

STUDIES ON THE AREA VASCULOSA OF THE EMBRYO CHICK

I. THE FIRST DIFFERENTIATION OF THE VITELLINE ARTERY

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I. INTRODUCTION

IN 1893, the *Untersuchungen über die Histogenese und Histomechanik des Gefässsystems* of R. Thoma was published, which describes the development of the area vasculosa of the chick blastoderm up to the third day of incubation. This was by no means the first time that the origin of blood vessels in the chick blastoderm had been traced, and Virchow (1891) had already described the anatomy of the area vasculosa, which was again studied by Popoff in 1894. Thoma was the first, however, to study the effect of the circulation on the configuration of the vessels of the area vasculosa.

Blood normally begins to circulate at the stage of sixteen somites, i.e. after about thirty-seven hours of incubation, previous to which a more or less uniform network of vessels has differentiated. By the end of the second day of incubation, from those sections of the vessel network through which the flow of blood has been most rapid, larger vessels or arteries have differentiated, in particular from those sections of the network next to the embryonic axis communicating with the primitive aortae.

Experimental proof that the cause of arterial differentiation in the area vasculosa was the stimulus of blood flow was given by Chapman (1918), who destroyed or removed the heart at the sixteen-somite stage from blastoderms in the egg, and continued the incubation of the operated eggs. No differentiation of the arteries of the area vasculosa took place under these conditions.

Similar results were obtained by Clark (1918), who observed the vessels in the transparent fin of the tadpole's tail, and watched the differentiation of particular capillaries into arteries and veins, which were at first in no way morphologically different from surrounding capillaries but which happened preponderatingly to transmit the blood flow in that particular region.

That this conception of arterial differentiation is generally applicable to the development of the intra-embryonic vascular system, was made probable by the results obtained from the study of embryonic material in which the vascular system had been injected. The general conclusion from such studies was that in ontogeny all differentiated blood vessels with the exception of the primitive aortae and aortic arches are preceded by a stage of capillary plexus, from which, it is reasonable to conclude, the various differentiated vessels arise under the influence of the circulation (Mall, 1904; Evans, 1909; Shearer, 1933; Woollard, 1921; Hughes, 1934).

Undifferentiated capillaries arise in ontogeny therefore by self-differentiation, whereas the development of differentiated arteries and veins belongs to the "functional period of development" (Roux, 1881; Huxley and de Beer, 1934).

The first differentiation of arteries does not, however, exhaust the influence of functional adaptation upon the form of the vascular system. As is well known, the angle at which branches are given off from the main artery can be related to the physical laws underlying the fluid friction generated in such systems (Roux, 1879; C. D. Murray, 1926), and indeed, as is shown from evidence in this paper and elsewhere, the form of all vessels is only maintained where blood continues to flow through them.

The present paper is a continuation of the work of Thoma on the area vasculosa of the chick. Thoma describes the histology of the developing area vasculosa only as far as the pre-circulatory differentiation of the vessels. It is the purpose of this paper (*a*) to extend the account of fine structure into the early post-circulatory period, (*b*) to fit into the description of arterial development the part played by the contractility of endothelium (Hammett and Zoll, 1928; Cohn and Lange, 1930), and (*c*) to describe the results obtained from the observation of living blastoderms in which arterial differentiation took place.

II. MATERIAL AND METHODS

The choice of the area vasculosa of the chick blastoderm for a study of the factors concerned with arterial differentiation depends on several circumstances. In the first place, we are following a comparatively rapid series of changes, in that the differentiation of the vitelline artery takes place within the first

twelve hours from the inception of circulation. Secondly, these changes are taking place in a thin sheet of tissue whose vessels are easy to observe in the living state, and which is sufficiently thin to study in the form of whole mounts after histological treatment, which was the method adopted by Thoma. The whole depth of an area vasculosa can be studied with an oil immersion lens of comparatively long working distance, which is an advantage not only in the time saved by avoiding the preparation of serial sections, but in the fact that the shape of nuclei, and the direction of mitotic spindles, are seen with such clearness in relation to their surroundings, a point on which much stress is laid in the present paper. Details of the histological methods used are given in the section following that on vital observation.

Observation of the living area vasculosa

There are two main methods of observing the living chick blastoderm, either in the unbroken yolk, or after explantation of the blastoderm to a non-yolky culture medium *in vitro*. In general, the advantages of the *in ovo* method are that it is less laborious, and that no aseptic precautions are necessary, while on the other hand *in vitro* cultivation makes it possible to perform elaborate operations on the blastoderm and to use transmitted light which is preferable from the photographic standpoint. Among workers on the earliest stages of development, Wetzel (1929), Gräper (1929), and Kopsch (1934), to mention only the most recent, work *in ovo*, while Waddington (1932, and subsequently) employs the tissue-culture technique.

Explantation has been used in the study of the vascular system of the blastoderm by McWhorter and Whipple (1912), by Sabin (1917, 1919) and by Patten and Kramer (1933). These authors have all used hanging drop methods, in that the blastoderms were explanted on to cover-slips with a nutrient medium. By this means, observation under high magnification is possible, but the time of survival is less than when the watch-glass culture vessel is used. The latter is in regular use in this laboratory for explants of large size, and permits of the use of a comparatively large amount of culture medium. Its use for whole blastoderms has been described by Waddington (1932). Such cultures can only be observed with objectives with a working distance of several millimetres.

McWhorter and Whipple used fowl plasma as a culture medium, either pure, or diluted with Locke's solution, and were able to grow blastoderms with three somites to the eighteen-somite stage or with ten-somite embryos for a further thirty-one hours. Sabin used a fluid medium in which her blastoderms survived for periods of five to eight hours, which was the experience of Patten and Kramer, using similar cultural conditions. When, however, the latter authors used larger culture chambers with the same fluid medium, the survival time became from twenty to thirty hours.

Waddington, using a mixture of fowl plasma and embryo extract in watch-glass cultures could grow primitive streak blastoderms for two to three days,

and nine-somite embryos for two days. This is the form of culture vessel which has been employed for the present work, and it has been found that the later the stage at which an embryo is explanted, the less is the total time for which cultivation is possible. The most advanced embryo which I have observed in culture was one of thirty-seven somites with well-marked fore and hind limb-buds, which had been explanted at twelve-somites forty hours previously. It is advantageous when growing embryos in culture to the latest stage possible to include in the medium the "subblastodermic fluid" of P. D. F. Murray (1933), which is the fluid within the vitelline membrane of eggs which have been incubated for three or four days. This when mixed with fowl plasma, and clotted with a trace of embryo extract, forms an admirable medium for the growth of explanted embryos in the second day of incubation, but it has the disadvantage of increasing the tendency of the clot to liquefy. Subblastodermic fluid must form a vehicle for the conveyance to the blastoderm of the white yolk which serves as the food material for the blastoderm in the first three days of incubation, for when centrifuged, a heavy deposit of white yolk results. It is even possible to use subblastodermic fluid alone as a culture medium by floating blastoderms on to discs of filter paper, the edges of which are kept immersed in the fluid. By this means it is possible to charge the culture medium at will.

Where, however, the aim of the investigator is vital observation of the latest stage possible, and where no extensive manipulation of the embryo is desired, the embryos, in the writer's opinion, are best left in the egg. The life of embryos in cultivation when the circulation has been well established is always terminated by an aggregation of the blood corpuscles, which appears to take place quite suddenly. This aggregation presumably does not involve the usual adult clotting reactions, for, according to Pickering and Gladstone (1925), chick blood contains neither fibrinogen nor prothrombin before twelve days of incubation. Embryos in cultivation at thirty somites have always an area vasculosa many times smaller in area than is normal in the egg. Yet multiplication of blood corpuscles seems to proceed at the normal rate, with the result that the vessels of the embryo and area vasculosa are over-full of corpuscles, which causes stagnation of the blood, and subsequent aggregation. Aggregation of corpuscles always seems to follow stagnation, and can be observed normally in the area vasculosa in particular places where arterial differentiation temporarily involves stagnation. Therefore, for the further maintenance in culture of explanted embryos, nutritional considerations do not appear to be the limiting factors.

Observation of embryos in opened eggs with whole yolks is by no means new. It is regularly undertaken in the teaching of chick embryology for a few hours, when the eggs are opened into warm saline, and it was found by Lange (1930) that eggs at the second day of incubation opened into Ringer kept saturated with oxygen when maintained at the proper temperature would continue their development for a further two days. Romanoff (1931) has in-

vestigated thoroughly the conditions for survival in an opened egg. Eggs are opened under sterile conditions, kept covered but well aerated, and maintained at 38° C. and 65 per cent. relative humidity. Under these conditions, when opened during the first day of incubation, all the eggs survive till the third day, half of them till four days, and some to the twentieth day.

I have found that under less carefully controlled conditions, merely by keeping dishes of water on the floor of the incubator, many eggs when opened in Romanoff's manner during the first day of incubation will continue to develop normally for a further four or five days, giving an area vasculosa of normal size. Aseptic precautions do not appear to be necessary. Development of the embryo in the opened egg, as Romanoff points out, permits of an enormous range of vital observation, full advantage of which yet remains to be taken.

For direct observation transmitted light can only be used for the area pellucida of explanted blastoderms; indirect illumination is necessary for the area opaca, and for the whole blastoderm *in ovo*. The "Ultropak" illuminator of Leitz is most convenient as a source of vertical illumination for the blastoderm *in ovo*. The objective and ring condenser system is fitted with a water dipping cone of parallel-sided glass, the lower surface of which is kept in contact with the vitelline membrane, which was kept wet by a slow stream of Ringer. The screw motion which varies the distance between the dipping cone and the objective forms a convenient fine adjustment for the microscope system. The series of photographs (Plate III, figs. 1-5) were taken *in ovo* with this illuminator, using the $\times 11$ objective.

For the observation of the vessels of the area opaca in explanted blastoderms a special method was devised, results obtained with which are shown in Plate I, figs. 1 and 2. The vessels were injected by means of a micro-manipulator with an Indian ink sufficiently non-toxic not to interfere with the usual life of the blastoderm in culture. This Indian ink was obtained in the first place by rubbing up the ordinary commercial stick of solid Indian ink in a phosphate buffer, and dialysing away toxic substances in a collodion membrane. This buffered solution of colloidal carbon withstood autoclaving, and could be made into a physiological saline by adding the necessary salts immediately before use. Later it was found that a stick of solid Indian ink free of the noxious substances of the commercial article could be made by mixing finely divided carbon in the form known as "gas black" with a strong solution of gelatin, and then desiccating the mixture.

This vital injection technique suffered from one main disadvantage, namely, that after injection a severe contraction of the injected vessels frequently resulted. Vascular endothelium, it is now recognised, is a contractile tissue, and a subsequent section is devoted to a consideration of this phenomenon. Moreover, since it was found that the state of expansion of vessels is of importance with regard to their differentiation into arteries, it was inadvisable solely to rely on vital injection to give a normal picture of the differentiation of

an artery. We shall see that the same objection applies even to the explantation of blastoderms in this connection.

Furthermore, since a mere description of the gross anatomical changes which take place in the differentiation of an artery loses much of its value if these changes are not correlated with the cell processes involved, attention is almost exclusively directed in the present paper to arterial differentiation in that region where the fine structure can be most readily and completely studied. This region is the area pellucida, where arterial differentiation first occurs, and where whole mounts treated in the manner described below can be examined with an oil immersion objective.

Histological methods

The suitability of the area pellucida for observation in whole mounts depends on the use of the Feulgen reagent for nucleic acid, which not only stains nuclei, but yolk in addition. Thoma's own preparations were stained in alum carmine, which allows the mesoderm of the area opaca to be observed to some extent, since the yolk is not stained so deeply as with the Feulgen reagent. In the area pellucida, however, where little or no yolk is present in the endoderm, nuclei stand out much more clearly in Feulgen preparations than in those stained in alum carmine, which is of great advantage where many layers of cells are to be observed, as in stages where arterial differentiation has taken place. Thoma's observations under high power leave off before this stage has been reached.

The blastoderms were fixed in Zenker, and washed in distilled water. For stages in which a fully developed exocoelom is present, the ectoderm and outer coelomic wall were dissected off when in water; this dissection near the embryonic axis involved the removal of large masses of mesenchyme dorsal to the coelom in the neighbourhood of the posterior somites, revealing the vessels below. By this means, the total depth of the preparations was reduced, and penetration by the various reagents employed was facilitated.

The preparation and use of the Feulgen reagent is described in Romeis (1932). Preparatory to its use, the material is hydrolysed in normal hydrochloric acid at 50° C., for which process various times are suggested by Romeis for different objects. I have found that for my material forty minutes' hydrolysis gave satisfactory results. Yolk stains in Feulgen without hydrolysis, whereas as the hydrolysis proceeds the nuclear material is rendered capable of taking a deeper stain. The staining of substances other than nucleic acid by the Feulgen reagent has been noticed by other workers. After hydrolysis, the blastoderms are washed in distilled water, and left over-night in the Feulgen. They are then washed in three changes of SO₂ water, as described by Romeis, dehydrated, and counter-stained in light green dissolved in absolute alcohol. They are then brought into 1 per cent. collodion dissolved in methyl benzoate, which is the clearing technique elaborated by Peterfi, and described in Romeis. The blastoderms are mounted in a second change of this medium, and the preparations

ringed round with paraffin wax with which methyl benzoate does not mix, when they form almost permanent preparations. Some last indefinitely, but others require the addition of more methyl benzoate at intervals. The advantage of this medium is that sectioning of the material is possible at any time subsequently, since no hardening takes place.

When an already well-stained blastoderm is to be sectioned it is brought into benzene for a few minutes, and then embedded in wax in the ordinary way. Sections are cut at 6μ and when mounted need only to be re-counter-stained in light green in absolute alcohol.

When studying whole mounts with an oil immersion lens, a less viscous immersion medium than the usual immersion oil must be used, and for this pure methyl benzoate is satisfactory.

A final point regarding the methods employed in this work is one concerning the relationship between the time of incubation of eggs, and the stage of development of the blastoderm. It is well known that in a group of eggs incubated for the same time, there may be considerable divergences in the degree of development of the embryos; these divergences are more marked in the first two days of incubation, and in eggs incubated during the winter months. In my experience, they are related to a variation in the period after the commencement of incubation before development is resumed, for once growth begins in the incubator it proceeds at the normal rate. Thus, in the winter especially, the phrase "embryo of twenty hours' incubation" has very little meaning.

Consequently, we are left with the number of somites as the only convenient measure of development. To reduce the number of somites to a standard time scale, the data in Lillie's *Embryology of the Chick* were plotted, and the resulting curve gives a measure of the time required for the addition to a living blastoderm of a certain number of somites, which holds true for blastoderms *in ovo*, and those *in vitro* when explanted after the ten-somite stage. Waddington (1932) describes the extent to which development *in vitro* is slower than *in ovo* when earlier stages are explanted. In estimating the number of somites in embryos in which there are at least twenty-four somites, their number posterior to the vitelline artery is counted, for this artery from this stage onwards is always opposite the twenty-third somite.

