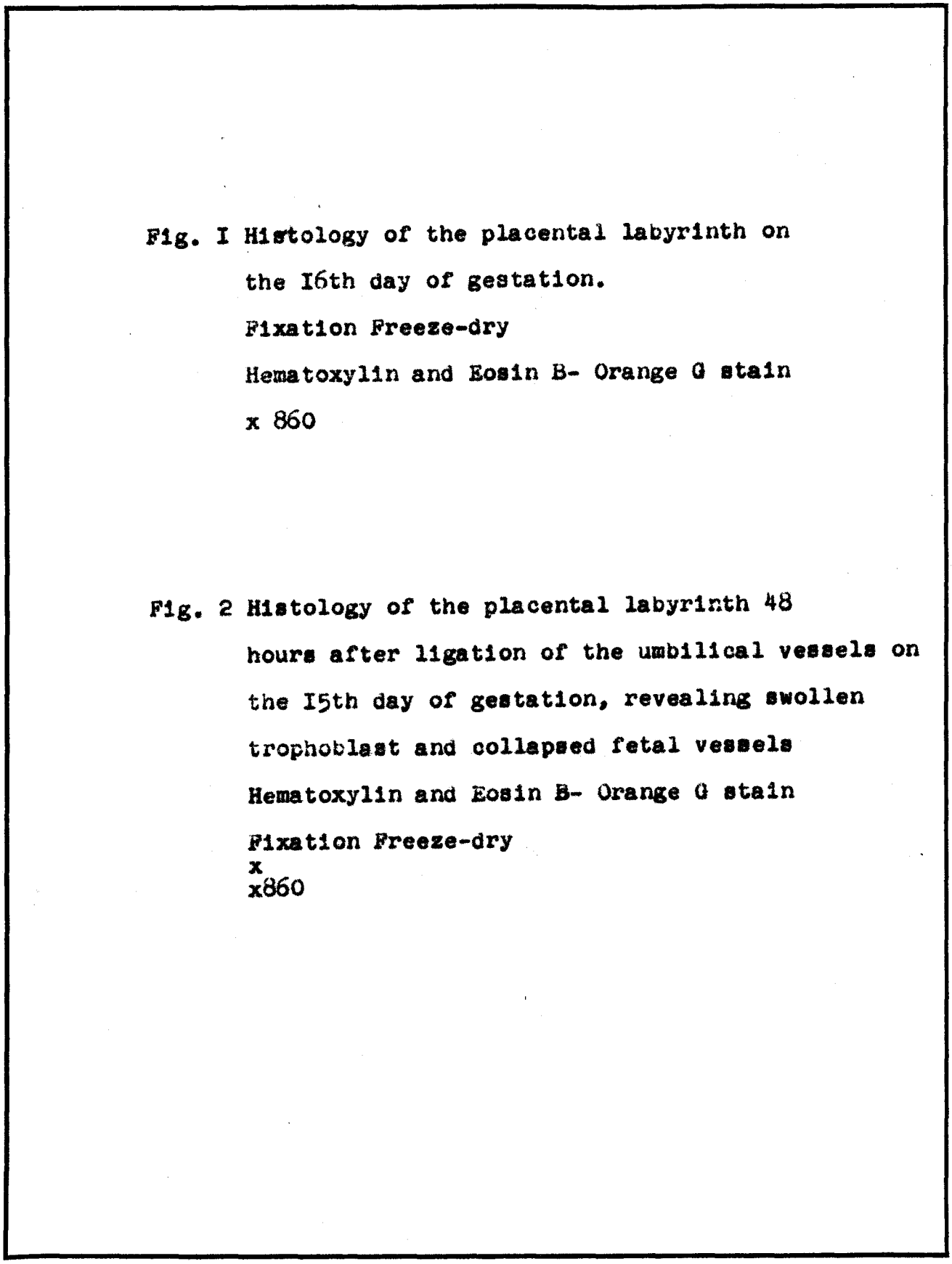
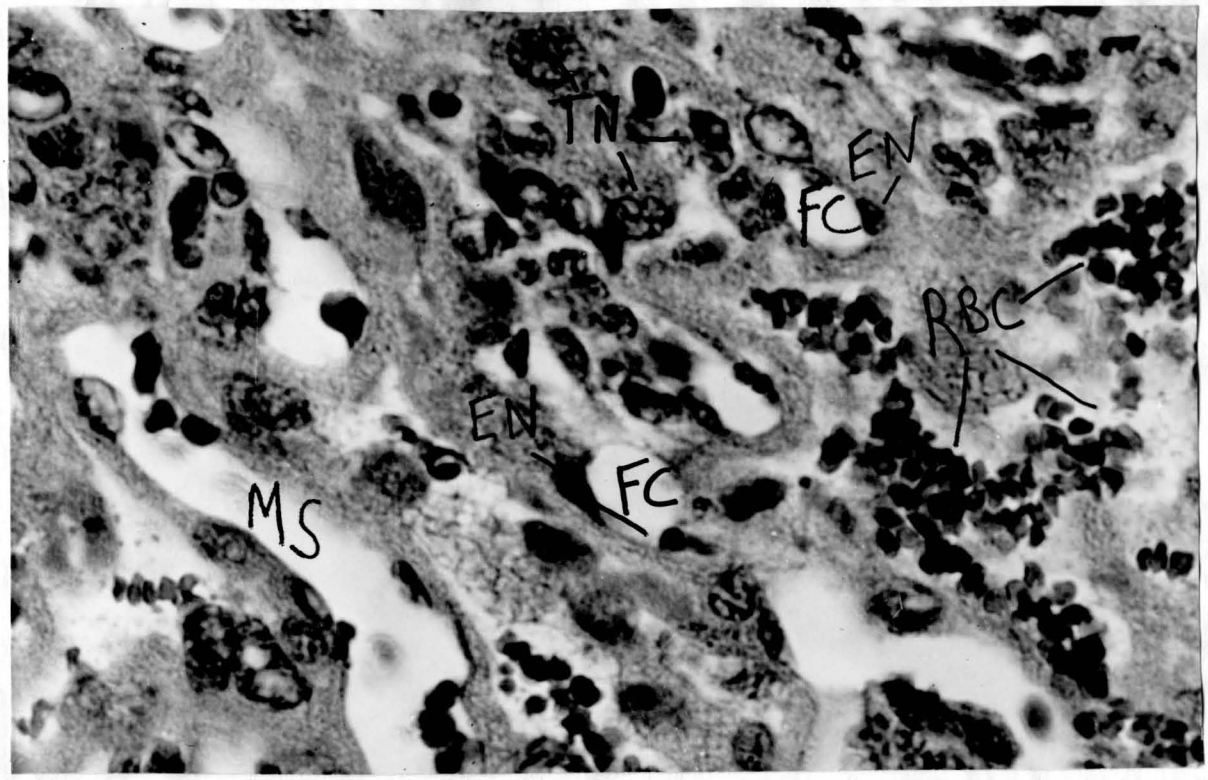


Fig. I Histology of the placental labyrinth on the I6th day of gestation.
Fixation Freeze-dry
Hematoxylin and Eosin B- Orange G stain
x 860

Fig. 2 Histology of the placental labyrinth 48 hours after ligation of the umbilical vessels on the I5th day of gestation, revealing swollen trophoblast and collapsed fetal vessels
