DIRECT OBSERVATION OF THE DEVELOPING MICROCIRCULATORY PATTERN IN LIMB BUDS OF FETAL MICE

by

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Enumeration of the countless realized factors in life which, combined with the unrecognized, stimulate one to achievement is not important; but acknowledgment of their existence provides purpose for achievement.

I would, however, like to express special thanks to my Professor, Dr. R. L. Bacon, for the privilege of working in his laboratory, for the opportunity of working according to my own interest, and for guidance when guidance was sought.

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INTRODUCTION

The adaptation of techniques used in microscopic observations of blood flow in organs of adult mammals has permitted me to observe the dynamics of blood flow in fetal mice. The results of this study, combined with earlier observations by others on the development of the vascular system, provide reasonable concepts of the process by which embryonic peripheral vascular morphology is transformed to the pattern seen in the newborn, of the relationships between dynamics of flow and vascular morphology, and of possible effects of changes in dynamics of flow on development of specific areas of the fetus.

Present concepts of peripheral vascular developmental mechanics in mammalian embryos are reasonably rational extrapolations from direct observations of capillary beds and the development of vascular channels in adult tissues, or are based on injections of embryonic vessels with opaque material. The former resembles the embryonic situation only in certain respects, and the latter, although it does indicate the presence and general pattern of vessels, gives no indication of the dynamics of flow and produces distortion and distension in the minute, sensitive, thin-walled vessels.

Two positions have been taken concerning the development of the vascular system. The older states that arteries and veins grow out of single trunks to their respective territories. This method of development implies that in a given area functional circulation can not exist until connection has occurred between capillary outgrowths from venous and arterial trunks.

The current position is that the initial vessels in any area are those of capillary size from which arteries and veins arise secondarily. Proponents for the capillary plexus ancestry of larger vessels view the vascular system as functioning from the beginning. The latter theory was first recorded by Aeby in 1868 (1), following a study of adult vascular variations and arterio-venous anastomoses. Krause (20) in 1876 accepted Aeby's hypothesis when discussing blood vessel variations. Nevertheless, Aeby's concept gradually fell into disrepute as a result of comparative investigations, which showed consistency in the number and distribution of vessels with respect to other structures such as muscles, nerves, etc. (12). Ruge in 1883 (29) explained the appearance of vascular variations by the over development of normally inconspicuous vessels rather than by the presence of capillary anlagen. In 1891 Hochstetter (16) denounced the Aeby-Krause theory and declared he could find no indifferent condition of the vascular system in the limb buds of Triton. Thoma (34) attempted to determine the method of development of arteries and veins by studying the development of vascular patterns in the yolk sacs of chick embryos at various stages of incubation. Early in development, he saw only an indifferent network of vessels in which no predominant channels could be identified. In successive preparations he showed the gradual formation of arteries and veins from this cpaillary-sized net. This transformation of capillary-sized vessels into larger ones was described as being representative of a functional adaptation of this network to the demands of the circulation. The decision as to which channels

of the net would become arteries and veins was considered to be dependent on the position with respect to the largest quantity of incoming arterial blood and to excurrent venous channels. Thoma's postulates have subsequently been shown by vascular injections to be applicable to the development of the vascular system of mammalian fetuses (25).

The presence of capillary nets peripheral to larger developing vascular channels in mammalian fetuses was first noted in 1894 by Zukerkandl (38). He described the early appearance of the median artery of the arm as a chain of capillaries accompanying the median nerve. Studies of the submaxillary gland and lung by Flint (14) and of the liver by Mall (24) reveal development of vascular trunks from pre-existing capillary plexuses. Rabl's studies (28) of the wing bud of the duck and Goppert's (15) studies of the developing fore limb in mice showed the formation of the subclavian artery and its branches from a capillary plexus.

Evans (11), utilizing a technique of injecting the vascular system of live chick embryos, traced the earliest vascularization of the limb buds. He saw an early capillary plexus arising from the lateral aortic wall from which the subclavian or sciatic arteries were formed by preservation and enlargement of specific vessels. Evans traced the development of the internal carotid artery and jugular veins from a stage when a capillary plexus connected with the aorta and vitelline vein composed the entire vascular system of the head of the chick embryo. Similar development of pulmonary arteries, gut arteries, caudal end of the aorta, and the aortic arches from

capillary anlagen was noted by Evans. Bremer (4) described an endothelial anlage for the entire cephalic portion of the aorta in five somite rabbit embryos.

Visualization of the finest radicles of the vascular system, made possible by the injection techniques noted above, gives strong evidence that blood vessels are formed from capillary anlagen. The apparent predetermination of the position of vessels (eg. the aorta) is shown not to result from the direct outgrowth of arteries and veins. Contrary to previous observations of investigators working with uninjected embryos, Evans saw no evidence in the chick of vessels not connected to the general system.

Direct observations of development of blood vessels have been performed on the transparent tails of living larval amphibia. Clark (7), working with the tadpole, noted specific peripheral capillaries in early stages which gradually became the arterial pathway to new capillaries in the expanding periphery.

Development of the inferior vena cava presents an interesting adaptation of the above described transformation of capillaries into larger vascular trunks. Lewis (21), working with rabbit embryos, showed that the inferior vena cava is formed through an inosculation between one of the posterior hepatic veins and the right subcardinal vein. The union between these two functioning venous trunks results in the formation of a channel through the caval mesentery. Davis, working on pig embryos, (9) demonstrated that the connecting channels across the caval mesentery were the result of fusion of capillary sprouts from the hepatic and subcardinal vessels.

As pointed out by Evans (11), tissue at one time permeated with a uniform capillary mesh may later show differences in amounts of blood supply to specific areas. Evans felt the variations in tissue capillarity were coincident with corresponding changes in the nature of the tissue and often positive evidence of these changes. He described premuscle and precartilage as being characteristically nonvascular areas circumscribed by capillary flow to the adjacent tissues. He used the anterior limb bud of the duck as an example of tissue which initially has a uniformly distributed capillary net and later has areas which capillaries appear to avoid.

Woolard (37), studying injected pig embryos, described three stages in the formation of an arterial supply to the anterior limb bud. Initially, he described the stage of the capillary net, second the retiform stage characterized by enlarged, coelescing vessels which have a tendency to fuse, and third the formation of a definite stem. He described each stage as a response to definite physiological demands, the first being an angioblastic response to tissue needs, the second representing the changes taking place according to the previously mentioned postulates of Thoma and which lead to the formation of a single arterial vessel (the third stage). Streeter (33) described a final stage of completion of histological differentiation of the walls of the vessels.

Hughes' study (17) of the histogenesis of the main arteries of the chick embryo clarifies the important determining factors in this later stage of development. He points out the existence of primitive endothelial tubes when circulation begins and the direct

correlation of histological differentiation of the mesenchyme around vessels with the pressure of flow within vessels. This correlation is in agreement with the principle of Thoma (34) that "the growth in thickness of a vessel wall is proportional to the tension in the wall which itself is determined by the diameter of the vessel lumen and by the blood pressure." The second determining factor for histological differentiation pointed out by Hughes is the role played by the tissues surrounding a given vessel.

Striking examples have been given of differences in structure between adjacent arteries which can not be explained by the dynamics of blood flow. In mammals, the ductus arteriosus is a muscular vessel whereas pulmonary and aortic arches are elastic. In birds, the ductus arteriosus is extremely thin in the region which closes at the time of hatching (5). Also in the chick, a thickening of the wall occurs in the common carotid just before the bifurcation and in the proximal portion of the internal and external carotids (5). An abrupt cessation of elastic fibers occurs in the omphalomesenteric artery as it passes beyond the body wall (8).

Much evidence for Thoma's hypothesis has been derived from the study of adult vessels and growth of tension-bearing, vascular elements in tissue culture. Fisher (13) described a thickening of veins when transplanted into arteries. Schaeffer and Radsch (31) demonstrated gradual loss of the normal structure of the common carotid artery following ligation and thereby interruption of circulation through the vessel. Tissue culture experiments (10, 27,

36, 26) have lead to the conclusion that mechanical tension within the medium is necessary for the formation and maintenance of the tension-bearing elements of fully developed vessels (that is, extracellular elastic and collagenous fibers, and smooth muscle).

Benninghoff and Spanner (3) in their study of an acardiac fetus, in which a normal twin was responsible for the circulation in both fetuses, noted all the main arteries of the acardiac twin could be physiologically peripheral arteries of the normal twin. The dorsal aorta and common carotid arteries were muscular rather than elastic in type.

The aortic arches of the chick embryo, although having similar gross structure and morphological position, nevertheless show marked differences in wall thickness which is largely attributed to differences in blood pressure and flow rate within them (17).