Even the number of somites is not a fully satisfactory criterion of the stage of development, for where the number of somites present is much less than that corresponding to the period of incubation in Lillie's table, the stage to which other structures, such as the blood vessels, are developed is ahead of that corresponding to the number of somites actually present.

III. THE PRE-CIRCULATORY DEVELOPMENT OF THE AREA VASCULOSA

As stated in the Introduction, blood normally begins to circulate in the chick blastoderm at the stage of sixteen-somites. By this time, a network of freely communicating blood vessels in the area vasculosa has developed, the

form of which is of the greatest importance in regard to the paths along which circulation will first take place, and for the ultimate differentiation of arteries within the vessel network.

A full discussion on the histogenesis of the extra-embryonic endothelium is postponed for a later communication. The main accounts of this process in the literature do not fully accord with one another (Thoma, 1893; Rückert and Mollier, 1906; Sabin, 1917, 1919). Modern workers, however, agree that endothelium is exclusively derived from the mesoderm, and originates *in loco* (McClure, 1921), contrary to the original angioblast theory of His (1900). There is further agreement, moreover, that the first vessel primordia take the form of isolated vascular spaces. They increase in size, and the walls of adjacent spaces become apposed, and then are resorbed at the point of contact.

The development of the vessel network

The first stage in vessel formation is the differentiation of isolated vascular spaces. Coalescence of adjacent spaces leads to the formation of a continuous vessel of comparatively large diameter, but where the first vessel spaces are far apart, they are joined together by means of the outgrowths known as "sprouts", the cells of which are derived by proliferation from the wall of an already formed endothelial space; the lumen of the sprout is formed largely by extension of the parent lumen, which may join up with small independent cavities which appear within the sprout. Vessels formed in this manner have at first narrow lumina, and in any part of the area vasculosa the general size of lumen depends on the relative proportion in which primary vessel formation and growth through sprouting have been concerned with the formation of the vessel network. In general, the elongation of vessels, or of vessel primordia, causes the vessel lumina to be of small diameter. These considerations are of great importance in relation to the paths in the vessel network along which blood will flow when the circulation begins, for the flow will be largely restricted to the largest vessels, which will offer least resistance.

Vessel differentiation proceeds antero-posteriorly, and from the area opaca to the pellucida. It is this latter circumstance, coupled with the fact that sprout formation is most marked in the anterior and middle regions of the pellucida, which led the early workers on the subject to formulate the doctrine of the vascularisation of the embryo from without.

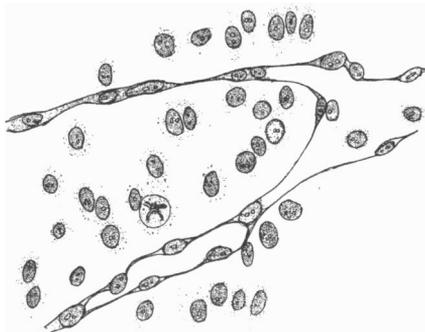
Isolated vessel primordia first appear in the area pellucida between the stages of three to six somites, first at the pellucida margin, and then nearer the embryo. Their first appearance and subsequent combination was most beautifully recorded in living material by McWhorter and Whipple in a series of photographs taken at a magnification of $\times 500$. My own photographs of a living blastoderm cultivated *in vitro* (Plate II, figs. 1-5) are taken at the much lower magnification of $\times 34$, and cover a period of about fourteen hours. From comparison with fixed material, it is apparent that the stage in the photographs at which the outlines of vessels are first distinctly seen, corresponds

with that of the full differentiation of the network, with fully communicating lumina. This stage is first reached in the region of the pellucida opposite the somites when their number is between ten and eleven (Plate II, figs. 2 and 3), when the network is made up of vessels of small calibre. Then, immediately behind the somites there differentiates an area of vessels of larger calibre, which unlike those more anteriorly which have already differentiated, communicate with the dorsal aortae on each side. These are the vessels from which the proximal sections of the vitelline arteries will ultimately arise. Their communication with the aortae is best shown in injected preparations, such as those figured by Popoff (1894), but they may be readily recognised at each stage by their large lumina. If they are followed from their first appearance onwards, it is apparent that they continue to occupy a position just behind the last somite, despite the fact that the number of somites is continually being added to from behind. This must mean that as the somites form they move forward as a whole, and that the anterior end of the embryo moves forward relative to the blastoderm. Now the heart connects with the vessels of the area pellucida through the sinus, and since from the ten-somite stage onwards the level of the sinus is in the neighbourhood of the first somite, this must mean that the network of vessels in the area pellucida elongates as a whole in the direction of the embryonic axis, though not to the same extent as the axis, for at seven somites the heart is well in front of the first somite, while at the twenty-somite stage the first somite occupies the same transverse level as the ventricle. But allowing both for this and for the longitudinal crowding of the somites which takes place during development, some elongation of the vessels of the area pellucida opposite the somites does take place, and is reflected in the details of their arrangement. The forward movement of the somites relative to the blastoderm can be seen in Plate II, figs 1-5, by comparing the position of the first somite relative to the yolk granule (*g*) lying on the surface of the ectoderm of the area pellucida.

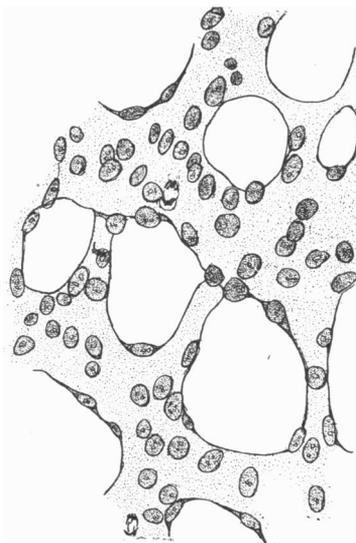
Plate II, fig. 6 is a photograph of a blastoderm at the eleven-somite stage, in which the three areas (*a*), (*b*) and (*c*) are again shown as high-power drawings in text-figs. 1, 2 and 3. Opposite the seventh somite (text-fig. 2) those components of the vessel network at right angles to the embryonic axis are of larger calibre than those parallel to the axis, and in the latter adjacent lumina are not yet fully patent, in that some resorption of adjacent endothelial walls has yet to take place. These narrower components of the vessel network are the late stages of "sprouts" from the larger, already differentiated transverse vessels, and these sprouts elongate with the general extension of the area pellucida vessels in this region.

The vessels opposite the seventh somite contrast strongly with the vessels of larger calibre of the future vitelline artery area behind the somites (text-fig. 3); posterior to this area, the vessels at this stage consist of unjoined vascular spaces surrounding blood islands.

The differentiation of the "vitelline artery vessels", as these may be termed,



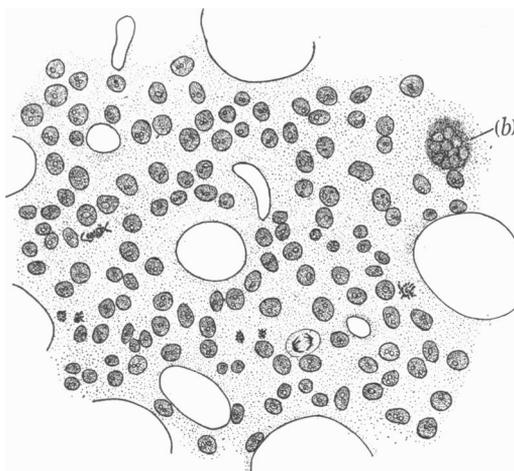
Text-fig. 1.



Text-fig. 2.

Text-fig. 1. Drawing of the area (*a*) of the eleven-somite blastoderm shown in Plate II, fig. 6. Area (*a*) is in the pellucida opposite the first somite. The drawing is largely in the plane of the vessel lumina. $\times 360$.

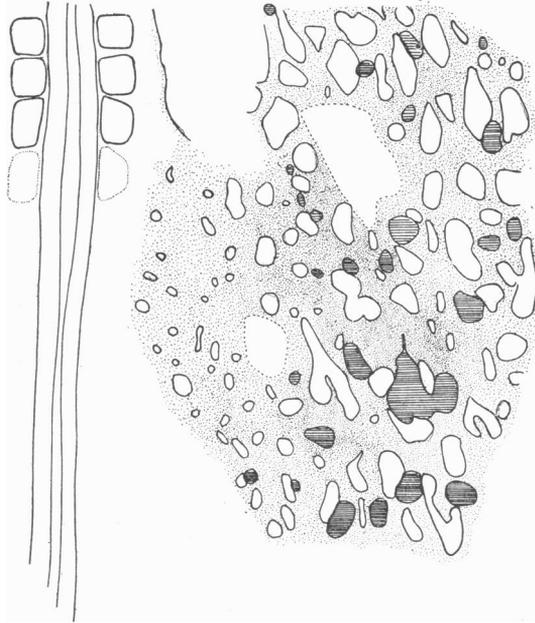
Text-fig. 2. Drawing of the area (*a*) of the eleven-somite blastoderm shown in Plate II, fig. 6. Area (*b*) is in the pellucida opposite the seventh somite. The drawing is largely in the plane of the upper endothelial wall of the network. $\times 400$.



Text-fig. 3. Drawing of the area (*c*) of the eleven-somite blastoderm shown in Plate II, fig. 6. Area (*c*) is in the vitelline artery area in the pellucida, behind the somites. The drawing is in the plane of the upper wall of the vessels, except for the blood island within the lumen at (*b*). $\times 400$.

may be followed in Plate II, figs. 3 and 4, in which it appears that they differentiate first at the margin of the pellucida, and are of large calibre from their first differentiation.

At the stage of sixteen somites, when blood first begins to circulate, differentiation of vessels from isolated spaces is complete throughout the area vasculosa, and vessel lumina are everywhere in free communication. Caudal to the "vitelline artery vessels" a vascular network has differentiated whose



Text-fig. 4. Drawing of the vitelline artery area of a sixteen-somite blastoderm taken from the egg. The blood islands are cross-hatched, and the depth of shading of the vessels is intended to represent the distance between the upper and lower endothelial walls of the vessel network, according to the key in text-fig. 15. The areas enclosed by dotted lines are gaps in the preparation. $\times 52$.

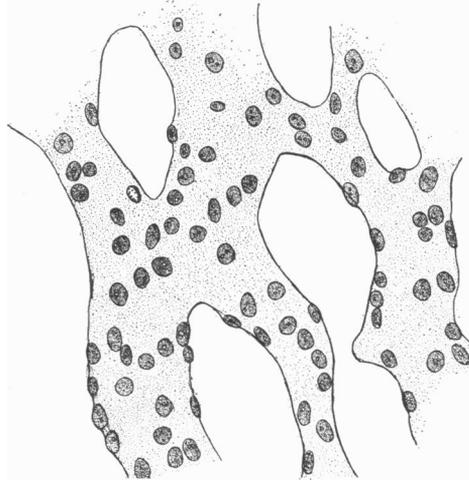
vessels are of medium diameter. The vitelline artery vessels have the largest lumina, and the smallest intervascular spaces in the whole area vasculosa, whereas that region of the vessel network anterior to the vitelline artery area and nearest the embryonic axis has the smallest lumina and largest intervascular spaces, the intervascular spaces being elongated still further with the subsequent extension of this area as a whole, consequent on the formation of the last five somites. Text-fig. 4 is a low-power drawing of the vitelline artery area at sixteen somites, and text-fig. 5 one at higher magnification showing elongated vessels of the same blastoderm opposite the seventh somite.

Thus, when the circulation first takes place, blood flows out from the aortae, through the vitelline artery vessels, and takes a wide path through the

area vasculosa before returning to the heart. Blood issuing from the aortae does not take the shortest path back to the heart through the vessels nearest the embryonic axis, because of the resistance offered to the flow by reason of their small calibre. Less resistance being offered by a longer path through larger vessels farther out in the area vasculosa, the circulation takes place through distal rather than proximal vessels, and in consequence affects as big an area of the whole vessel network as possible.

The path taken in the area vasculosa by the first circulating blood was described by Thoma, who saw that it is determined by factors other than those which cause the ultimate differentiation of an artery within the vessel network. Arterial differentiation is due to the effects of long-continued blood flow, but the operation of the afore-mentioned factors is necessarily antecedent to the later differentiation which is the result of circulation, in that they determine the path along which blood shall flow at a time before circulation begins. The differentiation of arteries has therefore two aspects, one of self-differentiation before the circulation takes place, and the other of functional differentiation in response to the circulation.

That this is true in a wider sense also, was pointed out by Thoma, namely in relation to the relative times in development at which the heart begins to beat effectively, and at which a fully formed vessel network is differentiated. We know that in the chick and elsewhere, the latter event precedes the former. Were this not so, blood would flow exclusively through those channels which were sufficiently differentiated to transmit a circulation, and which would thereupon begin to transform themselves by enlargement into arteries, while vessels of smaller calibre subsequently differentiated would offer too high a resistance ever to transmit blood under these conditions. It is essential for the ultimate development of a vascular system consisting of arteries, capillaries, and veins, that there should be presented to the first flow of blood a more or less uniform network of small vessels through which blood will not flow exclusively along one particular path.



Text-fig. 5. Drawing of a small area in the vessel network of the area pellucida, opposite the seventh somite, of the sixteen-somite blastoderm *in ovo* of text-fig. 10. The drawing is in the plane of the upper wall of the network. $\times 360$.

Embryonic endothelium as a contractile tissue

That vascular endothelium is of itself contractile was first demonstrated by Clark and Clark (1925) who showed that in the capillaries of the transparent fin of the tail of the frog tadpole, contraction can take place prior to the development of the adventitial Rouget cells, thus denying the general applicability of the conception of Vimtrup (1922) that the contractility of fully differentiated capillaries is solely due to these particular cells. The capillaries of the area vasculosa in the chick are devoid of Rouget cells, and were shown by Hammett and Zoll (1928) to react to the application of carbon dioxide in solution by a vigorous contraction, a response which was not evoked by changes in pH within the physiological range.

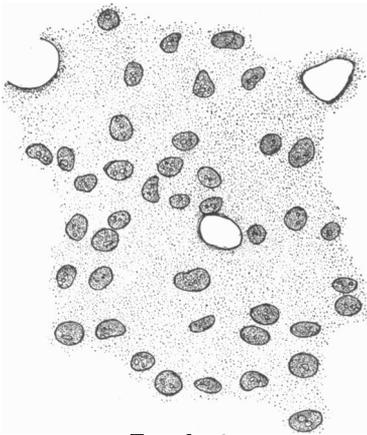
Work on the physiology of the vessels of the area vasculosa was continued by Cohn and Lange (1930). They showed that no innervation of the extra-embryonic vessels took place throughout the incubation period, that these vessels would react to mechanical and electrical stimuli, and to chemical stimuli such as ammonia. They further showed that the irritability of these vessels was in inverse proportion to their calibre, and that in general, weak stimulation caused dilation of vessels, which was followed by contraction on the application of stronger stimuli. However, in their experiments on the pre-circulatory area vasculosa "at the time when there are only blood islands and no regular flow of blood, contraction and dilation do not occur".