Hematoxylin and Eosin B- Orange G stain
Fixation Freeze-dry
x
x860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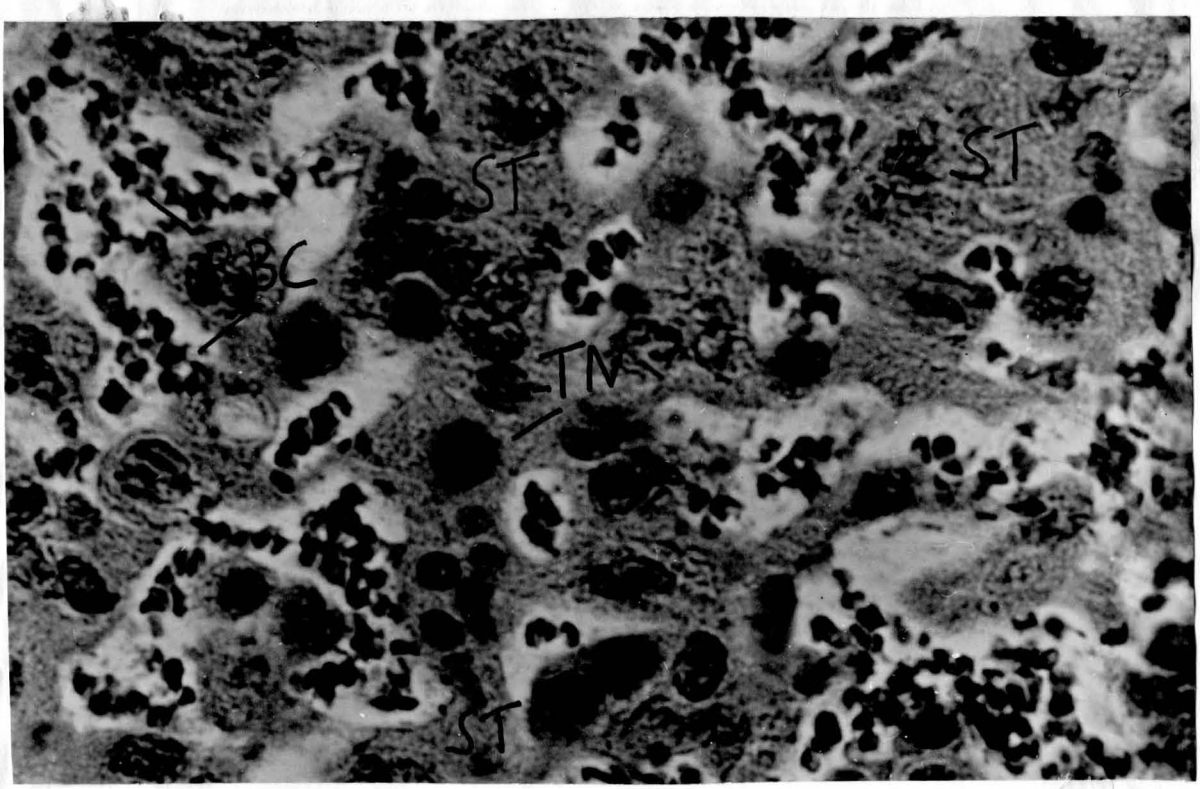
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Fig. 3 Histology of the placental labyrinth 72 hours after ligation of the umbilical vessels on the 18th day of gestation revealing severe