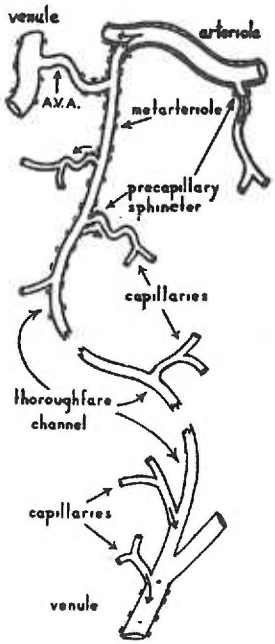
Lieter (23) and Silverman (32) first reported the use of fused quartz rods for illumination of microscopic material. Wearn, et al. (35) used a similar rod to conduct "cold" light in the study of intact mammalian lungs. Subsequently, Knisely (18) devised a method permitting direct microscopic observation of living organs in situ illuminated by a quartz rod while maintaining experimental conditions simulating the normal environment of the organ. Chambers and Zweifach (6), using this technique, have arrived at a concept of the structural vascular unit (Fig. 1) based on their microscopic observations of blood flow in the mesoappendix of the rat. Capillaries were shown to be the connecting links between the smallest ramifications of the arterial and venous trees (arterioles and venules). Occassional

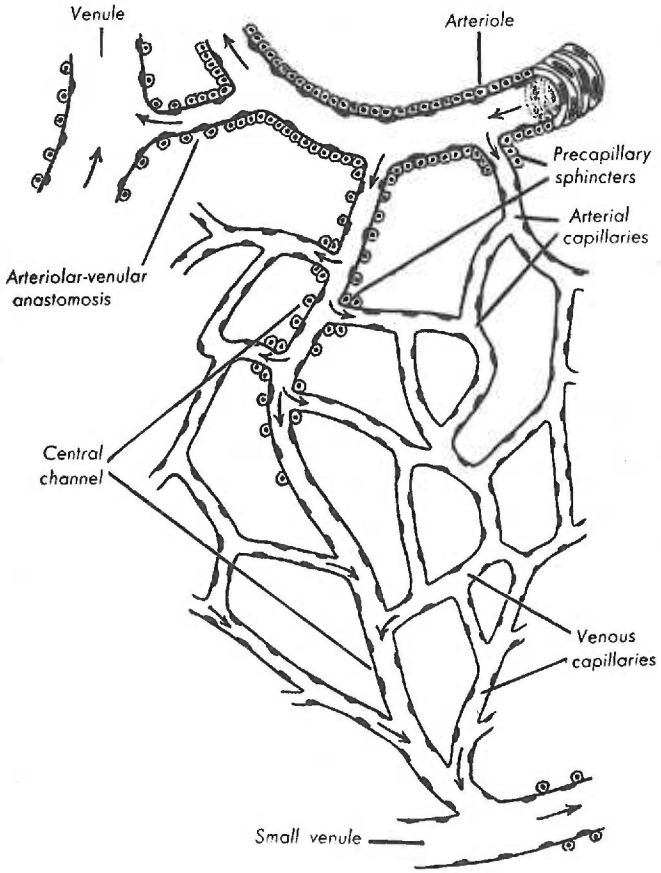
vessels of capillary dimensions were noted to transport more rapid flow than that which was occuring in adjacent capillaries. These latter vessels, termed central or thoroughfare channels, originated as branches or prolongations of terminal arterioles. The proximal portion of a central channel and the proximal segments of capillaries arising from this portion of the central channel were shown to contain contractile smooth muscle in contradistinction to the remainder of the capillary bed. The amount of flow through central channels and adjacent capillaries was noted to be a function of periodic contractions by these smooth muscle cells.

The observations to be reported below have been obtained by the adaptation of the technique described by Knisely (19) to the study of fetal mice.

Figure 1

These illustrations are diagramatic representations of Chambers and Zweifach's (6) concept of a functional vascular unit in the mesoappendix of an adult rat.


venule
arteriole
A.V.A.
metarteriole
precapillary sphincter
capillaries
thoroughfare channel
capillaries
venule


Venule
Arteriole
Precapillary sphincters
Arterial capillaries
Arteriolar-venular anastomosis
Central channel
Venous capillaries
Small venule

MATERIALS AND METHODS

Records of microscopic observations have been made of the dynamics of blood flow in fetal mice at various stages of gestation from 10 1/2 to 17 days. All fetuses were from the first litters of Webster strain albino mice which had been mated with Webster strain males. The mice were raised in small wire cages under uniform conditions. Their diet consisted of Purina Lab Chow supplemented with lettuce once a week.

Males and females were placed together for mating periods which varied from 1 to 48 hours. The age of individual fetuses was determined within the limits imposed by the length of the mating period, by elapsed time from observed mating, and were checked by measurements of preserved fetuses. The age of the mother mice varied from 3 to 12 months.

Anesthetic.

The pregnant mice were anesthetized by a mixture of Nembutal in 10% ethyl alcohol administered by intraperitoneal injection. Light anesthesia, judged by the type of respiration and the response to pain, was initiated by giving .063 mgms. of the anesthetic per gram of body weight. Additional small quantities of the anesthetic were occassionally given after the first hour in order to maintain a uniform level of anesthesia.

Dissection.

A 1 1/2 centimeter, oblique, abdominal incision through skin, muscle and peritoneum was made directly over and parallel to one of the uterine horns in the pregnant mouse. Major vessels of the

body wall (e.g. inferior epigastric) and of the peritoneum were avoided in order to prevent hemorrhage into the peritoneal cavity. Being careful to avoid unnecessary manipulation, a longitudinal incision adequate for easy delivery of the fetus was made through the antimesometrial side of the uterine horn in order to avoid the placenta which is located on the mesometrial side at this stage of gestation. This side of the uterus is also considerably less vascular; and, therefore, less loss of blood occurs by following this procedure. The fetus within its yolk sac and with its placental attachment in tact was thus exposed. Due to the great amount of blood flow within the yolk sac, the final stage in exposure is critical and must be done with the aid of a dissecting microscope (I used approximately 27X magnification). Because of the small total blood volume of the fetus, hemorrhage from yolk sac vessels greatly alters the hemodynamics of fetal circulation and thus must be avoided. The yolk sac incision was made above the particular part of the fetus to be observed, in this instance the posterior limb bud, while attempting to leave the underlying amniotic sac in tact. In order to maintain reasonably normal conditions for the fetus, observations were made when possible through this clear, bloodless membrane. Tension or pressure of any sort on the umbilical cord was specifically avoided because of the relative ease with which the umbilical vein can be occluded.

Maintenance of "Physiologic" Conditions.

By the use of the quartz rod technique as described by Knisely, the exposed fetus was kept in an environment as nearly as possible

simulating the normal fetal habitat. Precautions were taken to minimize trauma, control temperatures and adequately irrigate the exposed organs of the mother as well as the fetus itself with a physiologic salt solution, which had been warmed to 38.5°C. This solution contained 160 mEq./L of sodium, 6 mEq./L of bicarbonate, 4.3 mEq./L of calcium, 164 mEq./L of chloride and 5.6 mEq./L of potassium. The solution was directed to a position immediately beneath the fetus through hollow, fused quartz, light-conducting rod and dripped from above through a polyethylene tube (Fig. 2). Temperature of the ambient air was not specifically controlled but varied between 70 and 75°F. The anesthetized mother was placed on a wire frame which allowed the excess saline to drip away and thereby decrease the amount of cooling due to evaporation from her body surface (Fig. 2).

The fetuses were illuminated by light from a 750 watt projection bulb conducted through a fused quartz rod (Fig. 3). Again in order to avoid trauma to the fetus, great care was taken in easing the tip of the rod under it. Transmission of a "cold" light of the spectrum visible to the human eye, combined with the property of internal reflection which makes possible the transmission of light around curves by fused quartz, have been the principle factors in the success of this technique. The low thermal conductivity of fused quartz and division of the rod into two segments reduce heating of tissue to a minimum. Constant flow of saline through the hollow tip of the rod removes the heat formed by transformation of radiant energy within the tissue. The high specific heat of water

in the flowing saline enables the solution to take up heat as fast as it is produced with little change in its own temperature.

Due presumably to the high concentration of water in fetal tissues and the transparency and thinness of the posterior limb bud, the production of heat by "absorption" of light has not been sufficient to result in any visible alterations in the fetal circulation. The microcirculation is notoriously sensitive to such external stimuli, and it seems very likely that if significant amounts of heat had been produced, visible changes would have been observed. Fig. 2 depicts the quartz rod apparatus used in this study. The physiological saline contained within the 18-liter bottle at the top of the photograph was transported to the fetus through a small polyethylene tube by gravity. The course of flow of this saline was directed through thermostatically controlled, constant temperature baths. The water, which was heated to 38.5°C, was pumped through rubber hoses which encased the polyethylene tubes and thereby kept the physiological saline at the proper temperature until it was delivered to the fetus.

The tall pipe or "chimney" enclosing the light source functioned as a convector of the air warmed by the hot projection bulb. The heated air rising in the convector pulled in cool air from below and very effectively prevented over-heating of the light source and, at the same time, eliminated the problem of vibration which would be produced by motor driven, air cooling devices.

Observation and Recording Procedures.

With the aid of a dissecting scope (Fig. 3), the tip of the quartz rod was insinuated into position beneath the fetus. Adjustment of the amount of illumination was accomplished with a variable transformer.

Initially, observations were made for evidences of fetal or maternal hemorrhage, regularity and rate of fetal heart beat, tension on yolk sac and umbilical vessels, impingment on the exposed portion of the fetus by the yolk sac and any abnormalities in the general peripheral circulation of the fetus. If conditions appeared normal, observations of vascular morphology and flow patterns were made with an American Optical stereoscopic microscope at magnifications varying from 27X to 72X; and the results of these observations recorded by drawings with accompanying notes and impressions and, in many instances, verified by later study of motion pictures taken during these observations.