In the course of the present work, contraction of area vasculosa vessels was first encountered in the use of the technique of vital injection described in the above section of methods. Some fifteen minutes after the injection of the dialysed Indian ink, the vessels in that section of area vasculosa which had received the ink least diluted by the blood gradually contracted until their lumina would no longer transmit fluid. Recovery generally took place during the next one or two hours. This could occur equally before or after the first appearance of the circulation.

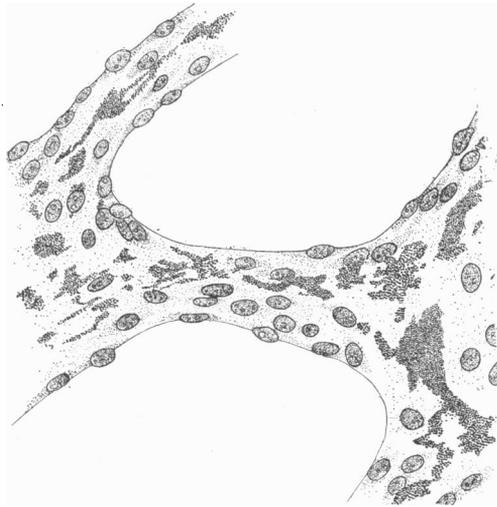
Cultivation of whole blastoderms or of fragments of area vasculosa without injection and under the conditions of tissue culture employed in this work leads in general to a dilation of the vessels, which persists and is not followed by a contraction to the normal state unless circulation of blood through them takes place. This fact has had considerable influence on the progress of this work, for since the state of expansion of vessels is of the greatest importance for their differentiation into arteries, it has not been possible to rely exclusively on the use of explanted blastoderms for the observation of arterial differentiation.

Dilation of extra-embryonic vessels is shown equally in explanted blastoderms in which the whole embryo or the heart has been removed, or in those in which the heart although beating, fails to propel blood through the vessels. Failure of the heart beat to cause circulation in blastoderms grown *in vitro* from the earliest stages has been noticed by Waddington (1932). Text-fig. 6 is a drawing of a small section of the area pellucida of a blastoderm in which the embryo was

removed at the stage of sixteen somites, and the rest of the blastoderm cultivated *in vitro* for a period of 27 hours, and subsequently mounted whole, as described in the section on histological methods. The dilation appears in surface view as a decrease in the area of the intervacular spaces, and on focusing from the upper surface of the vessel network through the lumen to the lower surface in contact with the endoderm, it appears from comparison with undilated vessels, that dilation has also increased the dimension of the vessel network at right angles to the plane of the blastoderm. This increase in the area of the endothelium does not involve any increase in the number of cells present, and throughout the endothelium mitotic figures are exceedingly rare.



Text-fig. 6.



Text-fig. 7.

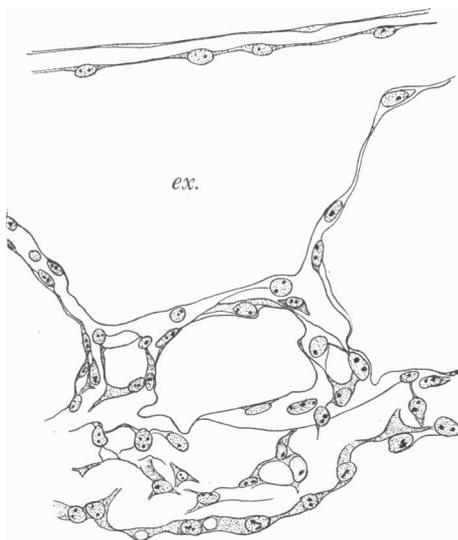
Text-fig. 6. Dilated vessels of a small area of a blastoderm cultivated *in vitro* without the embryo for twenty-seven hours. The upper wall of the vessel network is shown. $\times 400$.

Text-fig. 7. Vessels of a small area of a blastoderm cultivated under the same conditions as that of text-fig. 1, but in which the injection of Indian ink caused contraction. The ink has precipitated on the inner walls of the vessels, and the endothelial nuclei have elongated. $\times 400$.

Moreover, these dilated vessels can be made to contract equally with those of blastoderms which have not undergone this process of dilation through cultivation *in vitro*. Plate III, fig. 6 is a photograph of a blastoderm cultivated without the embryo under the same conditions and for the same time as that of text-fig. 6, but in which thirty minutes before fixation an injection into the vessels of the Indian ink used in the vital injection experiments had been made. Contraction of the heavily dilated vessels began a few minutes after injection, and gradually became more severe. The ink precipitated on the walls of the vessels, and shows the extent of the contraction, and the corresponding increase in the size of the intervacular spaces. A drawing of this blastoderm under high power is seen in text-fig. 7, which shows the effect of this contraction on

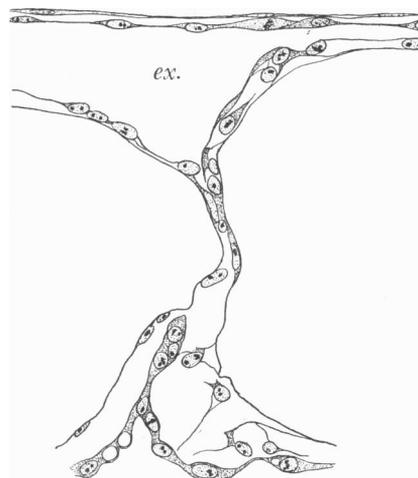
the shape of the endothelial nuclei, a matter which is discussed at length below.

Dilation of the vessels of explanted blastoderms in which no circulation takes place finally results in the obliteration of the intervascular spaces, which gradually become smaller until the bordering endothelial wall becomes a solid vertical strand. The stages in this process, as seen in transverse section, are illustrated in text-figs. 8–11, which are drawn from a section through a blastoderm cultivated *in vitro* in which circulation failed to take place at the appropriate time, and in which, in consequence, dilation of all vessels took place. Dilation in this blastoderm happened to be least anteriorly, and progressively



Text-fig. 8.

Text-fig. 8. A small area of a section through the area pellucida of a blastoderm cultivated *in vitro*, showing three adjacent unfused vessels. *ex.* exocoelom. $\times 400$.



Text-fig. 9.

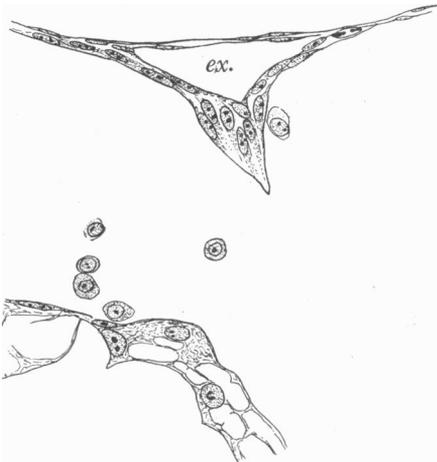
Text-fig. 9. Another part of the same section as that of text-fig. 3, showing two adjacent vessels, the intervascular spaces between being now an intervascular strand. *ex.* exocoelom. $\times 400$.

more severe in the caudal direction. Of these figures, text-fig. 8 is the most cranial in the section, and the others represent successive stages in the process passing caudally. After the apposition of the endothelia from opposite sides of the intervascular space as seen in text-fig. 9, a stage which I call that of the “intervascular strand”, the strand is resorbed as seen in text-fig. 10, where the dorsal end of the strand still hangs from the upper endothelial wall.

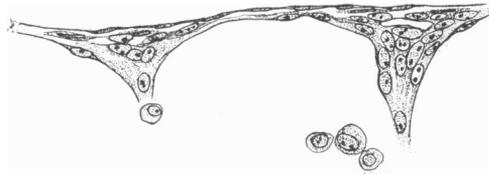
Meanwhile dilation has also been taking place in the dorso-ventral direction at the expense of the extra-embryonic coelom, which is gradually obliterated during the process, until in text-fig. 11, where the lower endothelial wall is now too far from the upper to be drawn in the same figure, ectoderm, somatopleure, splanchnopleure, and endothelium are all in contact, and small isolated spaces are all that remain of the extra-embryonic coelom. The same process takes

place wherever vascular spaces fuse. Within the differentiating artery, intervascular spaces are ultimately represented by intervascular strands (text-fig. 12).

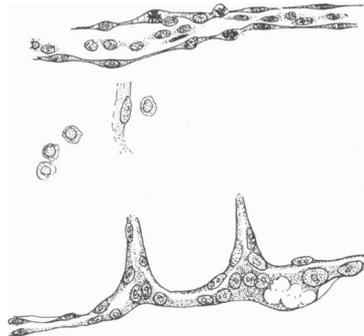
It is not clear what factor in the conditions of tissue culture is responsible for the dilation of these vessels where no circulation takes place. Dilation of vessels under these conditions has been noticed previously by Strangeways and



Text-fig. 10.



Text-fig. 11.



Text-fig. 12.

Text-fig. 10. Another part of the same section as that of text-figs. 3 and 4, in which the intervascular strand between two adjacent vessels has broken. The exocoelom (*ex.*) becomes smaller as the vessels continually dilate. $\times 400$.

Text-fig. 11. Another part of the same section as that of text-figs. 3, 4 and 5. This shows the end stage of the process of dilation and fusion of vessels, in which the exocoelom is almost entirely obliterated. $\times 400$.

Text-fig. 12. Section through the vitelline artery of a twenty-two-somite blastoderm cultivated *in vitro*, in which circulation took place for eight and a half hours. In the floor of the differentiated artery are shown two severed intervascular strands. $\times 400$.

Fell (1926) in their early work on the growth of chick limb-buds in tube cultures. This dilation affected spaces within the explants other than the lumina of vessels, such as cavities between ectoderm and mesoderm and within areas of necrosis within the mesoderm, and by the coalescence of these different cavities large cysts were formed. Cyst formation was most marked where media of a more fluid consistency were employed.

When blastoderms are explanted in the pre-circulatory period and successfully circulate at the appropriate time, after the circulation has been in progress for some hours a general contraction of all vessels takes place, after which the degree of dilation of the vessels corresponds with those of blastoderms at the

same stage *in ovo*. This general contraction only takes place *in vitro* where the circulation has been established, for otherwise progressive dilation results. Where circulation is prevented in blastoderms *in ovo*, as in the experiments of Chapman, all extra-embryonic vessels finally contract.

Since it was shown by Hammett and Zoll that carbon dioxide causes extreme contraction of the vessels, it might be possible that the normal carbon dioxide tension within the egg was responsible for maintaining a state of tonic contraction of the vessels, and that since explanted blastoderms are in contact with a free air surface at which the carbon dioxide tension might be lower than *in ovo*, this circumstance might be the cause of dilation of vessels *in vitro*. However, blastoderms which develop *in ovo* exposed to the air when the egg is opened show the normal degree of contraction, which is apparently also true when the yolk is decanted into warm Ringer through which oxygen is kept bubbling (Lange, 1930). Moreover, the contraction resulting *in vitro* from the development of the circulation cannot readily be related to carbon dioxide concentration.

The dilation of extra-embryonic vessels under the conditions of cultivation employed in this present work is probably part of a general tendency for the accumulation of fluid in intercellular spaces. The thickness of the layer of mesenchyme which forms above the differentiated vitelline artery is very much greater in blastoderms grown *in vitro* (Plate I, fig. 3) than in those at the corresponding stage taken from the egg (Plate I, fig. 4). This is due in part to a greater proliferation of mesenchyme *in vitro*, but even more to the greater distance between the mesenchymal nuclei, whose cells connect with each other by the anastomosis of long slender processes forming a meshwork with very large spaces in which fluid accumulates.

It remains to enquire whether anything corresponding to the dilation of vessels of blastoderms cultivated *in vitro* takes place normally *in ovo*. There can be no general tendency for the progressive dilation of vessels *in ovo*, where vessels through which no circulation ever takes place ultimately contract, as is seen in the experiments of Chapman (1918). But when the vessel network is first fully differentiated, the vessels tend to dilate until blood begins to circulate, as can be seen from Table I, and also from comparison of the text-figures illustrating particular regions of the area pellucida network at its first differentiation with similar figures of the same regions at the sixteen-somite stage. Thus in the vitelline artery area, although the intervascular spaces are small at eleven somites (text-fig. 3), they are still smaller at sixteen somites (text-fig. 4).

IV. THE EARLY POST-CIRCULATORY DIFFERENTIATION OF THE AREA VASCULOSA

In this paper the description of the changes in the area vasculosa which follow the development of the circulation is limited to those changes seen in the area pellucida in the neighbourhood of the "vitelline artery vessels" whose

first differentiation is described in the preceding section. These post-circulatory changes appear first in these vessels, and subsequently similar changes take place elsewhere in the area vasculosa along the path of differentiating arteries. Description is limited to this region, because the absence of yolk in the underlying endoderm facilitates observation both in the living and in the fixed condition. The differentiation of the vitelline artery is described first for blastoderms cultivated *in vitro*, and then for blastoderms *in ovo*. Certain differences are encountered between arterial differentiation under these two conditions, due to the tendency for vessels to dilate *in vitro*, a tendency which is one aspect of the contractility of endothelium.

Table I. *Vessels of the area pellucida.*

No. of somites	Vessels opposite middle somites	Amount of mitosis	Vitelline artery vessels	Amount of mitosis	Vessels in posterior region	Amount of mitosis
<i>Pre-circulatory in ovo</i>						
11 (Text-figs. 7, 8, 9)	Not dilated	Few	Dilation moderate	Frequent	Unjoined Anlagen	Frequent
13	Dilation small	Few	Widely dilated	Some	Differentiated, not dilated	Few
16 (text-figs. 10, 12)	Dilation small	Few	Widely dilated	Some	Dilation small	Few
<i>Post-circulatory in ovo</i>						
24 (text-fig. 19)	Dilation very small	Very few	Narrow irregular artery	Few	Dilation very small	Few
26 (text-figs. 20, 24)	Dilation very small	Some	Regular artery	Frequent	Dilation very small	Some
<i>Post-circulatory in vitro</i>						
19, 4 hours' circulation (text-fig. 13)	Dilation moderate	Very few	Fully dilated	Few	Dilation moderate	Few
20, 6 hours' circulation (text-fig. 15)	Dilation small	Very few	Fully dilated	Some	Dilation moderate	Few
27, 9 hours' circulation (text-fig. 16)	Dilation small	Very few	Wide artery	Some	Dilation moderate	Few
30, 13 hours' circulation	Dilation small	Very few	Wide artery	Some	Dilation small	Few
<i>In vitro</i> after 16 somites without circulation						
22, 7 hours after 16 somites (text-fig. 28)	Dilation moderate	Very few	Fully dilated	Very few	Dilation moderate	Very few

Arterial differentiation in blastoderms cultivated in vitro

In studying the effects of the circulation in an explanted blastoderm where the heart is beating effectively, it is first necessary to determine the part played by that tendency for the vessels to dilate which is always shown in blastoderms *in vitro*, but which is counteracted when circulation takes place.