swelling and obstruction of some maternal sinuses by the accumulation of hyaline appearing deposits
Hematoxylin and Eosin B- Orange G stain
Fixation Freeze-dry
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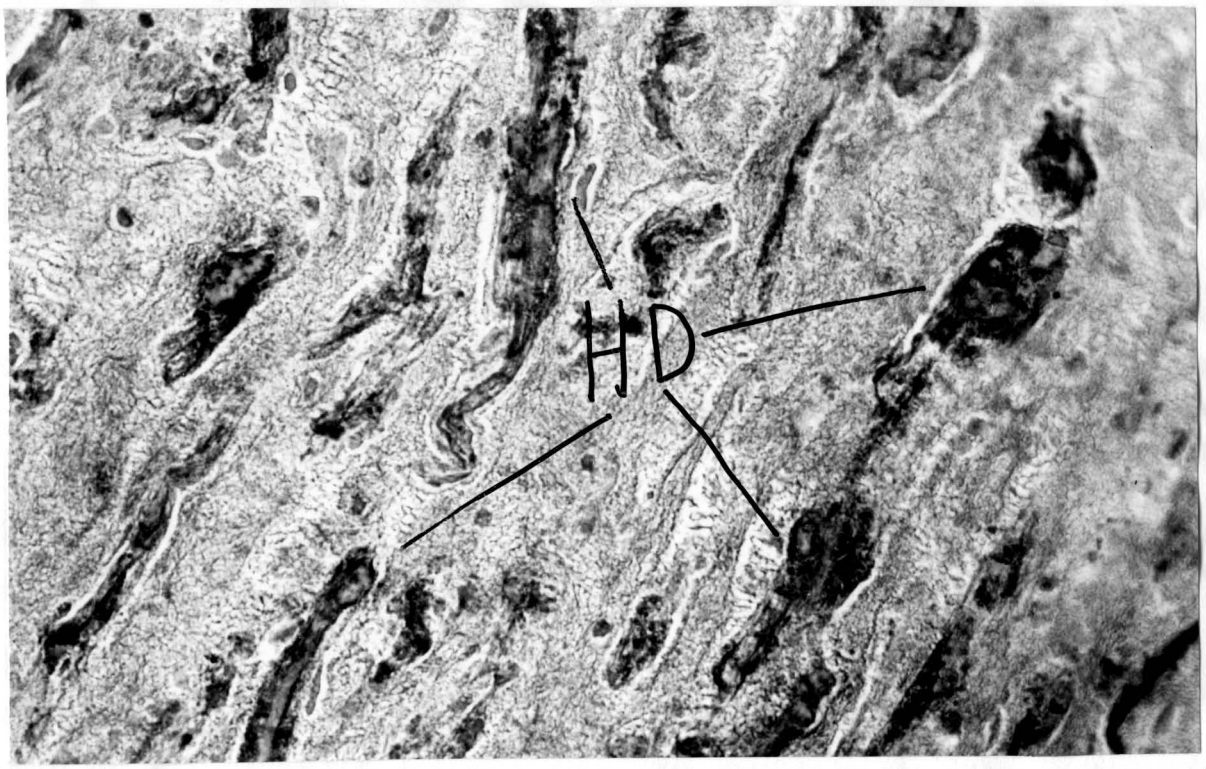
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Fig. 4 Example of negative calcium deposition by Hurst's method, in the placental labyrinth on the I6th day of gestation