Motion pictures were made with a 16 mm Cine Special II Kodak camera through a specially constructed monocular microscope (Fig. 2 and 3). The monocular microscope had a side arm viewer with a partially silvered mirror deflecting 10% of the light to the eye of the observer and passing the remaining 90% to the film.

Kodachrome and Kodak ER film were used. Camera speed was ordinarily 16 or 32 frames per second. By running the camera at the faster speed and showing the film at 16 frames per second, I was able to decrease the amount of apparent limb bud movements secondary to respiratory movements of the mother. Due to the

weight of the camera and microscope and the inherent vibrations of the movie camera, a rather massive, specially constructed stand was required to hold the photographic equipment. The optimal amount of illumination required for recording the dynamics of flow varies with the angle at which the light passes through the fetus, the thickness of the tissue, and the size of the vessels. Thus, the amount of detail visible and the general quality of the motion picture records are improved if the intensity of the light is varied gradually during the photography.

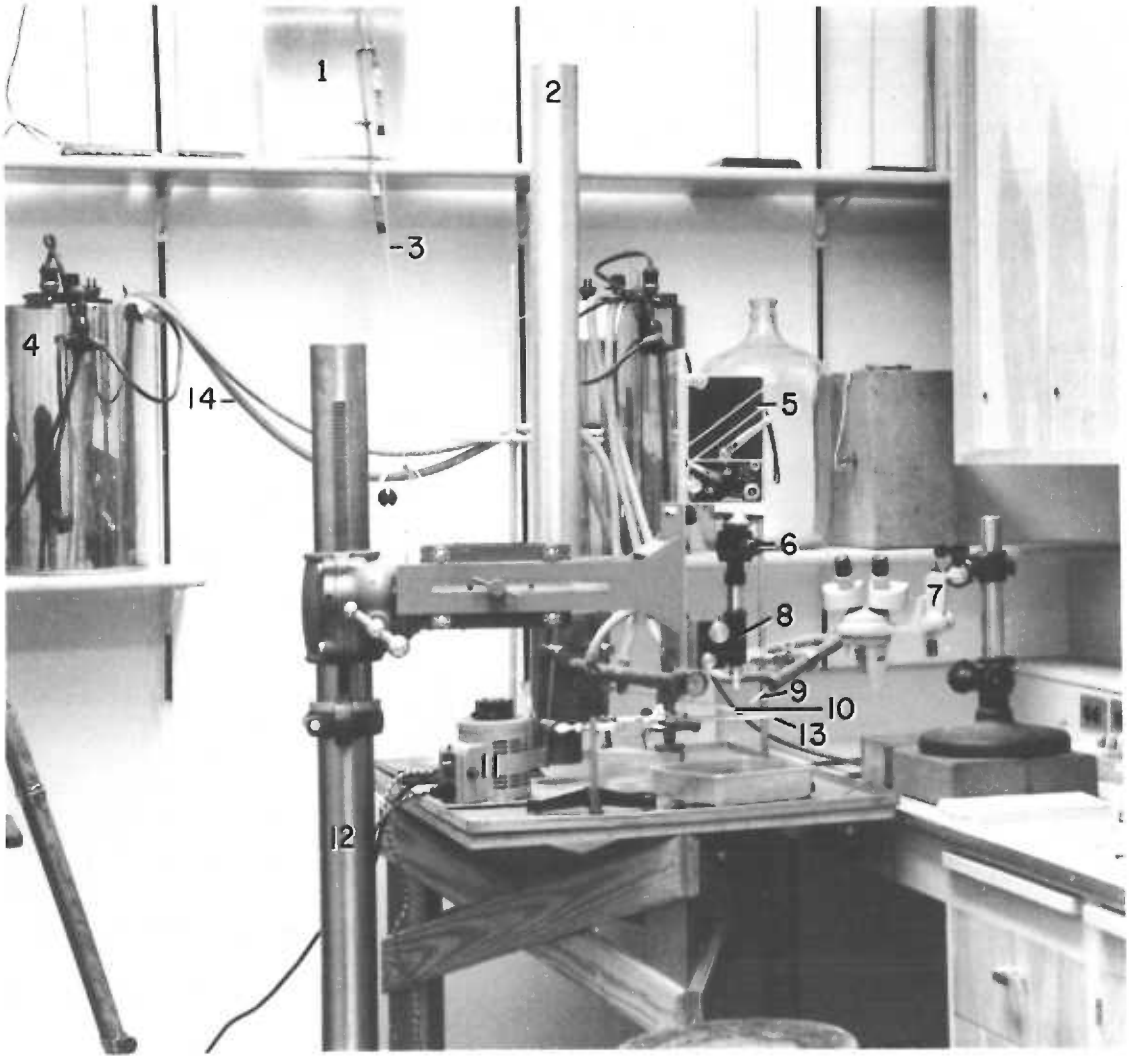
Duration of observations on a single fetus varied from 5 minutes to 90 minutes, although almost all data reported here were recorded from observations made within 10 minutes after exposure of a given fetus. Fetuses from a single litter were often exposed at varying intervals from 5 minutes to 21 hours apart. If the interval between exposures exceeded 3 hours, the mother's abdominal incision was closed with fine silk suture and the mother was allowed to regain consciousness while being kept warm with heat from a small lamp. The mother was then re-anesthetized at the appropriate time.

Records of all fetuses observed have been kept on coded data sheets. Representative fetuses from specific litters exposed at a given time have been numbered and preserved in 10% formalin. Approximately 200 fetuses from 61 litters are represented in the observations reported here.

Figure 2

Apparatus employed for observation and recording of microcirculatory patterns. Parts are numbered on the photograph and identified in the following list.

1. Reservoir of physiological saline
2. Convector chimney for cooling light source which is enclosed in its base
3. Polyethylene tube conducting saline through the warmed water bath and finally to the sight of the operation
4. Controlled temperature water bath
5. Kodak Cine-Special II 16 mm motion picture camera
6. Side arm viewer deflecting 10% of the light passing through the monocular scope and transmitting 90% to the film
7. Dissecting microscope on adjustable arm
8. Monocular scope
9. Terminal end of polyethylene saline conduit
10. Fused quartz rod drilled to deliver saline to lower surface of tissue
11. Variable transformer
12. Stand
13. Wire mesh operating stand
14. Rubber hose water jacket


1
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12

Figure 3

Detail of apparatus shown in Figure 2. This photograph shows a gravid mouse secured to the wire mesh operating stand. The fused quartz rod is under, and the terminal end of the polyethylene saline conduit is positioned over, an exposed fetus. The monocular scope which directs light to the movie camera is focused directly above the fetus.

OBSERVATIONS

Serial microscopic observations and motion pictures of vascular morphology and dynamics of flow in the posterior limb buds of fetal mice have provided objective evidence for alterations in the arrangement of patterns of capillary flow and venous drainage in this structure during development. The emphasis of this study has been on the changes occuring between 12 1/2 and 14 1/4 days of gestation. Earlier development was seen to be in accordance with observations on limb buds of injected pig embryos (37). Initially, blood flow coursed from a uniform capillary mesh to a sinus-like vein, usually referred to as the border vein, which lay along the entire periphery of the flattened limb bud. This peripheral vessel has been shown in rabbit embryos to drain cephalically into the umbilical network of veins and caudally into the posterior cardinal vein (22). In the mouse embryo, the proximal portion of the border vein of the anterior aspect of the limb bud disappeared at about 12 days; and the connection to the umbilical vein was thereby lost. The entire venous drainage then continued into the remainder of the border vein which coursed around the caudal aspect of the limb bud into the posterior cardinal drainage system (Fig. 4).

Early in development, the only arteriolar and capillary flow which could be seen emanated from an arterial trunk centrally located in the limb bud and drained to the periphery of the limb bud. In embryos of about 12 1/4 days gestation, superficial capillary flow was noted over the outer aspect of the proximal portion

of the posterior limb bud (Fig. 4). The multiple, parallel drainage pathways from this new capillary plexus constituted the anlage of what has been interpreted, on the basis of studies on injected rabbit embryos by Lewis (22), as the anterior tibial vein. The amount of drainage into this vessel gradually increased as the new capillary network grew out to the periphery of the limb bud. Between 12 1/2 and 12 3/4 days, the outgrowth of this new capillary network met the pre-existing peripheral mesh of capillary-sized vessels along the cephalic border of the limb bud (Fig. 5). Thus, confluence was established between two drainage systems. For a few hours after this anatomical connection was completed, specific vessels could be visualized which gave off capillary flow to both drainage systems (e.g. point x in Fig. 5). Fetuses from 13 litters (F-12, 16, 19, 20, 23, 25, 27, 29, 32, 33, 36, 55 and 57), gestation ranging from 12 1/4 to 13 days, have been observed to have a vascular architecture and pattern of flow similar to that denoted in Fig. 5.