Plate II, figs. 1-5 are from a series of photographs of a blastoderm cultivated *in vitro* in which no circulation took place. Circulation normally begins at the stage of sixteen somites, and, therefore, this series represents normal development up to this stage (Plate II, figs. 1-4). Plate II, fig. 5, shows the twenty-somite stage, four and a half hours after circulation would normally have taken place. It will be noticed that dilation, as viewed in superficial aspect, is everywhere complete, except in the vessels opposite the region extending from the twelfth to the nineteenth somite, in the area where elongation takes place, which, as explained in the section above, has the effect of restricting the size of the lumina *in ovo*. When no circulation takes place *in*

vitro, these vessels are the last to dilate. In another series of experiments where the heart was removed from a series of blastoderms before explantation, six and a half hours after the period when circulation would normally have taken place, dilation of vessels was everywhere complete except for two regions of the vessel network. The vessels which differentiate subsequent to the appearance of the circulation caudal to the vitelline artery area were not dilated, and those opposite the middle group of somites were only moderately dilated, much as in Plate II, fig. 5.

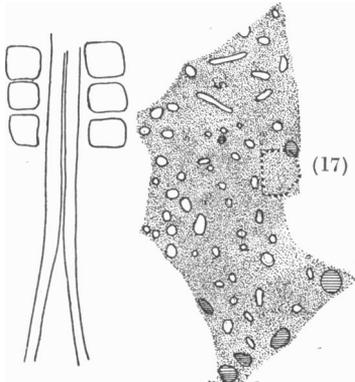
We are now in a position to compare the vessels of blastoderms cultivated *in vitro* where no circulation takes place with those in which blood begins to flow at the appropriate time, and in which a normal flow is maintained for known periods. Text-figs. 13 and 14 represent the vessels of the vitelline artery area of blastoderms which were fixed after circulation had been in progress for four and six hours respectively. Only the vitelline artery area is drawn, for the vessels anterior thereto are covered by a layer of mesenchyme so thick that accurate representation is impossible. It is possible to see, however, that whereas the vessels of the vitelline artery area are widely dilated, the vessels anterior to this area are of small diameter, and passing cranially, as soon as the connection with the aorta is lost, there is an abrupt transition to undilated vessels. Similarly, posterior to the vitelline artery area, and indeed generally throughout the area vasculosa, the dilation of vessels is less extreme when circulation has taken place. The first effect of the circulation, therefore, is to counteract in some measure the dilating influence of the conditions of cultivation. This influence, moreover, is progressive, for after six hours of circulation, the vessels of the area vasculosa generally are less dilated than those through which flow has been in progress for four hours.

Although in the blastoderms of text-figs. 13 and 14 the vitelline artery vessels anteriorly pass abruptly into very much smaller vessels, there is as yet free communication of lumina between these two regions of the area vasculosa. The loss of this free communication of lumina between the vitelline artery area and the vessels to either side leads ultimately to the differentiation of the vitelline artery itself. This process is complete after nine hours of circulation, and the appearance of the vitelline artery area at this stage is shown in text-fig. 16. The embryo in this blastoderm has twenty-seven somites, and circulation did not begin until the stage of nineteen somites. Normally, the circulation would have begun at sixteen somites, and there would be twenty-three somites by the time the circulation had been in progress for nine hours. By comparing blastoderms of slightly different stages in which, however, circulation has been in progress for the same time, it is seen that the time during which blood has flowed, rather than the age of the embryo, is the determining factor in arterial differentiation.

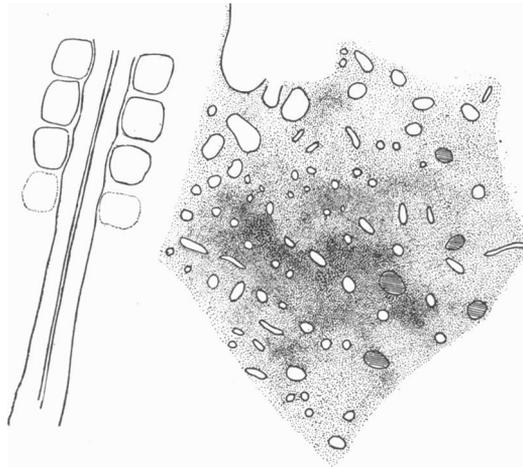
After nine hours' circulation, besides the isolation of the vitelline artery, there has been a general contraction of all other vessels throughout the area vasculosa. Only within the artery, which proximally has been formed from a region of the vessel network which is at least two network meshes wide, are

small intervascular spaces still to be found. Since the vessels adjacent to the vitelline artery area were somewhat contracted before this period, differentiation of the artery on its anterior border has meant only its isolation from neighbouring vessels. On the posterior border, however, neighbouring vessels have both contracted in diameter, and have undergone isolation.

The small intervascular spaces within the artery are all in process of forming intervascular strands, and ultimately disappear in exactly the same way in which neighbouring vessels fuse *in vitro* when no circulation takes place, as



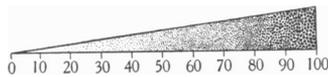
Text-fig. 13.



Text-fig. 14.

Text-fig. 13. Drawing of the vitelline artery area of a twenty-somite blastoderm cultivated *in vitro*, in which circulation took place for four hours. The area (17) is drawn at a higher magnification in text-fig. 17. $\times 52$.

Text-fig. 14. Drawing of the vitelline artery area of a twenty-one-somite blastoderm cultivated *in vitro* in which circulation took place for six hours. $\times 52$.



Text-fig. 15. Key to the depth of shading of the vessel network of text-figs. 4, 13, 14 and 20. The numbers represent distance in (μ) between the upper and lower endothelial walls. $\times 52$.

described above. In text-fig. 16, the intervascular space in which this process is farthest advanced is that marked (*i.v.s.*), which is now a strand, from which a long ridge leads in either direction along the artery, as shown in the section of which Plate I, fig. 3 is a photograph. Text-fig. 12 is a section through the differentiated vitelline artery of an explanted blastoderm in which circulation was maintained *in vitro* for eight and a half hours, and shows two severed intervascular strands whose ends are attached to the lower wall of the vessel.

The vessels to either side of the vitelline artery with which they have now lost their connection will form part of a plexus of vessels which is venous in

function, and will return blood from the caudal region of the area vasculosa, passing dorsal to the vitelline artery. The formation of this dorsal venous plexus in blastoderms cultivated *in vitro* is intimately related to the profuse production of mesenchyme under these conditions. For the dorsal venous plexus over the vitelline artery is formed not only by the growth of the severed vessels over the artery, but also by a linking up with small independent vascular spaces which have differentiated within the thick layer of mesenchyme dorsal to the artery.

It was mentioned above in passing that the thickness of the layer of mesenchyme formed under these conditions is due to two causes, viz. to the presence of a larger number of cells than in blastoderms at a corresponding stage *in ovo*, and to a greater accumulation of intercellular fluid, this being the more important factor of the two. The cell bodies within this mesenchyme give off long slender processes, which anastomose with those of neighbouring cells. In comparing the amount of mesenchyme present with the degree of dilation of the neighbouring vessels under different conditions, it is apparent that when dilated vessels contract fluid passes through the vessel wall from the lumen to the surrounding mesenchyme, and that in general dilation of vessels and volume of the mesenchyme are in inverse proportion to each other. In blastoderms which have been cultivated without circulation after the sixteen-somite

stage, and in which all the vessels are dilated, mesenchyme cells adjacent to the vessels are in close contact with the endothelium, from which they are not sharply demarcated by the long anastomosing processes which the cells possess when there is much intercellular fluid. Under these conditions the mesenchyme is similar to that of blastoderms *in ovo*, as seen in the neighbourhood of the differentiated vitelline artery in Plate I, fig. 4.

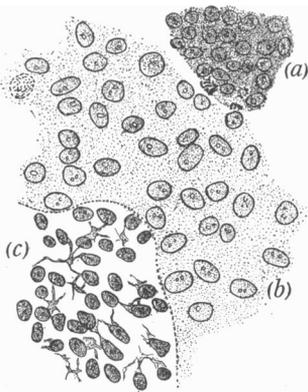
Blastoderms cultivated *in vitro* in which circulation takes place for periods less than that in which arterial differentiation takes place, show an intermediate condition of the mesenchyme. This is illustrated in text-fig. 17, a high-power drawing of the small region of the vitelline artery area of text-



Text-fig. 16. Reconstruction of the vitelline artery area of a blastoderm of twenty-seven somites which was cultivated *in vitro*, and in which circulation took place for nine hours. The blastoderm is the same as that from which the section in Plate I, fig. 3 was taken. The vitelline artery, here shown unshaded, has been isolated from the surrounding vessels, which are shaded; *i.v.s.* represents an intervascular strand within the artery; (v_1) and (v_2) are two isolated vascular spaces dorsal to the artery, and (3) represents the plane of the section of Plate I, fig. 3. $\times 76$.

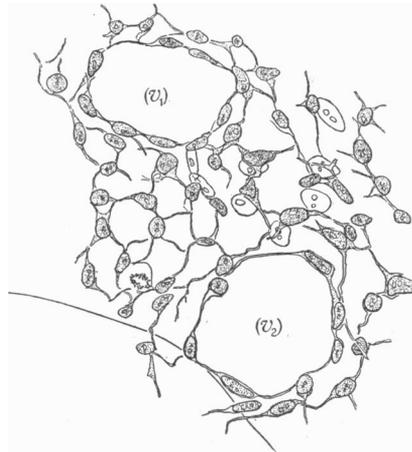
fig. 13, which is drawn from a blastoderm which circulated for four hours *in vitro*. In text-fig. 17, cells are drawn at three different levels, the most ventral at the level of a blood island, next at that of the upper endothelial wall of the vessel network, and most dorsal of all, at that of the mesenchyme above the endothelium. In this mesenchyme, the cells are at moderate distances apart, and the first stages in the formation of the cell processes are to be seen. In the moderately contracted vessels anterior to the vitelline artery area, the cells are much farther apart, and the thickness of the whole mesenchyme is therefore much greater.

Where arterial differentiation has taken place *in vitro*, the vessels of the whole area vasculosa are embedded in a uniformly thick layer of mesenchyme,



Text-fig. 17.

Text-fig. 17. Drawing of the area (17) of text-fig. 13. Portions (a), (b) and (c) are drawn at different planes. (a) represents part of a blood island within the vessels, (b) part of the upper vessel wall, and (c) part of the mesenchyme dorsal to the vessels. $\times 400$.



Text-fig. 18.

Text-fig. 18. Drawing of a region dorsal to the vitelline artery of the twenty-seven-somite blastoderm of text-fig. 16, made before sectioning. In the mesenchyme dorsal to the artery are seen isolated vessel spaces, of which (v_1) and (v_2) are the same as those similarly named in text-fig. 16. Mesenchymal nuclei are shaded, and the nuclei of the dorsal wall of the artery below are unshaded. $\times 400$.

with the cells far apart from each other, and connected by long processes through the intercellular fluid. Under these circumstances it is impossible to draw the vessel network with low magnification from a whole mount; text-fig. 16, representing a differentiated vitelline artery after nine hours' circulation, was obtained by reconstructing from serial sections. In the thick layer of mesenchyme above the vitelline artery, isolated vascular spaces differentiate, as mentioned above. Text-fig. 18 is a high-power drawing, made before the blastoderm was sectioned, of two such vascular spaces which are seen again in the reconstruction (v_1 and v_2); one of them is also shown in the section (Plate I, fig. 3). It was impossible to tell from inspection of the whole mount alone

whether this vascular space had any connection with the artery below, but the absence of such a connection is evident from the section. Occasionally, vascular spaces dorsal to the artery have an open connection with the artery, but where there is such a connection, the space has the same rounded form in cross-section; no typical forms of vascular sprouts from the artery are to be seen, nor does the differentiation of these isolated spaces involve any mitosis in surrounding cells. For these reasons, it has been concluded that these isolated vascular spaces differentiate *in loco* from mesenchyme cells which become endothelial by the conversion of their anastomosing processes into an apparently continuous protoplasmic membrane.

A similar differentiation of isolated vascular spaces above the vitelline artery at the same stage takes place *in ovo*, as will be described below, but *in vitro* the differentiation of the venous plexus dorsal to the vitelline artery from mesenchyme *in loco*, takes place earlier than *in ovo* for blastoderms of the same age, owing to the larger amount of mesenchyme present *in vitro*.

The differentiation of the dorsal venous plexus will not be followed in the present paper beyond its earliest stages. Later, the isolated spaces unite with each other, as in the earliest stages of vasculogenesis, and also with the severed vessels to either side of the vitelline artery. The plexus functions as a means of conveying blood from the hinder regions of the area vasculosa forward to the heart, and does so typically over the vitelline artery first at a point nearest to the embryonic axis, to the left of the embryo. Thoma relates its formation to an increase in pressure in the hinder part of the area vasculosa, where the greater efficiency of the newly formed vitelline artery in conveying blood to that region is not as yet compensated by a more efficient means of venous return than the existing path round the marginal sinus. However, there is first a phase of self-differentiation, as always in the vascular system, which is expressed in the differentiation of the isolated spaces, which appear dorsal to the vitelline artery area. Such isolated spaces are formed also in blastoderms in which no circulation takes place.

Arterial differentiation in blastoderms in ovo

At the time when circulation begins, the main difference between the area vasculosa of a blastoderm *in ovo* and that of a blastoderm *in vitro* is that in the latter the vessels have dilated. We know that *in vitro* when circulation does not take place, all vessels tend to dilate, whereas when circulation is prevented *in ovo*, as in the experiments of Chapman (1918), complete contraction of the vessels ultimately results.

It was concluded above, that in the vitelline artery area there is a slight tendency for the further dilation of the vitelline artery vessels after their first differentiation at the stage of ten somites when they are already of large calibre. By the stage of fifteen somites, some of the intervacular spaces of the vitelline artery area have been reduced still further, some to the condition of intervacular strands, which in some cases have already broken.

The vitelline artery area when circulation is about to commence is thus a network of vessels of large calibre, some of the intervascular spaces of which are of moderate size, while some are disappearing, as is shown in text-fig. 4. Anteriorly the vitelline artery area leads into a region of vessels of small calibre in virtue of their extension with the embryonic axis as explained above, and posteriorly into vessels of moderate calibre, which have been the last to differentiate. This is the condition of the living vitelline artery area photographed in Plate III, fig. 1, which is from a blastoderm *in ovo* of seventeen somites, in which the circulation had already been in progress for one hour. The next four photographs represent the same vitelline artery area during the succeeding six hours, while text-fig. 19 is a drawing of the same area after fixation of the blastoderm eleven hours after the time of the first photograph at the stage of twenty-four somites, and twelve hours after the beginning of the circulation. In this series we can follow the gradual isolation of the vitelline artery by the increase in size of the bordering intervascular spaces, and the contraction of the corresponding meshes of the network, one of which was seen to disappear. Whereas intervascular spaces to either side of the artery increased in size, those within the differentiating artery became smaller, and finally disappeared in the usual way. Thus the obliteration of intervascular spaces in the vitelline artery area *in ovo* is shared between the pre-circulatory and the post-circulatory periods. In the photographs, the dark areas represent blood islands, which had not been broken up entirely at the time of fixation, and are represented by shading in text-fig. 19.