Fixation Freeze-dry
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Fig. 5 Example of 2+ calcium deposition in the placental labyrinth by Hurst's method I2 hours after ligation of the umbilical vessels on the I8th day of gestation
Fixation Freeze-dry
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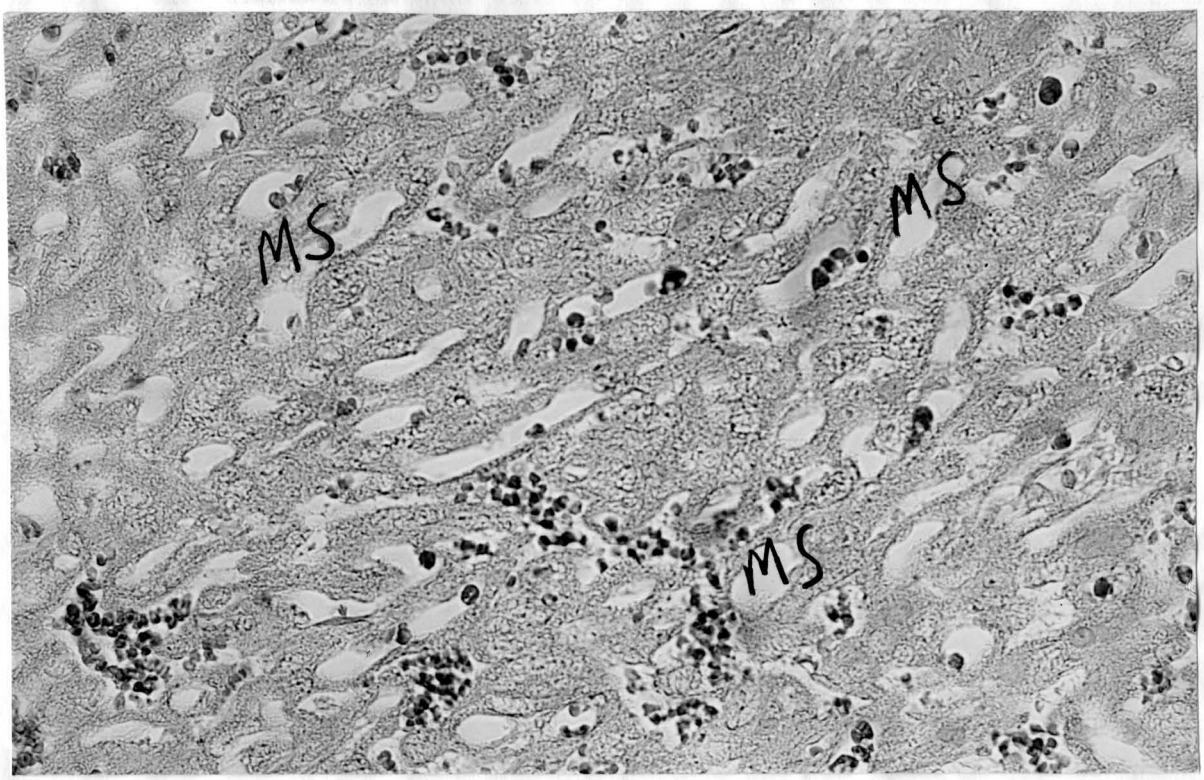