In fetuses from 18 litters (F-3, 7, 12, 19, 20, 24, 25, 27, 28, 29, 32, 33, 40, 44, 47, 50, 55, and 57), gestation ranging from 12 3/4 to 13 1/4 days, the direction of blood flow within the border vein along the cephalic border of the posterior limb bud was seen to have reversed (Fig. 6). From the cephalic segment of the border vein, blood flowed proximally into venules or capillaries which had previously carried flow from the outer aspect of of the limb bud distally to the border vein. By means of connections with the new capillary plexus, described above, blood flowed from the border vein to the anlage of the anterior tibial vein.

This flow pattern in which blood which has been gathered into a vein is dispersed to a capillary bed and finally to another vein is essentially a local "portal system". Initially, the reversal of flow within the cephalic portion of the border vein involved only a short terminal segment. (During the last 10 minutes of observation of the particular limb bud diagrammed in Fig. 5, reversed flow was noted to proceed from the cephalic termination of the border vein in the manner described above.) Within a few hours of the appearance of this altered system, the portion of the vein involved usually extended to the web between the developing second and third digits. The increase in flow through the capillaries of the outer aspect of the limb bud, subsequent to the reversal in the border vein, resulted in enlargement of certain channels. Figures 5, 6 and 7, all of fetuses from litter F-19 (fetuses 2 and 3 exposed 1 1/2 and 2 1/2 hours after fetus 1), show the transition in pathways for blood going from the cephalic termination of the border vein to the anterior tibial vein. In fetus 3, as depicted in Figure 7, the reversed flow was confined to one pathway which was enlarged to a diameter comparable to that of the border vein. However, much of this newly formed vessel, along with the border vein disappeared during the next 30 to 36 hours of development (Fig. 9). Coincident with the appearance of the new system, developmental changes in the limb bud were noted which, by exerting pressure on the border vein, may very well increase the resistance to flow through this large vessel distal to its junction with the anlage of the anterior tibial vein.

Expansion of the tips of the digits from the previously smooth contour of the limb bud established a sinuous course for the peripheral portion of the border vein (Fig. 8). Rotation of the limb bud, combined with more rapid thickening of the proximal portion of the bud than that which occurs at the distal end (which was to become the foot and remain relatively flat), resulted in further distortion of the initial pathway of the border vein (Fig. 5). No specific constriction or degeneration of the border vein was noted between the second and third digits prior to the establishment of that point as the bifurcation in direction of flow (Fig. 6). Distinct narrowing of the border vein, however, did occur in this area following the reversal of flow. In fetuses from 5 litters (F-35, 37, 42, 48, and 49) of 13 1/2 to 14 1/4 days gestation, the mid-portion of the border vein was absent (Fig. 9).

Observations of fetuses from 3 litters (F-7, 24 and 32) of about 13 days gestation revealed capillary drainage from the inner aspect of the limb bud to the border vein on the cephalic edge of the limb (Fig. 8A). Reversed flow from the cephalic termination of the border vein over both the inner and the outer aspects of the limb bud was noted in fetuses from 2 litters (F-28 and 40) of 13 1/4 days gestation (Fig. 8B). Reversed flow from the border vein to the inner aspect of the posterior limb was noted in fetuses from 12 litters (F-10, 14, 22, 31, 34, 42, 43, 44, 48, 49, 51 and 52) of 13 1/4 to 14 1/4 days gestation (Fig. 8C). In the more mature fetuses, the flow going to the inner aspect of the limb seemed to be confined to a single vessel which entered the border

vein on the caudal aspect of the bud near the junction with the anlage of the anterior tibial vein.

In one fetus of litter F-19 (gestation 12 3/4 days), capillary flow was noted to proceed from the inner aspect of the posterior bud to the border vein on the caudal margin of the bud opposite its junction with the anterior tibial vein. Attempts were made to duplicate this observation but were abandoned because of the great difficulty in getting adequate exposure of this area without trauma and consequent distortion of normal dynamics of flow. It was felt, however, that these observations indicated that, on the inner aspect of the posterior limb bud, capillary networks draining in opposite directions met and thereby formed a vascular pathway connecting the cephalic and caudal segments of the border vein.

As the superficial vascular network on the outer aspect of the posterior limb bud which drained into the anlage of the anterior tibial vein continued to expand, more and more communications were established with capillaries draining to the border vein (Fig. 8C). Following these connections, capillary flow which had originally gone to the border vein reversed and drained into the developing anterior tibial vein (Fig. 9). With the decrease in volume of flow, the border vein decreased in size (Fig. 9B). Conversely, following an increase in flow over the outer aspect of the limb, enlargement of specific vessels has been observed (Fig. 9B). Figure 9 depicts "portal systems" which have resulted from this tapping of capillary supply to various portions of the border vein.

Further evidence for relation between volume of flow and vessel size is provided by an isolated observation on a fetus of litter F-60 (13 1/2 days gestation) in which a small puncture wound was inadvertently made in the border vein between the fourth and fifth digits. Flow, seeking the path of least resistance, once again coursed through the narrowed portion of the border vein between the second and third digits and resulted in dilation of this segment to a diameter comparable to adjacent portions of the vein.

Exposure of fetuses from the same litters at intervals between the ages of 12 1/2 and 14 1/4 days gestation gave evidence of the rate of change in vascular morphology. Figures 5, 6 and 7, which are described above, provide an example of the morphologic changes that occur at 12 3/4 days gestation.

Figure 9 (F-35, fetuses 3 and 1) denotes vascular patterns in fetuses from the same litter as that shown in Figure 8C (F-34, fetus 1). The fetuses (gestation 14 1/4 days) represented in Figure 9, which were exposed within the same hour, are good examples of the final stages in the complete disappearance of the border vein. Another fetus (F-35, fetus 2), from the same litter as those shown in Figures 8C and 9, which was exposed at the same time as the fetuses represented in the latter figure and 18 hours after the fetus shown in Figure 8C, had no remnants of the border vein.

Variability in vascular patterns in the posterior limb buds of fetuses of the same litters exposed at the same time (as described

above in litter F-35) has been noted in most of the 61 litters investigated.

The original connection between the border and anterior tibial veins on the caudal border of the limb bud also disappeared at about 14 1/4 days. Prior to the disappearance of this junction, tapping of flow from the border vein into the anterior tibial drainage system in the manner described above resulted in local "portal systems" over the fifth digit. Fetuses from 3 litters (F-8, 17 and 39) of gestation 13 3/4 to 14 1/4 days showed the role of these "portal systems" in the disappearance of the original connection between these two veins. The transition correlated exactly with the transition, noted in Figure 9, in the disappearance of the border vein on the cephalic border of the limb bud.

"Portal systems" have also been noted in the anterior limb bud (Fig. 10) and in the posterolateral body wall.

Figure 10 (F-32, fetus 3) shows flow proceeding from the caudal termination of the border vein into a capillary network on the outer aspect of the anterior limb bud. The venous channel into which these capillary-sized vessels direct the blood flow is apparently the developing median vein. Fetuses from 3 litters of about 13 1/4 days gestation were observed to have similar flow patterns in their anterior limb buds.

Fetuses from two litters (F-23 and 25) of 12 3/4 days gestation had a "portal system" at the proximal caudal margin of the outer aspect of the posterior limb bud. Blood flow was seen to

course out of a vein of the posterolateral body wall into capillaries which drained to the border vein.

No "portal systems" were noted in the posterior limb after the disappearance of the border vein at about 14 1/4 days gestation. Fetuses from 11 litters (F-9, 13, 15, 17, 26, 30, 37, 38, 41, 46 and 53) of 14 1/4 to 15 1/2 days gestation were observed to have no remnants of the border vein. After 14 1/4 days, venous flow on the outer aspect of the limb bud was gradually encompassed within fewer and fewer vessels until the anterior tibial vein extended as a single vessel to the dorsum of the developing foot.

A seven minute film has been prepared as a portion of this thesis which provides good examples of the dynamics of flow and morphologic pattern at specific stages of development of the posterior limb bud. A copy of this movie will be kept in the Anatomy Department at the University of Oregon Medical School. The following fetuses are represented in this film:

(1) F-57, fetus 1 (gestation 12 3/4 days). The yolk sac circulation is shown in order to point out the great amount of flow which courses through the yolk sac. As indicated in the section on Materials and Methods, this network of vessels presents a major technical problem. Avoiding the yolk sac vessels when incising the yolk sac is critical since even minute hemorrhages might represent a high enough proportion of the total fetal blood volume to alter normal hemodynamics.

(2) F-50, fetus 1 (gestation 12 3/4 days). The vascular pattern and direction of flow on the outer aspect of the posterior limb bud is similar to that diagrammed in Figure 6.

(3) F-55, fetus 1 (gestation 12 3/4 days). The vascular pattern and direction of flow on the outer aspect of the posterior limb bud is similar to that noted in Figure 5. Flow from small vessels coming toward the surface near the cephalic termination of the border vein can be seen to give off capillary flow to both the anterior tibial and border veins.

(4) F-55, fetus 2 (gestation 12 3/4 days). The vascular pattern and direction of flow on the outer aspect of the posterior limb bud is similar to that diagrammed in Figure 6. A "portal system" consisting of flow proceeding from the cephalic termination of the border vein to capillary-sized vessels and finally to the anlage of the anterior tibial vein is in focus.

(5) F-48, fetus 1 (13 1/4 days gestation). Bifurcation of the direction of flow in the border vein between the second and third digits of the posterior limb bud is clearly shown.