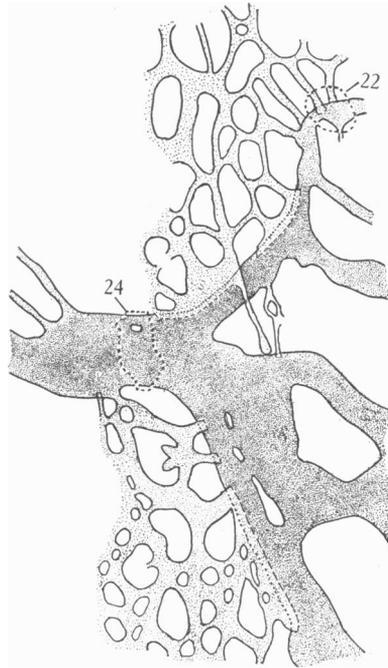
When the circulation had been in progress for seven hours, that is at the time of Plate III, fig. 5, the flow of blood was markedly more rapid through the differentiating artery than elsewhere, whereas previously the flow had been less unevenly distributed through the area. Two hours later, the circulation was mainly through the differentiating artery, but also in part through vessels running parallel on either side. No blood was passing through the contracted vessels to either side of the artery.

This series represents the first stages in the differentiation of the vitelline artery *in ovo*. No venous return over the artery was observed, and at the end of the period the outline of the artery was still irregular. A later stage in the differentiation of the artery is represented by text-fig. 20, which is drawn from a blastoderm *in ovo* which was fixed at the stage of twenty-six somites, some fourteen hours after the sixteen-somite stage. Here the artery is attaining some regularity of outline, and the dorsal venous plexus proximally is fully differentiated. We shall see later that striking changes occur within the endothelium. In this section we shall refer only to details which can be seen in the differentiation of the dorsal venous plexus. In text-fig. 20 no attempt is made to represent the plexus, but where vessels reaching the artery are cut off from connection with it the line of the artery is continued unbroken, and where vessels pass dorsal to the artery they are represented as cut off just inside the line of the artery.

Isolation of the differentiating vitelline artery can take place in two ways, either by the contraction and obliteration of the lumen of bordering vessels, a process which can be followed in the series of photographs mentioned above, and of which a high-power drawing from another blastoderm *in ovo* is given in text-fig. 21, or by the formation of a septum across the opening of the bordering vessel into the differentiating artery. This latter process is illustrated in text-fig. 22, a high-power drawing of the area (22) of text-fig. 20. At (s), a septum has formed across the opening of the vessel, and this process has taken place without apparently causing cell division within the artery. Of the other two vessels joining the artery, one is still in free communication, while in the



Text-fig. 19.



Text-fig. 20.

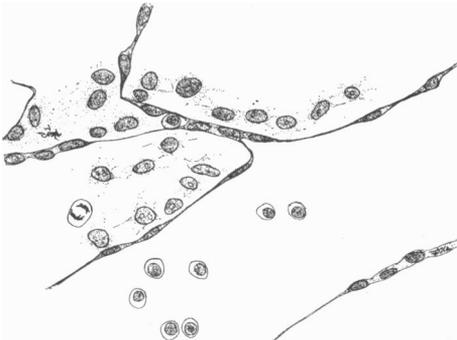
Text-fig. 19. Drawing of the vitelline artery area of a twenty-four-somite blastoderm, the earlier stages in the differentiation of which are shown in Plate III, figs. 1-5. The shading in the drawing represents blood islands. $\times 37$.

Text-fig. 20. Drawing of the vitelline artery area of a twenty-six-somite blastoderm *in ovo*. The dorsal venous plexus is in process of formation, but is not here shown above the artery. The areas (22) and (24) are drawn at higher magnification in the text-figures bearing those numbers. $\times 52$.

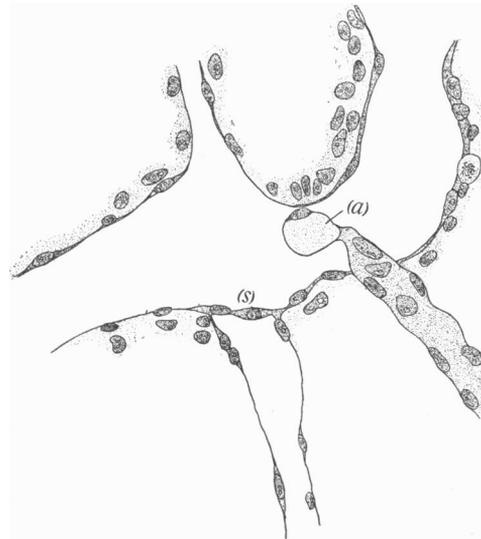
other, in which the endothelium is drawn in surface view, a septum has been formed at a level lower than that which is drawn, and the vessel runs over the artery, to join with an independent vascular space, with which free communication of the lumen has yet to be made. The existence of independent vascular spaces dorsal to the vitelline artery *in ovo* shows that the corresponding phenomenon *in vitro* is of real significance.

Thoma's account of arterial differentiation

The main difference between the outlook of Thoma on the histogenesis of the vascular system and that which is presented here is that since Thoma's time, the contractility of embryonic endothelium has been recognised. The recognition of this property removes some of the difficulties which Thoma encountered, but also suggests the need of caution in interpreting observations on developing vessels, particularly where quantitative treatment is attempted. Thus, Thoma assumed the vessel wall to form an elastic membrane, the tension in which is equal to the sum of the surface tension of the fluid contents and that of the "Zellverbänden getragenen Druckes". The surface tension of a cylinder of fluid is different in the circumferential and longitudinal directions, the



Text-fig. 21.



Text-fig. 22.

Text-fig. 21. Drawing of a small region of the vitelline artery of a twenty-five-somite blastoderm *in ovo*, showing the isolation of the artery by obliteration of the lumen of a bordering vessel. The drawing is made at a level within the lumen of the artery. $\times 360$.

Text-fig. 22: Drawing of region (22) of text-fig. 20. The drawing is made at a level within the lumen of the artery, and shows stages in the isolation of the artery. One side branch has a fully communicating lumen, in another a septum (*s*) has formed across the opening, and in a third, drawn at a higher level, has joined with an independent vessel space (*a*). $\times 400$.

former being twice the latter. Hence Thoma concluded that, in order that the form of the vessel may remain constant, the elasticity of the vessel wall must be different in the two directions. Now, in the first place we have as yet no evidence whether the vessel wall is elastic, and secondly, since the endothelium is contractile, the tension in the wall must depend on the state of contraction over and above the effect of physical forces.

To Thoma's description of the area vasculosa at the stage when circulation begins we have been able to add only a description of the means whereby the

narrow calibre of the vessel proximal to the middle somites is attained, namely by their extension with that of the embryonic axis. Also the present account of the gross anatomical changes which take place in the differentiation of the artery is in agreement with that of Thoma, except for differences in detail. His high-power drawings, however, do not take us into this period, and stop at the stage of the pre-circulatory differentiation of the network, although he has noticed the cell division in the wall of the differentiating artery which as described below follows on its growth under the influence of the circulation.

Thoma interprets this influence as essentially a nutritive one. "Die vom Blutströme bevorzugten, rascher durchströmten Capillaren wachsen stärker und werden demgemäss zu weiteren Röhren als die minder reichlich gespeisten Capillaren". Roux (1879) in his classical work on the angle of branching and form of cross-section of an arterial system in equilibrium with the blood flow which the system was transmitting, showed that the physical forces generated by the blood stream were the essential factors in moulding the form of the arteries, and that the form assumed was such as to offer least fluid resistance to the stream. It is therefore interesting to enquire how soon after the development of the circulation do physical forces play a part in the moulding of the vascular system, and what is the nature of the means through which this influence operates. In the succeeding section, evidence is given that physical forces generated by the blood stream play their part in the moulding of the vitelline artery. This evidence is based on the elongation of the endothelial nuclei which takes place in the wall of the differentiated artery, and its effect on the mitotic spindle at division. It is suggested that nuclear elongation is an index of tension action within the cell, and that tensions in the wall of the artery are set up by the fluid friction generated by the blood flow.

It can here be stated, however, that on general principles it seems improbable that a blood vessel through which blood is flowing rapidly should enjoy better nutritive conditions than one in which the flow is slower. Were this so, the endothelium must extract its nutritive substances from the blood stream almost instantaneously. Moreover, it would have to be possible for adjacent areas of endothelium to enjoy very different nutritive conditions without influence on each other. When the artery is differentiating, its lumen is enlarging, whilst the diameter of adjacent vessels is contracting, a process which we have spoken of as the isolation of the developing artery. Now isolation does not only take place by the narrowing of the bordering vessels, there is also the formation of septa across their openings into the artery to be considered, which is the process illustrated in text-fig. 22. This is incomprehensible on a purely nutritional explanation.

This leads us to the general account of the formation of the dorsal venous plexus which is given by Thoma. He states that the plexus is first formed proximal to the embryo, as described above and during its formation, "Gefässneubildung" and "Gefässschlund" go on side by side. He did not observe, however, the formation of those isolated vascular spaces dorsal to the artery, which

join up with the severed vessels, and thus the self-differentiating aspect of the formation of the dorsal venous plexus was obscured in his account. This aspect is further emphasised when isolated vascular spaces are seen dorsal to the vitelline artery area when no circulation takes place at all.

The formation of the dorsal venous plexus, according to Thoma, is in relation to an increase in pressure in the small vessels in the hinder part of the area vasculosa, which takes place when the artery has differentiated, and the resistance offered to the circulation on its way to this region is diminished in consequence. This excess pressure is relieved when the dorsal venous plexus is formed, which functions as a correspondingly more efficient means of venous return for this region. Thoma links this with the third of his histomechanical principles, which is put forward on a different footing to that of the other two, since it is left as a hypothesis. Thoma's three histomechanical principles are as follows:

(1) The increase in size of the lumen of a vessel, which is proportional to growth in surface of the vessel wall, is dependent upon the rate of the blood flow.

(2) The growth in thickness of the vessel wall is dependent upon the tension in the wall, which in turn is determined by the diameter of the vessel, and by the blood pressure.

(3) Increase of blood pressure above a certain limit, which limit is defined by the metabolism of the particular tissue, leads to the new formation of capillaries.

The first of these principles is incontestable, and has been proved by Thoma both in the work on the chick blastoderm, and in his earlier researches on pathological material. The second principle in its application to the chick area vasculosa falls outside the stages studied by Thoma and by the present writer, but the fact that increase in blood pressure leads to a thickening of the tunica media was proved by Thoma's earlier work, and also by the experiments of Fischer and Schmieden (1909), who transplanted a length of a vein into the path of an artery, and observed a great increase in thickness of the tunica media of the implanted vein.

The third histomechanical principle was formulated entirely from the work on the chick blastoderm. Thoma maintained that the extent of the initial vascularisation of an organ depended on the metabolism of the tissues of that organ, and the fact that some organs are more vascular than others was regarded as evidence for this view. Later increase in blood pressure can, however, increase the density of vascularisation. It is certainly true that the area vasculosa does increase in size, and that as it does so, new formation of capillaries goes on at such a rate that the average size of the intervascular spaces steadily diminishes. It is also true that during the first few days of circulation the output from the heart steadily increases. However, the possibility of a causal connection between these two facts was destroyed by Chapman, who showed that when no circulation takes place, the area vasculosa continues to increase

in size, although not so regularly as normally; that the new formation of capillaries takes place at about the normal rate is shown by inspection of Chapman's figures of the injected area vasculosa which has continued to grow when deprived of the circulation.

The fact concerning the relations between self- and functional differentiation of the vascular system which should be elevated into a histomechanical principle is the following: in a vascular system, the regular tubular form of the vessels is only indefinitely maintained when blood continues to circulate through them, although if the circulation ceases the endothelium persists as a tissue different from other mesoderm after the tubular form of the vessels is lost. This was demonstrated by Chapman's experiments, in that while new formation of vessels goes on at the periphery of the area vasculosa without circulation, those at the centre contract and undergo regression. Where isolated whole organs such as limb-buds are cultivated for a considerable time *in vitro*, it is easy to see that while the endothelium within the explant persists as a definite tissue, it no longer takes the form of a system of tubes. Some vessels expand into sinuses, while others are reduced to strands of cells without a lumen.

Results which have been obtained in the course of the present work from the cultivation of fragments of area vasculosa will be described in the succeeding subsection. They illustrate further the influence of the shape of a vessel on the form of the endothelial nuclei.

V. THE FORM OF THE ENDOTHELIAL NUCLEI

So far our account of vascular differentiation has been limited mainly to a description of the changes in the gross anatomy of vessels which can be seen with low magnifications. It remains to consider whether these changes can be further elucidated by the study of the individual cells which compose the walls of the vessels.

As the cell boundaries in embryonic endothelium are usually not seen, the account of the changes which take place in the endothelial cells is limited to the alterations in shape of the endothelial nuclei.

Moreover, since the tunica media of the extra-embryonic arteries does not develop until considerably later than the period of the first differentiation of the artery with which this paper is concerned, these observations on the shape of the endothelial nuclei apply solely to the period when the wall of the artery consists only of a single layer of endothelial cells.

The endothelial nuclei and vascular differentiation

The purpose of this section is to describe the relationships which exist between the histogenesis of vessels, the form of the endothelial nuclei, and the relative amount of cell division which takes place at each stage. The mesoderm of the area vasculosa which has migrated out from the primitive streak at a stage prior to the first appearance of vessel spaces, consists of strands of cells in close contact with each other, in which cell boundaries are readily recognised.

The nuclei are spherical or ovoid, and everywhere mitotic figures are exceedingly abundant. When the first isolated vascular spaces appear, the form of the cells does not immediately change, and mitosis continues as before, but later the cells differentiate into two types. The cells round the vascular spaces which form the first endothelium flatten, and cell boundaries are no longer distinguishable with ordinary cytoplasmic stains, while elsewhere cells become mesenchymatous in that they then consist of separate cell bodies united by anastomosing processes. The views of various authors differ on the exact method whereby the endothelial cells become flattened; in this section only the changes which take place in their nuclei will be described. The endothelial nuclei when seen in surface view remain rounded or oval, but become larger in diameter, and less deeply staining; when seen from the side it is apparent that they have flattened, and their lighter stain is due to their thinness. This is the stage of the fusion of the now enlarged contiguous spaces, and mitotic figures are still plentiful, though less so than before. When division of flat endothelial cells takes place, the nucleus remains flat when the nuclear membrane disappears, and in late prophase, the chromosomes are spread out over an area larger than that of the non-dividing nucleus, whilst the plane of the equatorial plate is at right angles to that of the vessel wall. During the later stages of division the cytoplasm of the dividing cell is thicker than that of the surrounding cells, and the cell boundary is easily distinguishable.

When the vessel network has everywhere a patent lumen and is fully differentiated, mitosis gradually diminishes, until by the time the sixteen-somite stage is reached, there are comparatively few mitoses throughout the endothelium; cell division still goes on in the blood islands however, where pycnotic nuclei are also frequently found, while in the endothelium nuclear degeneration is hardly ever seen at any stage.