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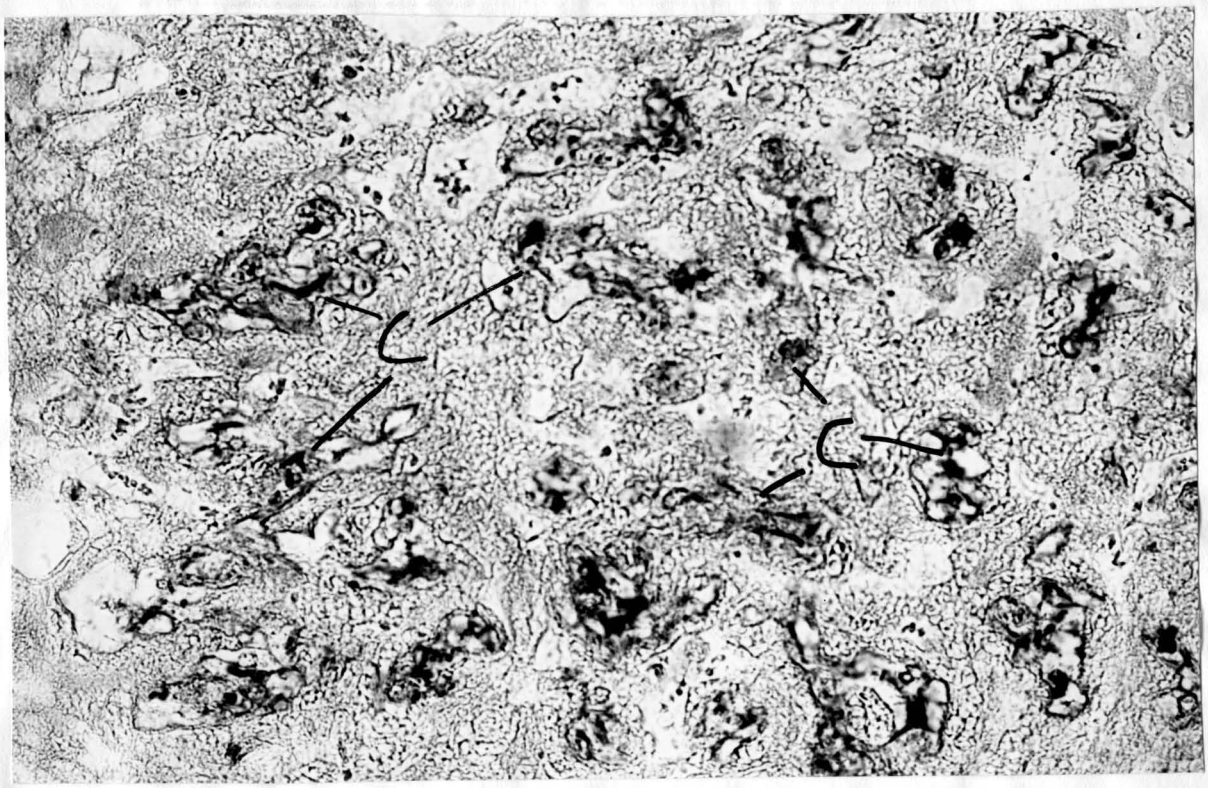
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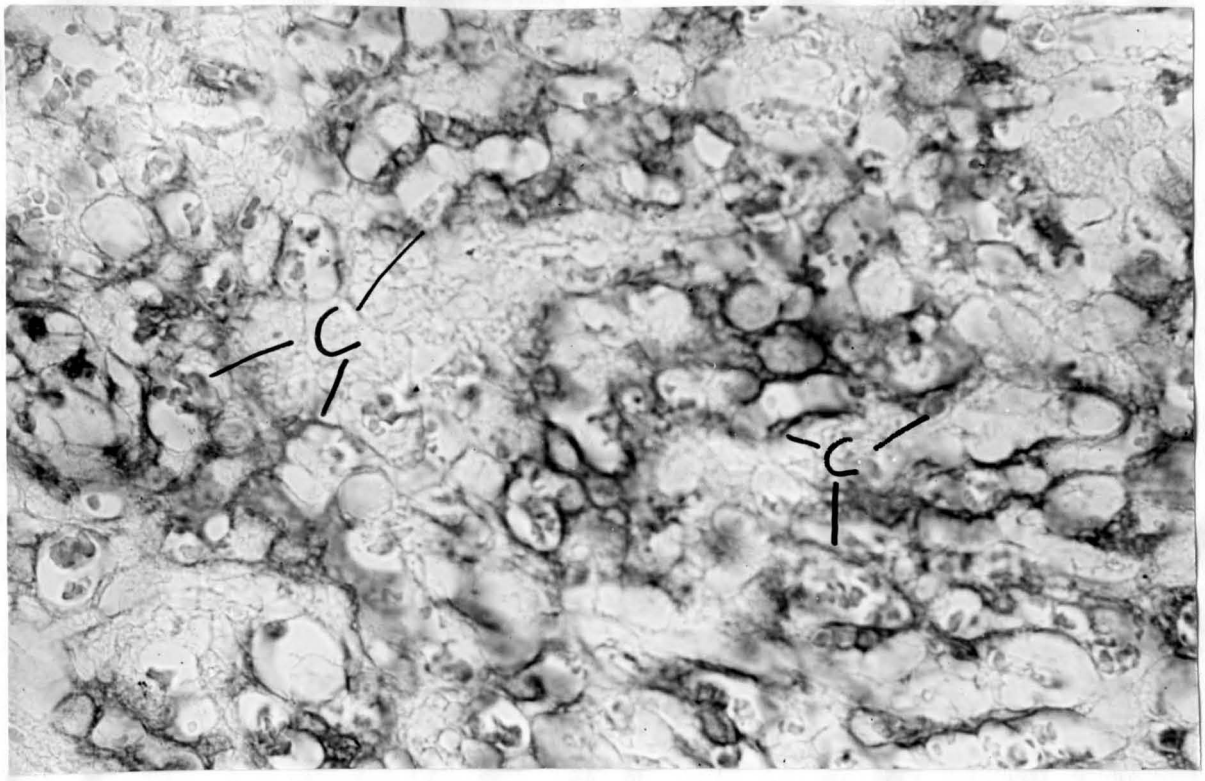
Fig. 6 Example of 4+ calcium deposition by the method of Hurst, in the placental labyrinth 48 hours after ligation of the umbilical vessels on the I7th day of gestation.