(6) F-47, fetus 1 (14 days gestation). The vascular pattern and direction of flow on the outer aspect of the posterior limb bud shown in this part of the film is similar to that noted in Figure 9A. Small vessels coming toward the surface near the periphery of the limb bud can be seen giving off flow to the border vein and also in the direction of the anterior tibial vein. The border vein is quite narrow at this stage.

(7) F-49, fetus 1 (13 3/4 days gestation). The border vein is seen to be divided into two distinct segments by loss of continuity at the periphery of the limb bud. Flow proceeds from the cephalic portion of the border vein to the inner aspect of the limb bud (as in Figure 9A), and a small remnant of the border vein is noted to collect flow along the caudal border of the fifth digit.

(8) F-53, fetus 1 (14 1/2 days gestation). No remnants of the border vein of the posterior limb bud can be seen. The anterior tibial vein extends as a single vessel to the dorsum of the developing foot.

ILLUSTRATIONS

Each drawing is a diagrammatic representation of the vascular pattern in a limb bud of a particular fetus of the age indicated in the caption. Patterns of flow pertinent to interpretation of vascular development in specific areas are represented. Thus, although figure 5 is relatively complete, the others show large areas where vessels have not been drawn in.

Figures 6 and 7 represent patterns of flow seen on the outer aspect of the posterior limb buds of fetuses from the same litter as that from which the specimen in figure 5 was obtained. Figure 6 is of a fetus exposed 1 1/2 hours and figure 7 is of a fetus exposed 2 1/2 hours after the fetus shown in figure 5.

Figures 9A and 9B represent patterns of flow seen on the outer aspect of the posterior limb buds of fetuses from the same litter as that from which the specimen in figure 8C was obtained. Figures 9A and 9B are of fetuses exposed 18 hours after the fetus shown in figure 8C.

The symbols are as follows:

a. border vein of posterior limb bud

b. anterior tibial vein

c. area of termination of central arterial trunk

d. point of division of flow and eventual structural division of vein

e. cephalic termination of border vein

f. vascular connection between capillary-sized vessels on inner and outer aspects of limb bud

g. cephalic border of limb bud

h. median vein

i. border vein of anterior limb bud

j. caudal border of limb bud

x. point of emergence of a deep vessel which divides its flow between anterior tibial and border vein drainage systems

y. caudal termination of border vein

Figure 4

This illustration depicts the outer aspect of the posterior limb bud of a mouse fetus of 12 1/4 days gestation.

Capillary drainage from the outer aspect of the proximal portion of the limb bud is directed into the border vein (a). The multiple, parallel drainage pathways from this capillary network constitute the anlage of the anterior tibial vein (b).

Arterial flow to the peripheral portion of the flattened limb bud emanates from a central arterial trunk (c) and drains into the border vein (a) through vessels of capillary size.

(F-28, fetus 3)

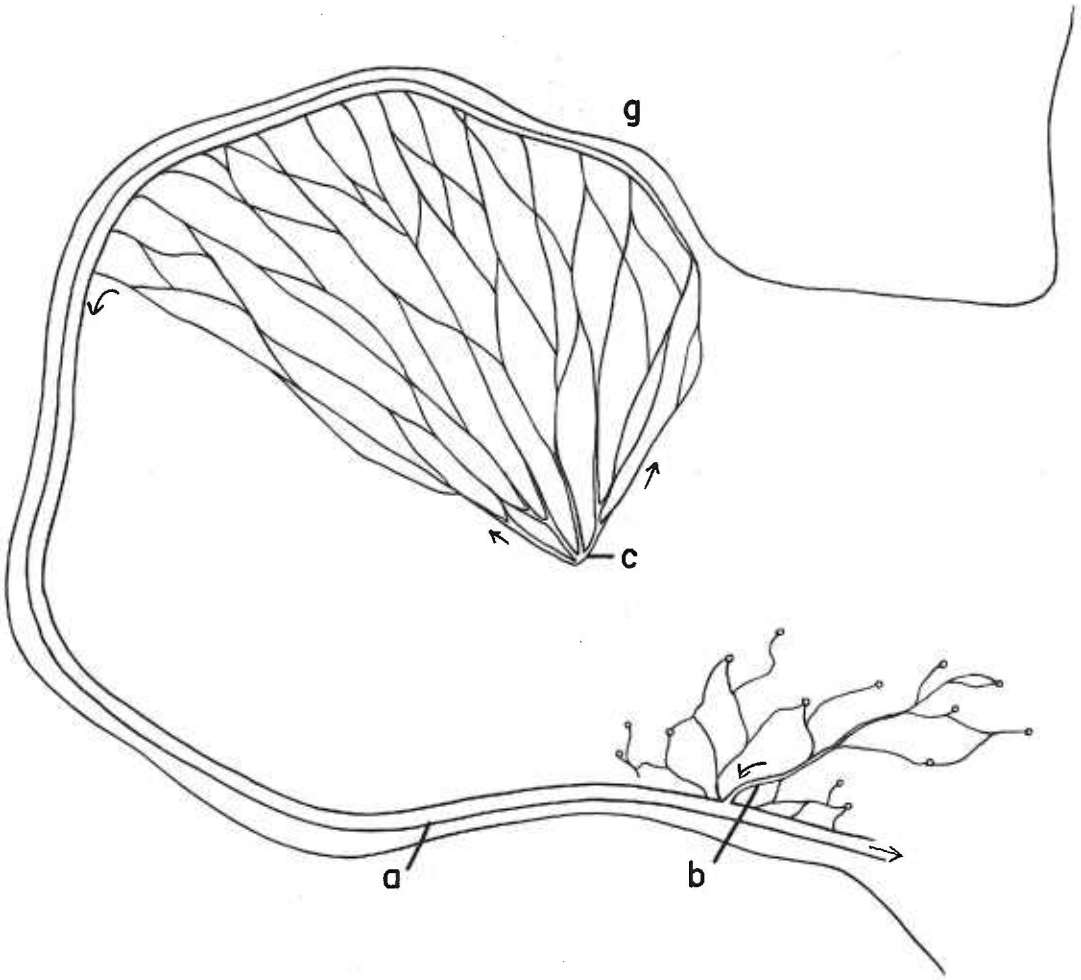

g
c
a
b

Figure 5

This illustration shows the outer aspect of the posterior limb bud in a mouse fetus of 12 3/4 days gestation. It depicts the vascular confluence between the anterior tibial (b) and border (a) veins. The vessel approaching the surface at the cephalic aspect of the flattened bud (x) contributes flow to both drainage systems.

Flow to the anterior tibial vein (b), although primarily from the outer surface of the proximal portion of the bud, is also shown to be rising from small vessels near the area in which arterial vessels can be seen emanating from a central arterial trunk (c).

Junction of the anterior tibial and border veins on the caudal aspect of the limb bud is obscured. Arterial and capillary flow to the distal portion of the limb bud is uniformly distributed at this stage.

(F-19, fetus 1)