An exception to the rule that with the full differentiation of the vessel network there is a great reduction in cell division is afforded by the vitelline artery vessels. They are, as explained above, of large calibre from their first differentiation at the stage of ten to eleven somites, and at that time (text-fig. 3) there are still numerous mitotic figures present, which, however, have become fewer in number by the sixteen-somite stage, by which time the endothelial nuclei in the vitelline artery vessels are closer together and more numerous than elsewhere. During the first few hours of circulation, and before there has been any effect on the anatomical disposition of the vessel network, cell division in the vitelline artery vessels falls to the same low level as that elsewhere in the vessel network, and at this time there is less cell division throughout the area vasculosa than at any other period.

When vessels increase in length, at first no cell division takes place in the wall, and the endothelial nuclei become farther apart. It has been explained above how the vessels in the area pellucida opposite the middle somites extend parallel to the embryonic axis with the increase in length of the embryo. At ten somites (text-fig. 2) this extension has begun, but there are still some mitotic

figures in the endothelium which represent the last traces of the cell division accompanying differentiation. At sixteen somites (text-fig. 4), although the shape of the intervacular spaces in this area implies a great extension of the vessel network, there is hardly a mitotic figure to be found in the vessel walls, and the endothelial nuclei are farther apart from each other than elsewhere in the vessel network.

Meanwhile, the area vasculosa is increasing in size owing to the increase in circumference of the marginal sinus, and the first effect of this process within the vessel network is a progressive increase in length of the network meshes. Something of this process can be seen in Plate I, figs. 1 and 2, photographs of the same vitally injected blastoderm. Not until the area vasculosa has increased considerably in size is this extension of the vessel network correlated with the appearance of endothelial cell division. This occurs at about the stage of twenty-six somites.

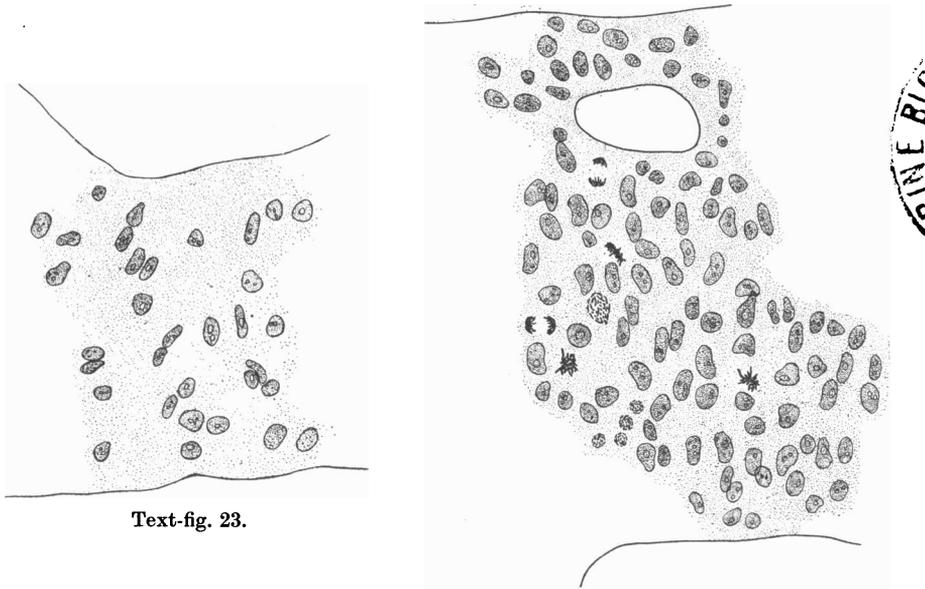
Another gross change which can take place in the vessel network without being accompanied by mitosis is the dilation *in vitro* in the absence of a circulation. A high-power drawing of this process is given in text-fig. 6; the endothelial nuclei are far apart, and mitotic figures are very rare.

The changes in the vitelline artery area due to the maintenance of the circulation, namely, the differentiation of an artery, are not at first accompanied by cell division within the endothelium. The difference in gross appearances between arterial differentiation *in vitro* and *in ovo* is reflected in the extent to which cell division takes place in the endothelium in each case. As mentioned above, the stage at which the circulation normally begins is one at which mitosis is at a minimum throughout the vessel network. When a blastoderm has been explanted some time previous to this stage, the extra-embryonic vessels are generally more dilated than *in ovo*, and the absence of mitosis in the endothelium corresponds to that seen in dilation where no circulation takes place. After the first few hours of circulation vessels other than those of the vitelline artery area contract somewhat, and in the dilated vitelline artery area occasional mitotic figures now appear. In text-fig. 17, a high-power drawing of a portion of the vitelline artery area of a blastoderm in which four hours' circulation *in vitro* took place, only one prophase is to be seen in the area of endothelium drawn. In the vitelline artery area drawn in text-fig. 14, where six hours of circulation took place, mitotic figures are slightly more numerous than before. The isolated artery after nine hours of circulation *in vitro* consists of a tract of dilated vessels with contracted vessels to either side. In the dilated vessels which form the artery, the endothelial nuclei are still far apart, and this condition is maintained even after twenty hours' circulation. Mitotic figures in the endothelium never become very numerous. In text-fig. 23, a drawing of an area of the vitelline artery of a thirty-somite blastoderm in which thirteen hours' circulation *in vitro* took place, there were no mitotic figures in the area chosen.

The differentiation of the artery from dilated vessels *in vitro* is to be con-

trasted with arterial differentiation *in ovo*, where the vitelline artery area is not dilated and contains some large intervacular spaces. In the photographs (Plate III, figs. 1-5) it can be seen that the vessels which differentiate into the vitelline artery are more contracted after their differentiation than before. In this blastoderm (text-fig. 19) the vitelline artery after twelve hours' circulation is of irregular outline, and in its wall there are very few mitotic figures.

Large numbers of dividing cells first appear in the wall of the vitelline artery *in ovo* at the stage when the dorsal venous plexus is in process of formation, and the artery is acquiring a regular outline. This stage is reached usually when the embryo has twenty-six somites, fourteen hours from the time when



Text-fig. 23.

Text-fig. 24.

Text-fig. 23. Drawing of a region of the upper wall of the vitelline artery of a thirty-somite blastoderm, cultivated *in vitro*, in which circulation took place for thirteen hours. This field is exceptional in showing no mitotic figures. $\times 360$.

Text-fig. 24. Drawing of region (24) of text-fig. 20. It represents this part of the upper endothelium of the artery. $\times 400$.

circulation first begins, and the relative number of dividing cells in the wall of the artery at this time is illustrated in text-fig. 24, a high-power drawing of the area (24) of text-fig. 20. In comparing blastoderms *in ovo* between the stages of twenty-three and twenty-six somites, it is evident that the artery when first differentiated must offer as much resistance to the circulation as did the vessels from which it has been formed, for as yet there has been no enlargement of the artery (Plate III, figs. 1-5, text-fig. 19). If the effectiveness of the heart beat and the force of the circulation continually increases during the first twenty-four hours of circulation, as is certainly suggested by inspection, then

in the period immediately before growth of the differentiated artery begins, the frictional resistance offered by the whole vitelline artery area must then be at a maximum. During the next few hours, the artery increases in diameter comparatively rapidly, and cell division within the wall begins, and more than makes up for the increase in diameter of the artery, so that in the end the endothelial nuclei in the arterial wall are closer together than ever before.

The vitelline artery after differentiation *in vitro* remains in the dilated condition of vessels from which it has been formed, and is thus comparatively large in diameter from the beginning, and at its first differentiation the frictional resistance which it offers to the blood stream cannot be maximal. Accordingly, the endothelial nuclei within the wall of the vitelline artery *in vitro* remain comparatively far apart from each other, and much less cell division takes place than in the vitelline artery *in ovo* in the first few hours after differentiation.

We have now to consider a related phenomenon in the fine structure of the vessels, which sheds further light on the changes which take place in the artery after the first differentiation. This phenomenon concerns the shape assumed by the non-dividing nuclei, and the part which this plays at cell division. The nuclei in differentiated endothelium are flattened, but when seen in surface view are generally rounded or oval. However, when vessels are either strongly contracted or widely dilated, the endothelial nuclei tend to be distorted from their rounded shape, and to become elongated. In contracted vessels the elongation of the endothelial nuclei is along the axis of the vessel, and takes place wherever vessels of small calibre are found. Such are the sprouts which grow out from the first formed vessels in the area pellucida (text-fig. 1), the elongating vessels in that region of the area pellucida which extends with the growth in length of the embryo (text-fig. 20), or the contracted vessels bordering on the differentiating vitelline artery, which either contract until the lumen is obliterated (text-fig. 21), or are cut off from the artery by the formation of a septum.

Where the intervascular spaces are of medium size, and the vessels are moderately dilated, all the endothelial nuclei are either rounded or oval, and the range of vessel calibre over which this holds good extends from the moderately contracted vessels of the area pellucida opposite the middle somites at the sixteen-somite stage, to a degree of dilation rather less than that of the vitelline artery vessels at the stage of ten somites (text-fig. 3), when nuclei begin to elongate in response to dilation. Where vessels dilate *in vitro*, nuclear elongation is observed at the stage in the process when the intervascular spaces are becoming so small that they are forming intervascular strands, when the elongation tends to be along lines joining these intervascular strands. Text-fig. 25 is a drawing of a small area of the vessel network of a blastoderm which was cultivated *in vitro* without circulation taking place for a period of seven hours after the sixteen-somite stage, and shows elongation of endothelial nuclei in this manner. A similar elongation is seen in blastoderms in cultivation where circulation has taken place. There is some nuclear elongation in the

vitelline artery area of the blastoderm in which circulation took place for four hours which is drawn in text-fig. 17, but this is more extensive when circulation has been in progress for six hours. With the differentiation of the vitelline artery, nuclear elongation becomes more pronounced, as is shown in text-figs. 18 and 23.

Elongation of the endothelial nuclei also accompanies arterial differentiation *in ovo*. It is less marked in the irregular artery at twenty-four somites than at twenty-six somites when the outline of the artery is regular, and when mitotic figures have appeared in large numbers in the endothelium (text-fig. 24).

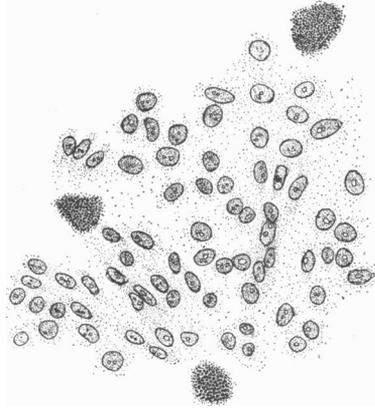
At twenty-six somites (text-fig. 24), at which stage both elongation and mitosis are considerable, the endothelial nuclei are frequently elongated in a direction at right angles to the axis of the artery. Not all the elongated nuclei point in this direction however, and a general trend for orientation to be at right angles to the axis of the vessel is only seen where the artery has the form of a straight tube without side branches. Where the individual nuclei are most elongated, that is with a ratio of length to breadth of 2.5 : 1, all the surrounding nuclei tend to be orientated in the same direction within the endothelium, but the direction of orientation of nuclei immediately overhead within the mesenchyme may be quite different.

Endothelium in tissue culture

In the preceding subsection, an account was given of the influence of the form of vessels in the area vasculosa on the shape of their endothelial nuclei. The personal observations described in this section refer to the different forms assumed by vessels which outwander from explants of fragments of area vasculosa in tissue culture, and their relation to the shape of their endothelial nuclei.

The cultivation of vascular endothelium *in vitro* has been frequently described, and the literature relating to this subject has been reviewed by Levi (1934) in his survey of the whole subject of tissue culture.

Most of the earlier accounts refer to the outgrowth of endothelium not in the form of vessels but as a plexus of cells, which under favourable circumstances can be distinguished from other types of mesodermal cells. In general, the outgrowth of endothelium comes from vessels present in the explant, and only in the work of Rienhoff (1922) and of Murray (1932) was an actual dif-



Text-fig. 25. Drawing of a region of the upper wall of the vitelline artery vessels of a twenty-two-somite blastoderm cultivated *in vitro* without circulation. Complete dilation of the vessels took place, and the three dark areas represent intervascular strands. There is a tendency for the elongation of endothelial nuclei between these strands. $\times 360$.

ferentiation of endothelium observed, which in both cases assumed the form of hollow vessels. Rienhoff explanted fragments of embryonic metanephros, and obtained the differentiation of sinus-like vessels, while Murray explanted fragments of chick primitive streak, which underwent a differentiation into blood cells and vessels similar to that undergone by the primitive streak mesoderm *in vivo*.

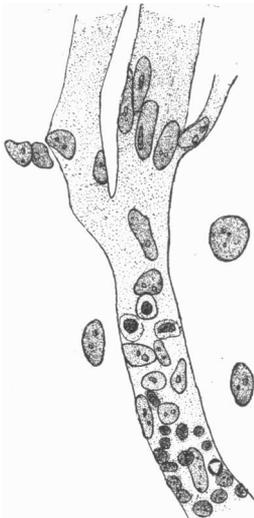
Where the outgrowth of hollow vessels is obtained as in the work of W. H. Lewis (1922), and of Scriba (1935), the form of the endothelium as hollow tubes is never indefinitely maintained, but the extension of the vessels in the zone of outgrowth leads to the breaking up of the lumen into isolated spaces, and finally to the outwandering of the individual endothelial cells in a fibroblast-like form (Ephrussi, 1930; W. H. Lewis, 1931; Scriba, 1935).

In the present investigation, fragments of area vasculosa were explanted at a stage subsequent to the differentiation of the vessel network, so when an outgrowth of vessels was obtained it had come from pre-existing vessels. The embryos used ranged from the primitive streak stage to that of twenty-two somites, and explants were taken from the area pellucida, or in one experiment from the area opaca.

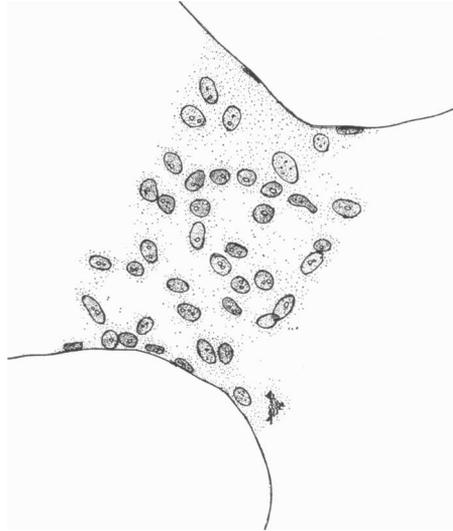
Out of eighty-four cultures, only seven showed outgrowing vessels in the form of hollow tubes. When the outer wall of the exocoel was not dissected off, as in the majority of the experiments, the culture formed a rounded cyst, the interior of which, when vessel outgrowth took place, became lined with a network of vessels similar to those of a whole area pellucida. Usually, however, comparatively little new formation of vessels occurred; the original vessels of the explant dilated as previously described in the case of explants of the whole area vasculosa in the absence of circulation, and finally all that was visible were the long intervascular strands which represent the intervascular spaces when complete dilation takes place.