Fixation Freeze-dry
x 430

Fig. 7 Example of 2+ calcium deposition by the method of Kashiwa, in the placental labyrinth 24 hours after ligation of the umbilical vessels on the I7th day of gestation.
Fixation Phosphate buffered formalin
x 430



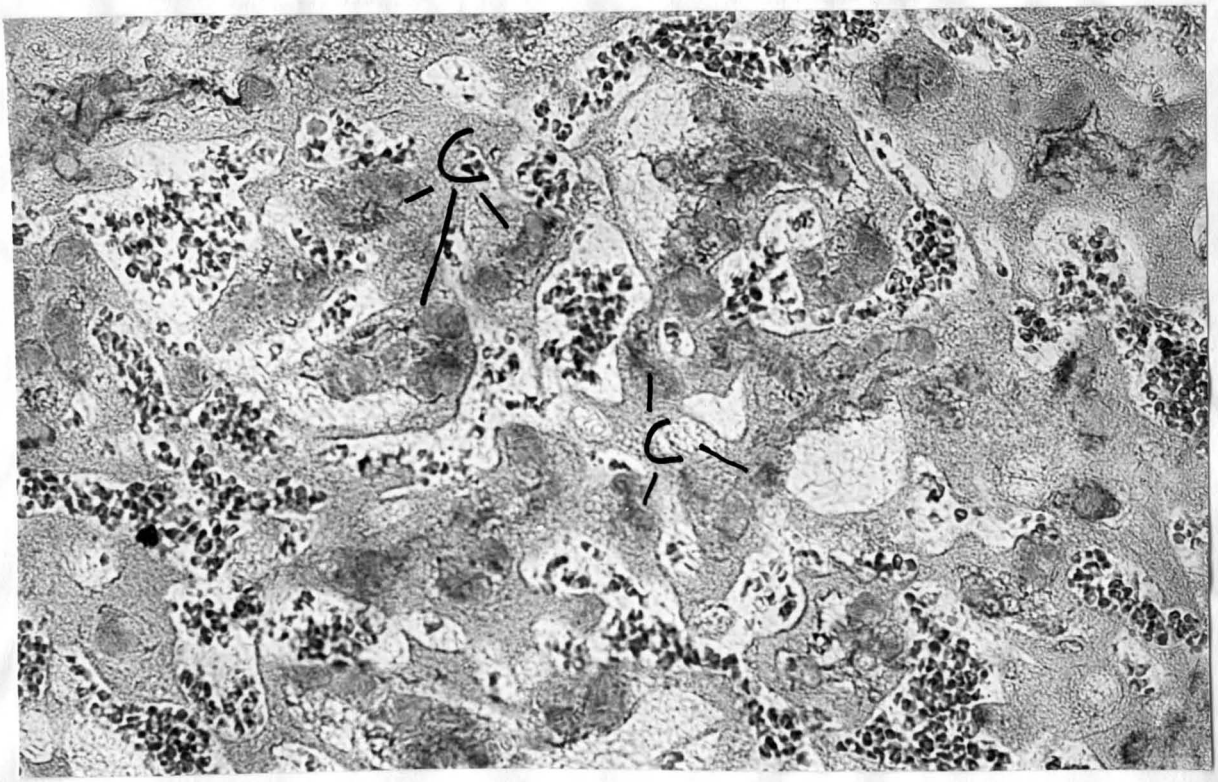


Fig. 8 Alkaline phosphatase activity demonstrated by Burstone's method, in the placental labyrinth on the I7th day of gestation