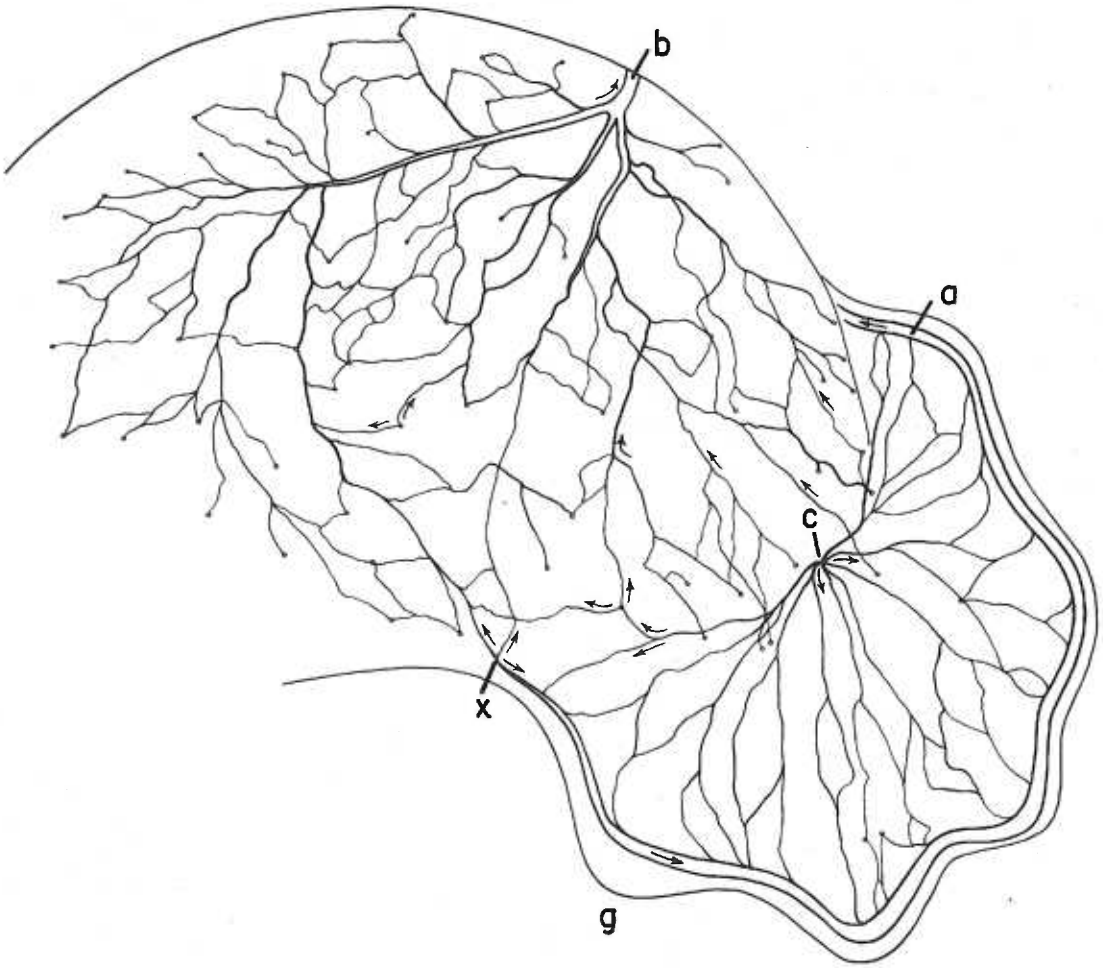

b
a
c
x
g

Figure 6

This illustration shows the outer aspect of the posterior limb bud in a mouse fetus of 12 3/4 days gestation. Flow proceeds from the terminal portion of the border vein (e) on the cephalic aspect of the limb bud (g) to smaller vessels including channels of capillary size on the outer aspect of the limb bud. These in turn drain into the anterior tibial vein (b). The segment of the border vein at the bifurcation of flow (d) is shown to be patent although somewhat narrower than adjacent segments of the vessel.

(F-19, fetus 2)

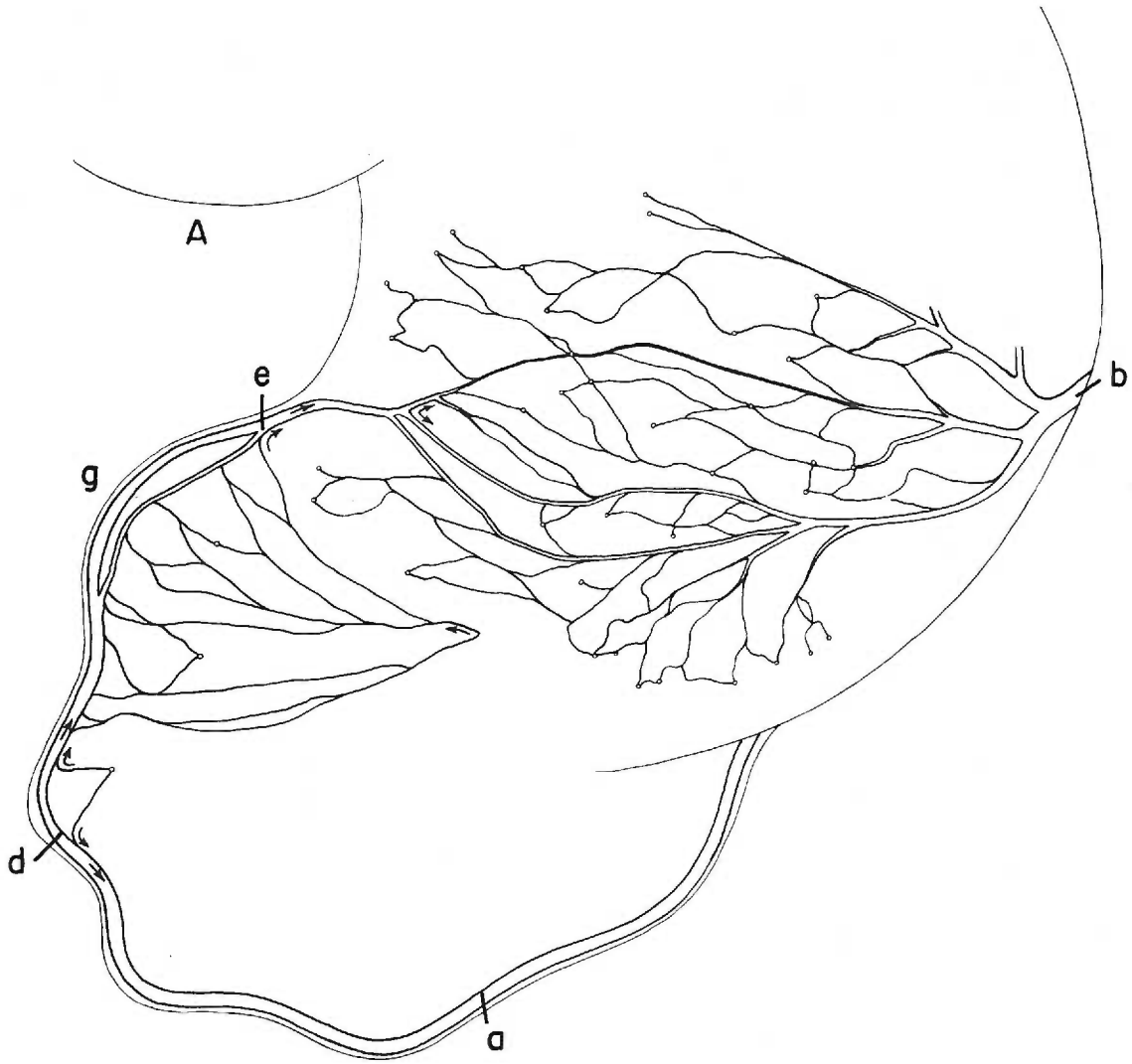

A
e
b
g
d
a

Figure 7

This illustration shows the outer aspect of the posterior limb bud in a mouse fetus of 12 3/4 days gestation. Flow from the cephalic termination of the border vein (e) to the anterior tibial vein is almost completely confined to one pathway, which is enlarged to a diameter comparable to that of the border vein.

(F-19, fetus 3)

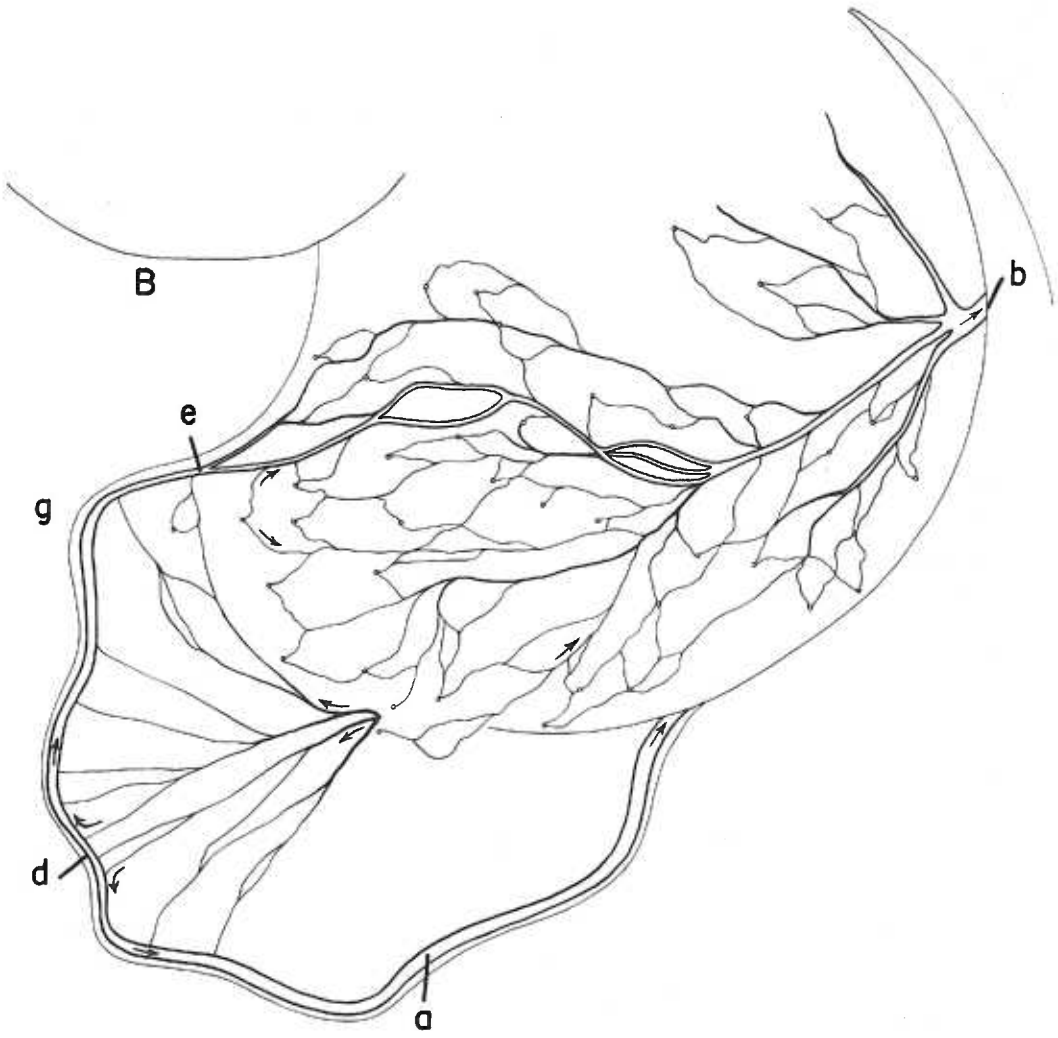

B
b
e
g
d
a

Figure 8

These diagrams are of three posterior limb buds viewed from the cephalic aspect.