In one experiment in which area opaca was explanted at the ten-somite stage, the outer coelomic wall was dissected off, and the endoderm spread out as a flat epithelium. In one culture out of ten, new vessels sprouted from those of the original explant forming a network of vessels over the surface of the endodermal outgrowth, as shown in Plate III, fig. 7. The vessels in this photograph are conspicuous owing to their heavy content of erythroblasts, formed presumably by multiplication of the cells of the blood islands of the original explant. The vessels in Plate III, fig. 7 have not dilated, for the outgrowth of vessel sprouts has been so extensive that the lumen has everywhere collapsed. The upper and lower walls of the vessel network are only separated by a layer of erythroblasts, many of which are degenerating. A drawing of the area (26) of Plate III, fig. 7 is shown in text-fig. 26; it can be seen that the large extension of the vessel network and the obliteration of the vessel lumina are reflected in the shape assumed by the endothelial nuclei, which have become very large, and tremendously elongated in the direction of the length of the vessel. Their ratio of length to breadth is, in some cases, as much as 4 : 1.

In another series of cultures which were made from fragments of the area pellucida of a twenty-two-somite blastoderm, extensive outwandering from the whole explant took place, and the mesenchyme surrounding the vessels migrated into the medium as cells with the typical form of tissue culture fibroblasts. On examination of the fixed culture it is seen that the vessels of the explant, which are identified by their content of degenerating blood vessels, no longer possess definite walls, but the nuclei of the cells bordering the lumen are elongated in the same direction as those of the surrounding fibroblasts. It appears that the endothelium has here differentiated into fibroblast-like cells.



Text-fig. 26.



Text-fig. 27.

Text-fig. 26. Drawing of the area (26) of the culture of area opaca of Plate III, fig. 7. The large nuclei within the vessel are endothelial, and the smaller darker nuclei are those of erythroblasts. $\times 400$.

Text-fig. 27. Drawing of a region of the upper wall from a vessel from a network in a cyst-like culture of area pellucida, in which the vessel lumina remained of normal dimensions. $\times 360$.

Where extensive outwandering of the vessels of the explant is prevented by the formation of a cyst, the vessels usually dilate eventually, but before this stage is reached normal tube-like vessels can be seen. Text-fig. 27 is a drawing of such vessels in a culture in which the lumen is still of moderate dimensions. It will be noticed that nuclear elongation in the wall at this stage is not extensive.

There is therefore considerable evidence that the form of vessels as uniform tubes is not indefinitely maintained in the absence of a circulation. De-differentiation of the endothelium does not necessarily follow the loss of the external form of a vessel, but where alteration of external form occurs then the shape of the endothelial nuclei reflects the changes which take place.

Nuclear elongation as an index of tension, and its effects on mitosis

It has been shown above that the alteration in the form of a vessel from that of a uniform tube of moderate diameter to one with a smaller or larger lumen is accompanied by elongation of the endothelial nuclei in particular directions depending on the nature of the change. In this section, evidence is considered which suggests that such nuclear elongation is an index of mechanical tensions acting on the cell.

Most of the evidence which has accumulated within recent years regarding the consistency of the living nucleus indicates that the nuclear contents are of a fluid nature, while the nuclear membrane is solid (Chambers, 1924; Gray, 1927). Consequently we should expect that a living nucleus would be deformable by physical forces. That endothelial nuclei can change their shape comparatively rapidly can be demonstrated by causing the vessels in a region of an area vasculosa to contract. A number of blastoderms were cultivated *in vitro* after removal of the embryo. The vessels dilated, with the result that the endothelial nuclei were comparatively far apart, and displayed no general distortion. Text-fig. 6 shows a part of such an area vasculosa. One of these blastoderms with dilated vessels was then injected with the Indian ink that was used in the vital injection method, which was described in the second section. The injection caused the vessels to contract, and the precipitation of the ink on the inner surface of the vessel wall rendered visible the condition of the vessel network as shown in Plate III, fig. 6. The vessels at injection were in the same condition as those shown in text-fig. 6, but began to contract a few minutes later. Thirty minutes after injection, the contraction had become more severe, and this condition persisted at fixation, ten minutes later. Text-fig. 7 is a drawing of part of this blastoderm under high power, and it can be seen that there has been a general tendency for the endothelial nuclei to elongate. Now when the endothelium contracts it does so in an isometric manner, since the distance between the mid-points of the intervascular spaces remains approximately the same, and the tension developed within a mesh of the vessel network may be considered proportional to the length. Where the circumference of a contracted vessel is less than the length, we should expect that the lines of maximum tension will be along the vessel.

Now nuclear elongation is uniformly along the axis of the vessel in the longer meshes of the network, as shown in text-fig. 7, and the only sites where elongation is not uniform are at the nodes where several vessels meet. This is precisely what would be expected on the hypothesis that nuclear elongation is an index of tension.

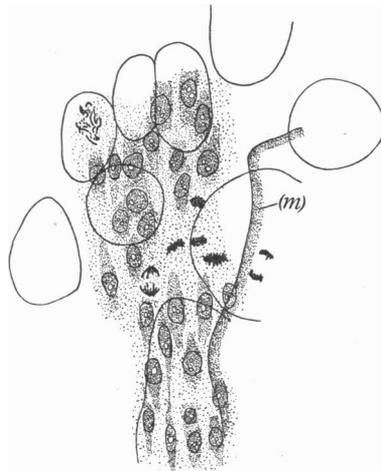
The next question to be considered is whether there is any effect on the orientation of the mitotic spindles in an area of endothelium or epithelium in which the resting nuclei show a general elongation in one particular direction. For this purpose, the area vasculosa of a blastoderm cultivated *in vitro* without circulation will not serve, for there is very little mitosis in the endothelium. In

early development, the most striking example of a tissue in which both general nuclear elongation and mitosis occur simultaneously is the endoderm of the area pellucida of blastoderms *in ovo* during the formation of the first ten somites. Here, nuclear elongation at the edge of the area pellucida in a direction parallel to the border of the area opaca is a very marked feature, and the evidence for the influence of this elongation on the direction of the mitotic figures is presented in the accompanying Table II. That the area pellucida tends to be under a state of tension is indicated by its behaviour when a portion of a blastoderm is explanted *in vitro*, when contraction takes place along a cut edge of the area pellucida.

Nuclear elongation in the pellucida endoderm is much more marked at the margin than near the embryo, where the endodermal nuclei are generally rounded. Where in the margin the elongation is generally greatest, there the axes of the division spindles are almost exclusively in the direction of the elongation. Such an area is illustrated in text-fig. 28. The elongation of the non-dividing cells is seen to affect not only the nucleus but also the cytoplasm, which is much denser opposite the two ends of the extended nucleus. Where elongation is less pronounced, the number of mitotic spindles in line with the general elongation still predominates, but a certain proportion are at right angles to the direction of elongation. In Table II the dividing nuclei are classified under three headings, apart from prophases which although usually extended in the direction of elongation, offer no certain indication of the direction of the future spindle. The three categories depend on the angle at which the spindle is orientated with relation to the margin of the pellucida.

From the results given in Table II, it can be seen that in areas of general nuclear elongation, the number of mitotic spindles within 15° to either side of the general direction of elongation is nearly twice the number of those occupying the remaining 150° of the half circle. There can therefore be no doubt that nuclear elongation influences the direction of the mitotic spindle in these particular tissues, and presumably also in all unilayered pavement epithelia.

A correspondence between nuclear elongation and the direction of the mitotic spindle is also seen very clearly in the fibroblast-like cells which outwander into the medium from an explant of embryonic chick heart, when a speeded-up cinema film of such a culture is observed. This correspondence has



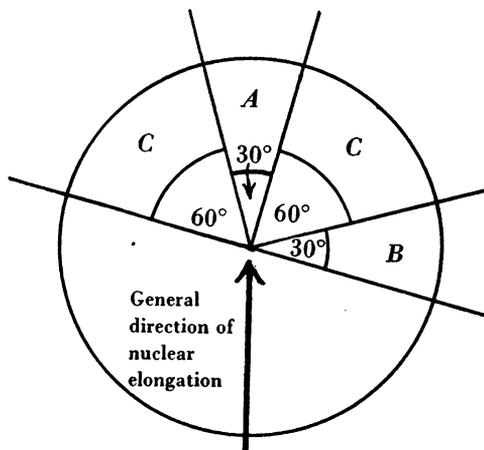
Text-fig. 28. Drawing of a region of the endoderm of the area pellucida of a seven-somite blastoderm at the boundary between area pellucida and area opaca. The outlines are those of spaces within the mesenchyme dorsal to the endoderm. (m), area opaca margin. $\times 400$.

been incidentally investigated by Paul Weiss in his work on the cultivation of fibroblasts on a film of medium coagulated across a small triangular or oblong frame (1929, 1933). In such cultures, the outgrowth is most abundant along certain directions, which, according to Weiss, are the lines along which the micellae of the plasma coagulum are orientated by the tensions set up within

Table II.

Nuclear Elongation and Mitosis

The mitotic figures in areas of nuclear elongation are classified into four groups; prophases, and later stages according to the axis of the spindle into the groups (A), (B) and (C) as follows:

*The Area Pellucida Endoderm*

Nuclear elongation is seen at the opaca border in a direction parallel to the boundary between pellucida and opaca.

Blastoderm	Prophases	A	B	C
3 somites	7	22	7	8
7 somites (text-fig. 27)	17	45	12	21
11 somites	4	25	6	5

The Vessels of the Area Pellucida

At ten somites onwards, the vessel network in the area pellucida extends as a whole with the embryonic axis, and nuclear elongation is seen in the endothelium parallel to the axis.

	Prophases	A	B	C
Vessels in the pellucida in an area opposite somites five to eleven in an eleven-somite blastoderm (text-fig. 8)	1	18	4	8

the coagulum either during clotting, or by the weight of the culture, or by the abstraction of water by the culture. Where outgrowth is most luxuriant, the long axes of the fibroblast nuclei all tend to be orientated in the same direction as that of the general outgrowth, and when division takes place, the mitotic spindles are similarly arranged. Nuclear elongation tends to be more pronounced in the orientated region, and the mitotic figures more abundant.

Weiss explains his results not as the result of tension acting directly on the cells themselves, but as the result of their tendency to grow along orientated protein micellae. The pattern of the culture reflects the invisible pattern of the "ultrastructures" within the plasma film.

The main importance of Weiss' results in relation to the present study is his observation of the correlation between nuclear elongation and the direction of the spindle at division. His explanation, however, although possibly correct for his cultures, can hardly be applied to such a case as the area pellucida endoderm, where a whole circular tract of epithelium undergoes a general extension. In these endodermal sheets we are far more likely to be dealing with the effect of tension on the cells themselves.

In prevascular "einreihige Zellsträngen" (Rückert and Mollier, 1906), there is elongation of the nuclei in the direction of the whole strand, and the axes of the mitotic spindles also lie in this direction. In the vessels opposite the middle somites at the ten-somite stage, when extension of the whole network in this region keeps pace with that of the embryonic axis, many of the endothelial nuclei are elongated parallel to the embryonic axis, and the endothelial mitotic figures show a strong tendency for a similar orientation. In Table II, a count is given of the mitoses in the specified region of the area pellucida of the eleven-somite blastoderm of which Plate II, fig. 6, is a photograph, and text-fig. 2 a high-power drawing of the area (b).

Where embryonic vessels elongate, therefore, the orientation of the endothelial mitotic figures tends to correspond with the direction of the elongation of the resting nuclei. But the present study is primarily concerned with arterial differentiation within the vessel network, and we must therefore enquire whether the nuclear elongation within the differentiated artery has a similar influence on the endothelial mitotic spindles.

In ovo, when the differentiation of the artery reaches the stage shown in text-fig. 20, at which the dorsal venous plexus is in process of formation and the outline of the artery is becoming regular, nuclear elongation for the first time becomes a marked feature of the endothelium, and at the same time mitotic figures increase in number. Although many of the endothelial nuclei are elongated at right angles to the axis of the vessel, others are elongated in other directions, and there is no general preponderance of mitotic spindles at right angles to the axis of the vessel.

If it is true that the nuclear elongation which accompanies the differentiation of the vitelline artery is due to physical forces generated by the circulation, the wall of the vitelline artery should be under tensions which would be relieved if the flow of blood were interrupted. Experiments were therefore made in which the whole posterior region of an area pellucida was explanted at the stage of thirty somites, when there is a fully differentiated dorsal venous plexus over the proximal part of the vitelline artery; it was found that whereas the vessels of the dorsal venous plexus dilated as do other vessels of the area vasculosa under similar conditions, the vitelline artery did not dilate, but slowly contracted to a

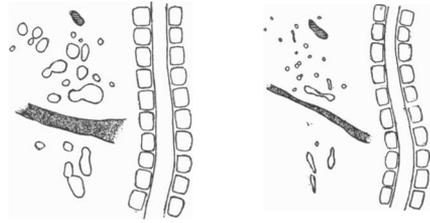
thin cord, which was made visible by reason of its content of blood cells. Text-figs. 29 and 30 are camera lucida drawings of a living explant of the hinder part of an area pellucida at thirty somites and Plate I, fig. 5, is a photograph of a section through the explant, which was fixed ten minutes after text-fig. 30 had been drawn. The section shows that much degeneration took place in the blood cells within the artery, and in the mesenchyme round the vessels, but not in the endothelium itself, or in the walls of the extra-embryonic coelom. As far as can be judged from sections, the nuclei in the wall of the vitelline artery are no longer elongated. The dilated venous plexus is seen dorsal to the contracted artery.

If the hinder part of the area pellucida is explanted at an earlier stage, either before the process of isolation of the artery is completed or when the isolated artery is still irregular in outline, dilation occurs, and not contraction, as in the case of the regular artery in which nuclear elongation and mitosis are taking place in the wall. Correlated with the nuclear changes in the wall of the artery is a change in the reaction of the whole vessel to the cessation of the blood flow.

If we apply the results shown in Table II to the vitelline artery at twenty-six somites, and assume that when an elongated nucleus divides, the axis of elongation and the axis of the spindle will correspond, we shall be postulating a mechanism for the relief of tensions within the endothelium which will give rise to a directional growth. The tensions are set up within the endothelium by the fluid friction generated by the circulation, and directional growth will mould the form of the vascular system to that which will offer minimal resistance to the flow of blood, which is the case in a fully formed arterial system, as Roux concluded.

Thoma, however, inclined more to the view that nutritional factors were those responsible for the first phases of arterial growth, for the reason that he could see serious objections to ascribing arterial growth to the influence of fluid pressure. Certain equally serious objections to his nutritional hypothesis are mentioned above, and since Roux has shown that fluid friction is the main force at work in the later functional differentiation of the vascular system, it is natural to enquire whether this force does not operate at earlier stages of vascular development.

Many points will have to be investigated further in such an inquiry. In particular, the exact relation between the operation of chemical and physical factors in causing mitosis must receive attention. It is obvious that tension



Text-fig. 29.

Text-fig. 30.