Fixation Freeze-dry
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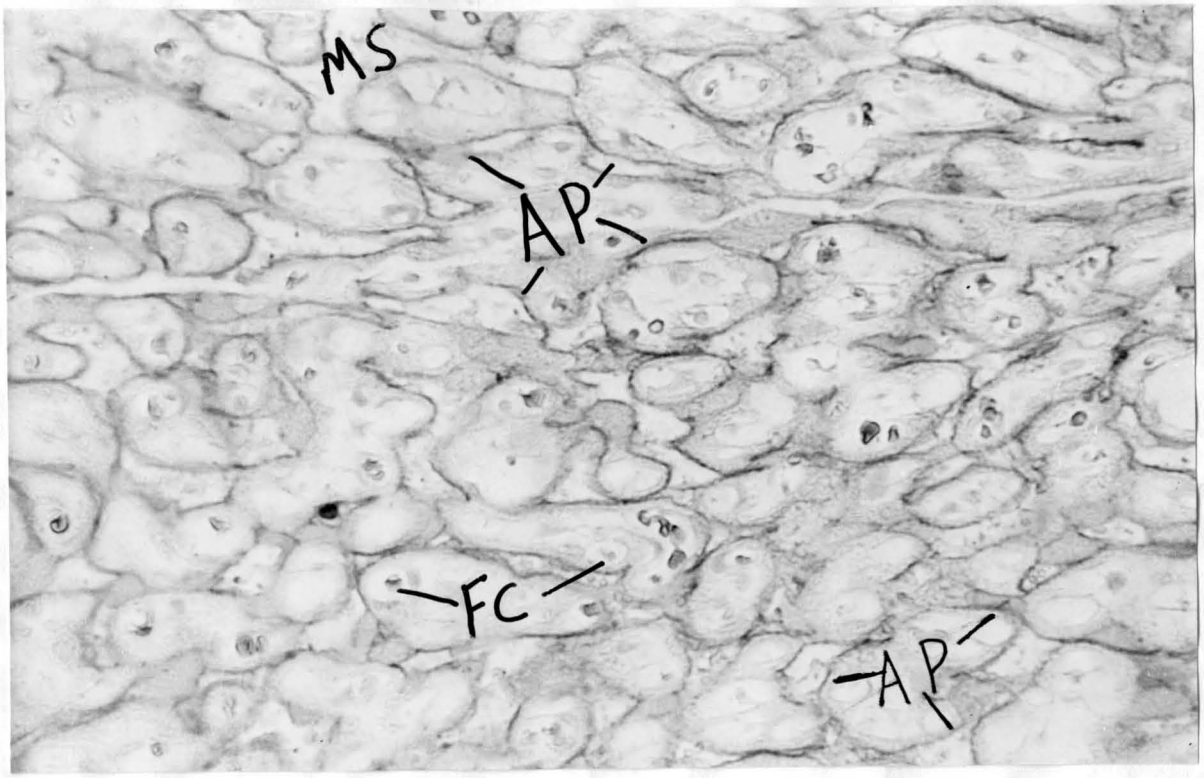
Fig. 9 Alkaline phosphatase activity demonstrated by Burstone's method, in the placental labyrinth 24 hours after ligation of the umbilical vessels on the I7th day of gestation
Fixation Freeze-dry