A. A fetus of gestation age 13 days. Flow from the cephalic termination of the border vein (e) is directed into vessels of varying sizes which lie on the outer aspect of the bud and connect with the anlage of the anterior tibial vein. The small vessels (f) near the cephalic termination of the border vein direct flow from the inner to the outer aspect of the limb bud.

(F-32, fetus 1)

B. A fetus of gestation age 13 1/4 days. Flow from the cephalic portion of the border vein (e) is directed in equal proportions to both the inner and outer aspects of the limb bud. Flow to the outer aspect of the bud drains into the anlage of the anterior tibial vein.

(F-40)

C. A fetus of gestation age 13 1/2 days. All flow from the cephalic termination of the border vein (e) is directed to the inner aspect of the limb bud and subsequently to the border vein on the caudal aspect of the bud as described above. Numerous vessels approaching the surface (denoted by small circles) are shown to give off flow both to the border vein (a) and to the developing anterior tibial vein (not shown in diagram).

Marked narrowing of the border vein has occurred in the segment in which the bifurcation of flow occurs (d).

(F-34)

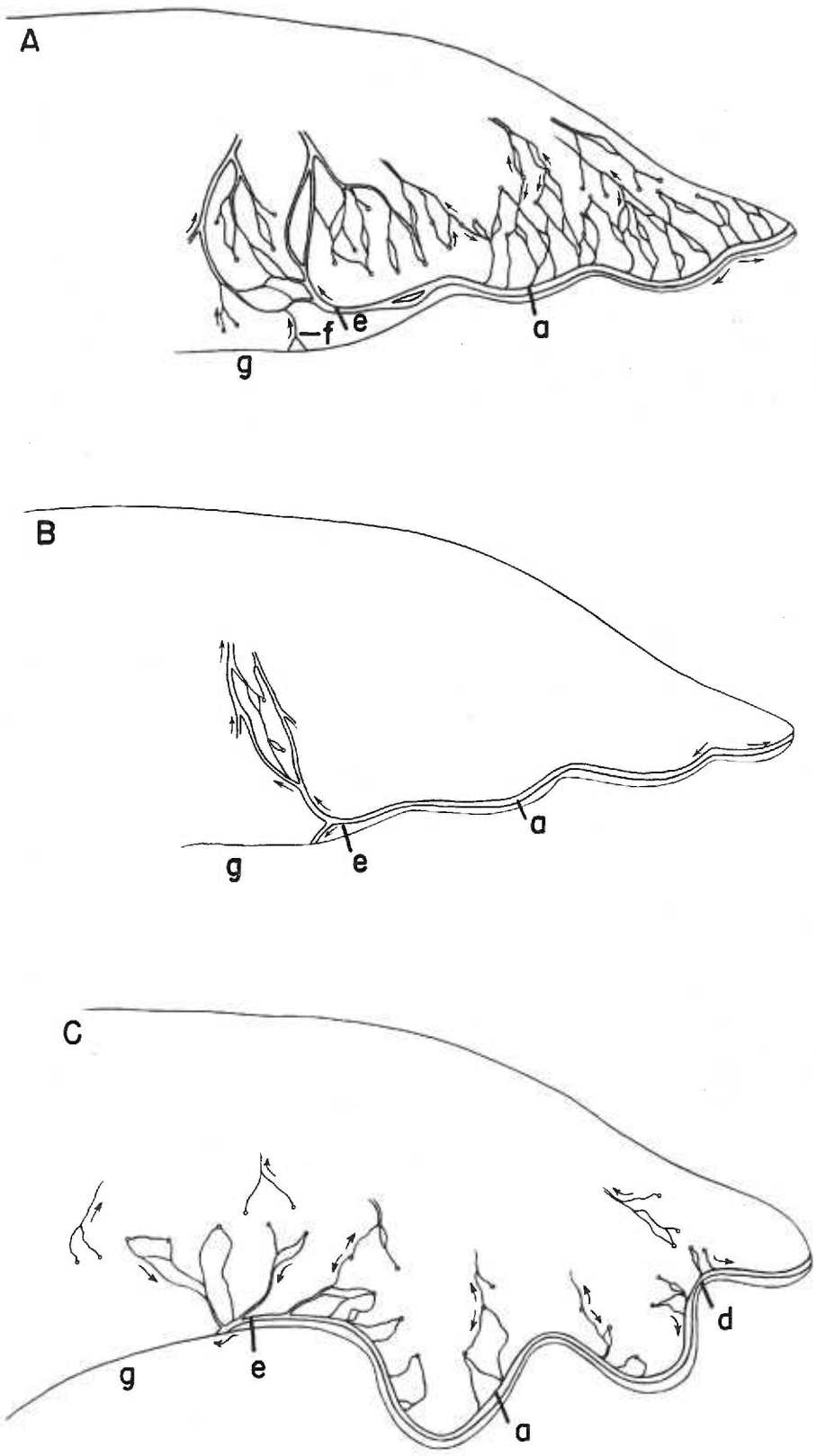

A
f
e
a
g
B
a
g
e
C
d
g
e
a

Figure 9

A. Gestation age 14 1/4 days. Continuity of the border vein (a) along the periphery of the limb bud has been lost between the second and third digits. Flow from the border vein to vessels of capillary size which eventually drain into ramifications of the anterior tibial vein is noted between the first and second digits. Flow through this narrowed, disconnected, cephalic portion of the border vein is directed to the inner aspect of the limb bud at the cephalic margin (g). No vessels other than those shown were seen to empty into this segment of the vein.

(F-35, fetus 1)

B. Gestation age 14 1/4 days. A small remnant of the border vein (a) is noted to extend between the first and fourth digits. The border vein branches into capillary-sized vessels in the inter-digital groove between the first and second and between third and fourth digits. These small branches direct flow to the developing anterior tibial vein (b).

(F-35, fetus 2)

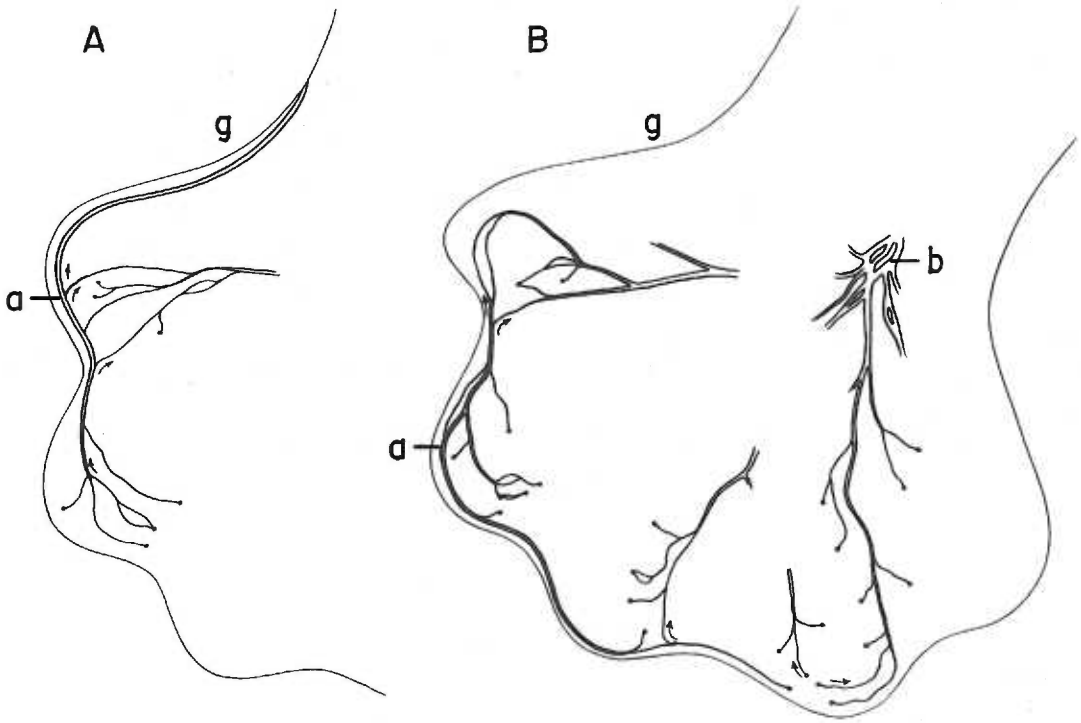
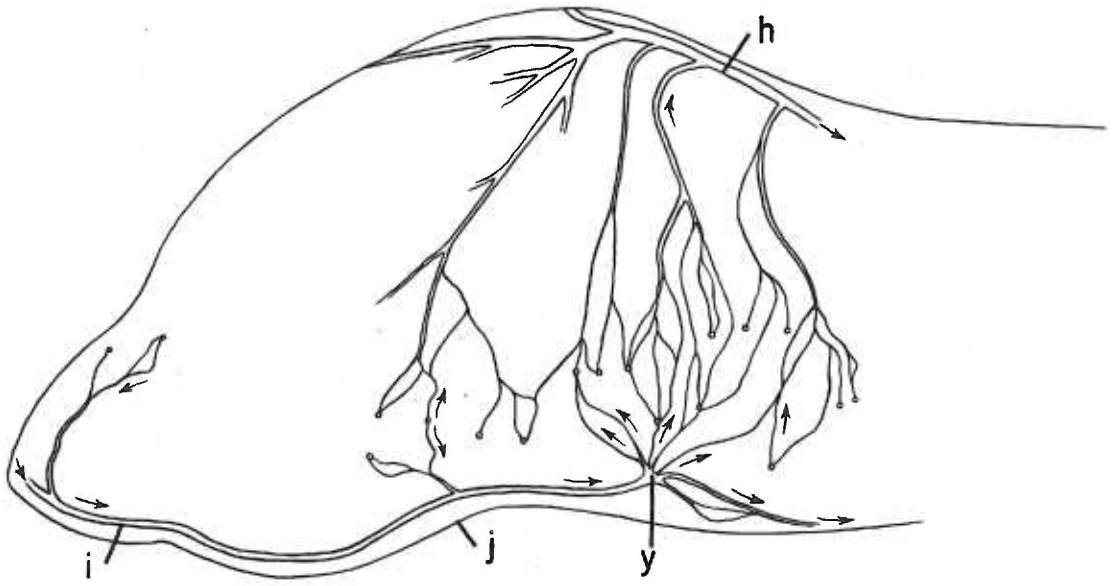

A
g
a
B
g
b
a

Figure 10

This illustration shows the outer aspect of the anterior limb bud of a mouse fetus of 13 days gestation. The limb bud is viewed from the caudal aspect.

A portion of the blood flow from the caudal termination of the border vein (y) on the caudal border of the limb bud (j) is shown to proceed to smaller vessels including channels of capillary size on the outer aspect of the limb bud. These in turn drain into the median vein (h).

(F-32, fetus 3)


h
i
j
y

DISCUSSION

Discussion of the results of this new approach for assessing the hemodynamic aspects of fetal circulation necessitates correlation with previous observations of fetal vascular patterns and theories of vascular development, consideration of significance, and projection of possible ramifications of this study.

It should be pointed out immediately that there are difficulties in categorizing the vessels which have been studied. The large vessels draining an area have been called veins although structurally they appear to be single endothelial tubes of varying diameter and probably should be termed sinusoids. The vessels referred to here as capillaries are undifferentiated vascular channels of capillary size which may exist only temporarily in this state, going on to become larger vessels, sprouting new vessels to keep pace with growth of the region, or disappearing completely to give place to new local systems. Only the terminations of what presumably are arterial, or potential arterial, channels can be seen in these preparations; but many of the vessels I have termed capillaries must eventually become arterial. The direction of flow in most channels seen during these studies appears to be variable and may change, possibly several times, in the history of a given vessel.

My observations appear to fit with Evans' distinction between primary and secondary veins (12). The shift from the border vein to the anterior tibial as the predominant venous drainage appears to be a clear example of the alteration of pattern from one of widely separated arterial and venous pathways to one of parallel and closely

approximated afferent and efferent channels characteristic of the definitive peripheral vascular system. The dynamics of flow during such a transformation have not been described previously. The border vein would be a primary vein and the anterior tibial a secondary vein in Evans' sense.

The establishment of the anterior tibial vein from a channel or channels in a previously uniform plexus, as the tapped border vein reverses flow and pours blood into the plexus, effectively demonstrated in a living embryonic system Thoma's postulate that capillary-sized vessels are transformed into larger ones as functional adaptation to circulatory requirements. The disappearance of large vessels such as the border vein following reduction in flow supports the contention of Hughes (17) that "...once the circulation has acted on a network in this way, the calibre of the larger vessels thus formed is only maintained if the circulation continues." The observations do not, however, eliminate the concept that genetic pattern may play an important part in the response of the vessels and the establishment of the definitive arrangement.

The interaction of limb bud mesoderm and the apical ectodermal ridge in the development of a normal limb seems to be well established (30, 39). I would like to suggest that the system of capillaries, which at an early stage drains distally into the border vein (or sinusoid) in intimate relation to the base of the ectodermal ridge throughout its length and later the reversal of flow and establishment of "portal systems", provides a vascular channel for the

reception, transportation and diffusion of agents controlling normal morphogenesis of the limb. Blood which flows slowly through the border vein, nearly in contact with the base of the ectodermal ridge, is for a time distributed through a mesh of capillaries to the rapidly growing proximal portion of the bud before being gathered again into the anterior tibial vein. If the ectodermal ridge is producing a diffusable substance to which relatively distant mesoderm is to react, these vascular pathways seem to constitute a reasonable mechanism for its transportation. It may be that similar vascular arrangements will be found to exist in other interacting systems of the embryo to account for continued organization of complex parts until differentiation is complete. On the basis of preliminary observations, it seems reasonable to predict that such a system will be found to exist in the eye.

Since this paper represents the initial recording of direct microscopic observations of mammalian fetal circulatory hemodynamics, the possible ramifications of this study are numerous. Transparency of the fetal epidermis at an early stage permits observation of the vascular system in the tail, body wall and brain as well as in the anterior and posterior limb buds. Comparison of circulatory development in these areas, and of the relation of vascular development to hemodynamics, is the most obvious study which might be performed.

Observation of cerebral hemodynamics without the necessity of dissection of skin, cranium and dura offers an excellent opportunity for testing the effects of various substances (e.g. drugs, hormones, etc.) on cerebral vessels. I have noted constriction of cerebral

arteries and artericles in response to nor epinephrine (paper to be published).

Comparison of fetal microcirculation before and after administration of a substance to the mother, which has previously been noted to have a direct effect on fetal hemodynamics, will give evidence as to whether or not the substance will pass the placental barrier. I did not observe constriction of microvasculature over the outer aspects of the telencephalon and limb buds in fetal mice following intraperitoneal and/or IV injections of nor epinephrine to the mother mouse (paper to be published).

Relative avascularity of the chest wall permits dissection with negligible blood loss. Utilization of the fused quartz rod technique previously described has enabled me to place the tip of the quartz rod under the beating heart. Movies of the heart taken at high speed and projected at a slow speed might well delineate the manner of cardiac filling and the developmental morphology and hemodynamics of the coronary circulation. Likewise, examination of intrathoracic or intra abdominal organs can be carried out in a similar manner.

Observation of a given vascular network with high magnification should reveal the stage at which the vasculature begins to demonstrate periodic constriction and dilatation of precapillary sphincters (as described by Chambers and Zweifach).

The high concentration of vessels noted in areas of rapid fetal growth poses once again the question of correlation between these two interesting phenomena. Speculation on this question leads

me to wonder if rapidly growing tissue produces a substance which either stimulates existing undifferentiated mesenchyme to form vascular channels or stimulate existing vascular networks to extend capillary buds into newly formed tissue.

Algire (2), based on studies of vascularization of grafts, points out that vessel remnants in grafts of rapidly growing tumors are not active in forming connections with the host. He states that tumor stimulates the growth of new capillaries from the host, whereas blood vessels within grafts of normal tissues frequently play an active role in making connections with the host. Rapidly growing tumors, by stimulating outgrowth of new capillaries from the surrounding tissues, are shown to acquire a rich supply of afferent and efferent vessels. Limited differentiation into arterioles, irregular anastomosing of the capillary network, and steady rather than intermittent flow characterizes the blood supply to the rapidly growing tumors.

The characteristics of the vascular pattern and hemodynamics in rapidly growing tumor grafts described by Algire are also typical of microcirculation noted in fetal mice. I feel it is likely that a similar substance is produced by rapidly growing tumors of adult tissue and by rapidly growing embryonic tissue which stimulates vascular supply. I feel an attempt should be made to isolate such a substance.

Since the fetus is physiologically immature, it is necessary to constantly re-evaluate the methods by which the environment of the exposed fetus is controlled. For further studies on fetal

circulation, I suggest the addition of a colloid to the "physiological" saline solution, comparison of the effects on fetal circulation of various anesthetics with and without specific analgesics, use of a thermistor or thermocouple at the tip of the quartz rod beneath the exposed fetus, provision of a water bath for the fetus (e.g. plastic cup surrounding the tip of the quartz rod), and performance of the operation in a room in which the temperature and humidity can be controlled accurately.

SUMMARY

The equipment and techniques used in the study of adult microcirculation have been adapted to observations on mammalian fetuses.

This paper is mainly concerned with the changes in hemodynamics in the posterior limb buds of fetal mice occurring between 12 1/2 and 14 1/4 days of gestation. Serial microscopic observations and motion pictures of vascular morphology and dynamics of blood flow, combined with earlier observations on the development of the vascular system, provide reasonable concepts of the process by which embryonic peripheral vascular morphology is transformed to the pattern seen in the newborn, of the relationships between dynamics of flow and vascular morphology, and of possible effects of changes in dynamics of flow on development of specific areas of the fetus.

It is suggested that the temporary portal systems observed here may provide a mechanism for transfer of substances between interacting portions of the developing limb bud.

The possibilities for future studies of microcirculation of mammalian fetuses are numerous.

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