x 430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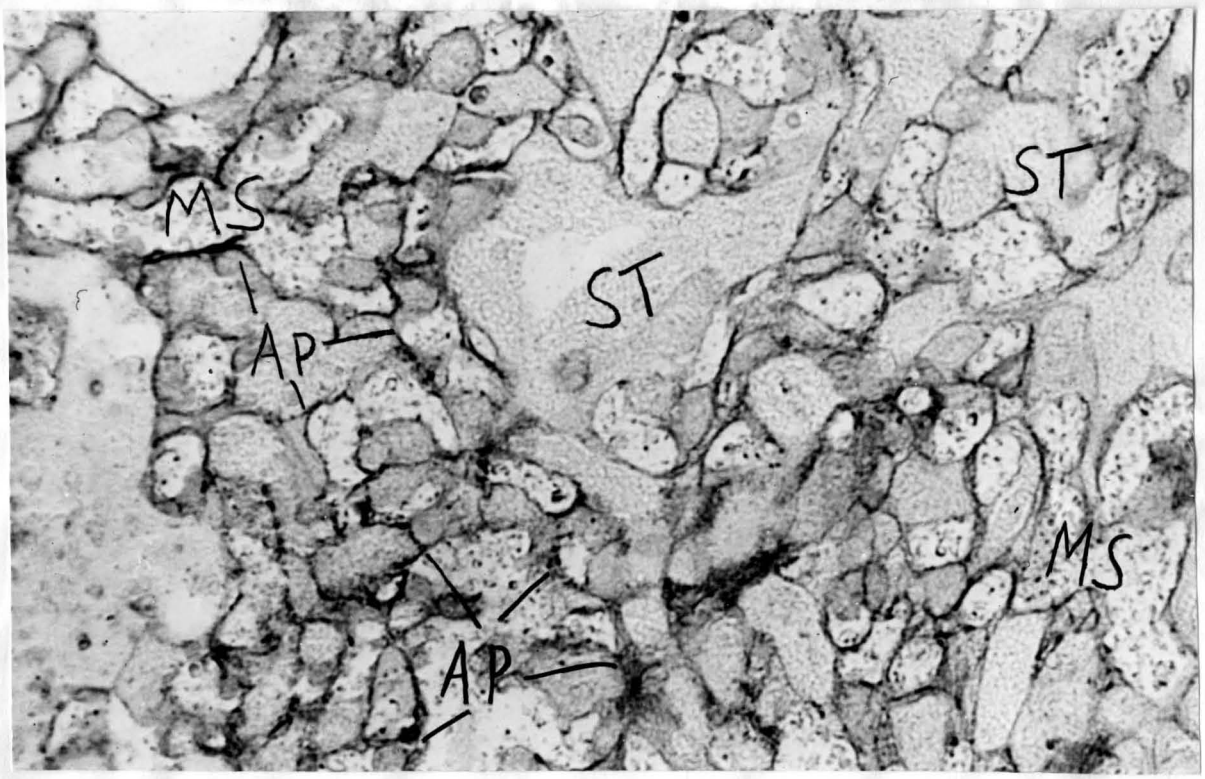
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Approval Sheet

The thesis submitted by William G. Schmitz has been read and approved by three members of the faculty of the Graduate School.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 27 1966
Date

Leslie H. Tennant
Signature of Adviser