Text-figs. 29 and 30. Drawings of the vitelline artery area of a thirty-somite blastoderm, in which the posterior region was isolated and cultivated *in vitro*. The vessels of the dorsal venous plexus dilated, while the vitelline artery contracted. Text-fig. 29 was drawn seven hours after explantation, and text-fig. 30 ten and a half hours after. The vitelline artery is shaded. $\times 24$.

acting on a cell cannot be regarded as the sole cause of mitosis, for nuclear elongation can be found in areas where no cell division takes place, and, in general, cell division is known to be stimulated by chemical factors. It may be that when cell division appears in the vitelline artery there is a change in its chemical environment which induces cell division; it is interesting to notice in this connection that the period when mitoses are first abundant within the artery is also the period when cell division in the general network occurs in response to the increase in size of the area vasculosa. One further point in relation to the special problems of the form of the vascular system is whether nuclear elongation can always be regarded as an index of tension, in particular after the development of the tunica media, when the endothelial nuclei of the arteries are all elongated in the direction of the length of the vessel.

That the physical aspects of the blood stream are important in moulding the shape of the first differentiated artery is strongly suggested by the alteration in cross-section which the artery undergoes during the first day after the beginning of the circulation. The change is from the flattened and somewhat irregular cross-section seen in Plate I, fig. 4, to the uniformly circular one seen in Plate I, fig. 6, which is taken from the vitelline artery of an embryo of thirty-three somites, twenty-seven hours after the circulation began.

VI. SUMMARY OF RESULTS

1. There are two periods in the differentiation of the area vasculosa. First, a period of self-differentiation before the circulation begins during which a more or less uniform network of vessels develops, and secondly, a period of functional differentiation during which arteries and veins differentiate within the network. This paper deals only with the first example of visible functional differentiation in the area vasculosa, namely the differentiation of the vitelline artery.

2. In the period of self-differentiation, the path along which the blood will mainly flow can be recognised some hours before the flow actually begins. This path is made up of vessels of larger calibre than elsewhere in the network, and when the flow begins, offers less frictional resistance than other alternative paths.

3. Vessel formation in the extra-embryonic mesoderm begins with the differentiation of isolated vascular spaces, which enlarge and then join up to form the vessel network. Where the first vessel spaces are far apart, they are joined by means of outgrowths from the spaces known as "sprouts". In this way a network of vessels of small calibre is formed.

4. Embryonic vascular endothelium is a contractile tissue; in general it dilates when small stimuli are applied, and contracts when strongly stimulated. When blastoderms are explanted *in vitro*, a dilation of the vessels takes place before the circulation begins, and persists should circulation not occur. One effect of the circulation *in vitro* is to stimulate the vessels to contract.

5. When vessels fuse, two layers of endothelium come into contact, and

are then resorbed. This is seen in the fusion of the first isolated vessel spaces, in the fusion which takes place when dilation *in vitro* persists, and where vessels fuse together in the formation of the artery. In the two last examples, the process leads to the disappearance of round intervacular spaces within a vessel network.

6. When circulation occurs *in vitro*, the process of differentiation of the vitelline artery can be followed. After six hours' circulation, the vessels outside the area in which the artery will form are less dilated than they would be if circulation had not occurred, but no other change has yet taken place.

7. The artery is differentiated after nine hours' circulation *in vitro*. It is formed proximally from a tract of the vessel network which, as shown by the intervacular spaces within it, is two or three network meshes in width. The differentiation is mainly effected by the disappearance of the connections between the differentiating artery and the smaller vessels to either side. This process is spoken of as isolation.

8. The vessels of the area vasculosa *in vitro* are more dilated than *in ovo* during the period before the differentiation of the vitelline artery. The vessels of the region in which the vitelline artery will differentiate are more dilated than those elsewhere in the area vasculosa both *in ovo* and *in vitro*. The vitelline artery is isolated at the same time after the beginning of circulation both *in ovo* and *in vitro*.

9. Isolation can take place either by the contraction of the vessels adjacent to the differentiating artery until the lumen is obliterated, or by the formation of a septum across the opening of the adjacent vessel into the artery.

10. The vitelline artery *in vitro* is differentiated from a tract of dilated vessels, and is large in calibre at isolation: no growth in diameter takes place in the next few hours of circulation. The vitelline artery *in ovo* is formed from less dilated vessels, and intervacular spaces are still seen within it after isolation. The whole artery then begins to grow in calibre, and becomes regular in outline.

11. The vessels to either side of the artery which are cut off from connection with it become venous in function. They form part of a plexus of vessels running dorsal to the vitelline artery, which returns blood to the heart from the hinder region of the area vasculosa. The formation of the plexus dorsal to the vitelline artery is preceded by a differentiation of isolated vessel spaces within the mesenchyme, which join up with the severed vessels on either side of the artery. When no circulation takes place, the isolated vessel spaces dorsal to the vitelline artery differentiate, but do not join up with other vessels.

12. *In vitro*, the layer of mesenchyme surrounding the vessels is much thicker than *in ovo*. This is due partly to the presence of a larger number of cells, but chiefly to a greater accumulation of intercellular fluid. The relations *in vitro* between the amount of intercellular space in the mesenchyme and the degree of dilation of the vessels show that when the vessels contract, fluid passes into the surrounding mesenchyme.

13. In the early pre-vascular mesenchyme, mitotic figures are very abun-

dant, and with the differentiation of the vessel network, the relative number of dividing cells steadily decreases. During the first few hours of circulation, mitosis is at a minimum throughout the vessel network, but as the vitelline artery differentiates, mitotic figures reappear in the wall. They become much more numerous in the artery *in ovo*, where a rapid growth in calibre takes place.

14. When vessels increase in length, this increase is not at first accompanied by endothelial cell division. The increase in circumference of the area vasculosa causes an increase in length of the individual meshes of the network, and not until the twenty-six-somite stage does compensatory mitosis in the general network take place.

15. When a vessel increases in length, and contracts in diameter, the endothelial nuclei elongate in the direction of the axis of the vessel. When the artery has differentiated and is increasing in diameter, many of the endothelial nuclei elongate, and are frequently orientated at right angles to the axis of the vessel.

16. There is evidence to show that elongation of a previously rounded nucleus may be caused by tensions acting within the cell. There is also evidence that when an elongated nucleus divides, the direction of the axis of the mitotic spindle tends to correspond with that of the elongation in the resting phase.

17. In virtue of this relation between tension, nuclear elongation, and mitosis a mechanism is suggested whereby the growth of an artery in its first stages of development would tend to decrease the resistance offered to the passage of the blood stream. At those points along the artery where the fluid friction generated by the circulation was greatest, excessive tensions would be set up within the wall, which would be manifested by nuclear elongation within the endothelium. When cell division took place, the directional increase in area of the endothelium under tension consequent on the orientation of the mitotic spindles would tend to relieve these tensions.

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EXPLANATION OF PLATES

PLATE I

- Fig. 1. Photograph of a twenty-two-somite blastoderm, cultivated *in vitro*, and vitally injected. The injection was made into the left side of the blastoderm, and has caused the vessels in that area to contract, as is seen anterior to the head of the embryo. $\times 22$.
- Fig. 2. Photograph of the same blastoderm as that of fig. 1, eight hours later. The embryo now has twenty-eight somites, and the differentiation of the vitelline artery is progressing. $\times 22$.
- Fig. 3. Section through the right vitelline artery of a twenty-seven-somite blastoderm cultivated *in vitro*, to show the thickness of the mesenchyme layer dorsal to the artery, in which isolated vascular spaces such as (v_1) are to be found. The plane of section is indicated in text-fig. 16, a reconstruction of the whole vitelline artery area. $\times 180$.
- Fig. 4. Section through the right vitelline artery of a twenty-six-somite blastoderm *in ovo*, to compare with the corresponding artery *in vitro* of fig. 3. The mesenchyme layer above the artery is much more compact. $\times 190$.
- Fig. 5. Section through the vitelline artery area of a thirty-somite blastoderm, in which the posterior region was isolated and cultivated *in vitro*. The vessels of the dorsal venous plexus have dilated, while the vitelline artery has contracted. Much degeneration has taken place in the erythroblasts within the vessels, and in the surrounding mesenchyme, but not in the endothelium. $\times 300$.
- Fig. 6. Section through the area pellucida of a thirty-three-somite blastoderm *in ovo*, in the region of the vitelline artery, and parallel to the embryonic axis, to show the change in form of the vitelline artery from a flattened (fig. 4) to a rounded cross-section. The artery is the more ventral vessel, dorsal to which is the venous plexus. $\times 74$.

PLATE II

- Fig. 1. Photograph of a blastoderm of nine somites, cultivated *in vitro*. The vessels of the area vasculosa are not yet fully communicating. The position of the first somite relative to (*g*) in this, and the succeeding four photographs shows the forward migration of the somites. $\times 23$.
- Fig. 2. Photograph of the same blastoderm as that of fig. 1, 2 hours 20 minutes after fig. 1. The embryo now has ten somites. $\times 23$.
- Fig. 3. Photograph of the same blastoderm as that of fig. 1, 3 hours 35 minutes after fig. 1. The embryo now has eleven somites. The area vasculosa consists largely of fully differentiated vessels, which have been formed within the last hour by the fusion of separate vascular spaces. The vitelline artery area behind the somites consists of vessels of larger calibre than elsewhere. $\times 23$.
- Fig. 4. Photograph of the same blastoderm as that of fig. 1, 4 hours 30 minutes from fig. 1. The embryo now has twelve somites. Further differentiation of vessels has taken place. $\times 23$.
- Fig. 5. Photograph of the same blastoderm as that of fig. 1, taken 13 hours 50 minutes later. The embryo now has thirty somites. The circulation failed to take place at the appropriate time, and the vessels progressively dilated. The dark spots represent all that remains of the intervascular spaces. $\times 23$.
- Fig. 6. Photograph of blastoderm of eleven somites. The vessel network is differentiated caudally as far as the vitelline artery area. The three areas (*a*), (*b*) and (*c*) are drawn on a larger scale in text-figs. 1, 2 and 3 respectively. $\times 32$.

PLATE III

- Fig. 1. Photograph of the left vitelline artery area of an embryo of seventeen somites *in ovo*. The circulation has been in progress for one hour. $\times 37$.
- Fig. 2. Photograph of the same vitelline artery area as in fig. 1, 50 minutes later. The intervascular spaces have been outlined. $\times 37$.
- Fig. 3. Photograph of the same vitelline artery area as in fig. 1, 2 hours 10 minutes later. Isolation of the vitelline artery is beginning, and the vessels to either side of the future artery are contracting. $\times 37$.
- Fig. 4. Photograph of the same vitelline artery area as in fig. 1, 3 hours 10 minutes later. Isolation of the artery is more advanced on its cranial margin. $\times 37$.
- Fig. 5. Photograph of the same vitelline artery as in fig. 1, 5 hours 45 minutes later. The artery is now isolated on the caudal margin. $\times 37$. The final stage reached by this area, 12 hours after the circulation had begun, is shown in text-fig. 19.
- Fig. 6. Photograph of a blastoderm cultivated *in vitro* without the embryo, in which the vessels were made to contract by the injection of Indian ink. $\times 24$. A drawing of a small area of this blastoderm under high magnification is shown in text-fig. 7.
- Fig. 7. Photograph of a culture made by explanting a fragment of area opaca at the ten-somite stage. A network of vessels has been formed, which are shown up by their content of erythroblasts. $\times 20$. The rectangle encloses the area drawn at higher magnification in text-fig. 26.

LIST OF ABBREVIATIONS

- d.v.p.* Dorsal venous plexus.
e.c. Extra-embryonic coelom.
g. Yolk granule on the surface of the blastoderm.
*i.v.s.*₁ Intersvascular space in the vitelline artery area, small in size from its first differentiation.
*i.v.s.*₂ Intersvascular space in the area opposite the middle somites, large in size at its first differentiation.
*i.v.s.*₃ Intersvascular space in the area opposite the middle somites, small in size after the dilation of the vessels.
*s*₁ First somite.
*s*₄ Fourth somite.
*v*₁ Isolated vessel space above vitelline artery, identical with (*v*₁) in text-figs. 16 and 18.
v.a. Vitelline artery.

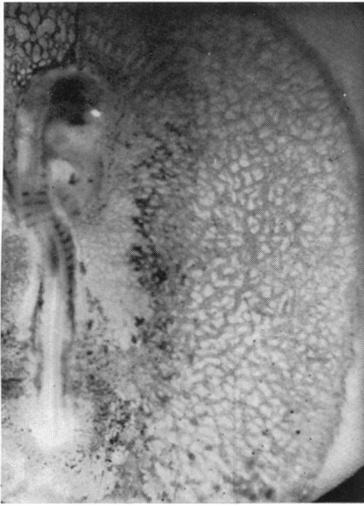


Fig. 1.

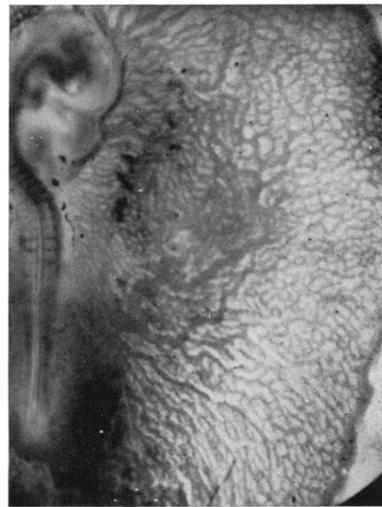


Fig. 2.

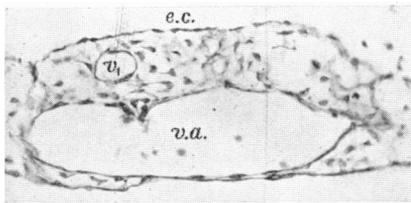


Fig. 3.

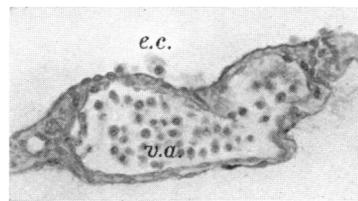


Fig. 4.

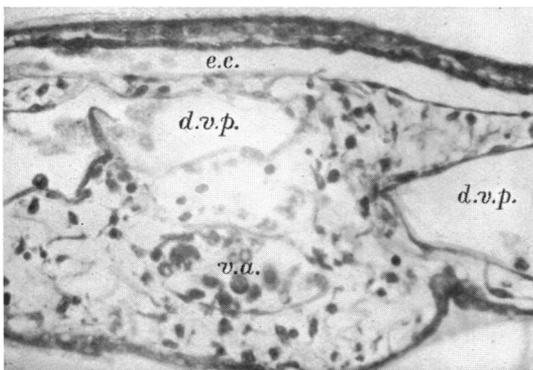


Fig. 5.

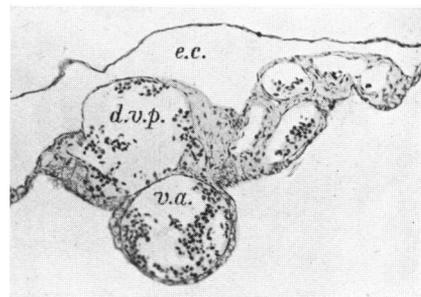


Fig. 6.

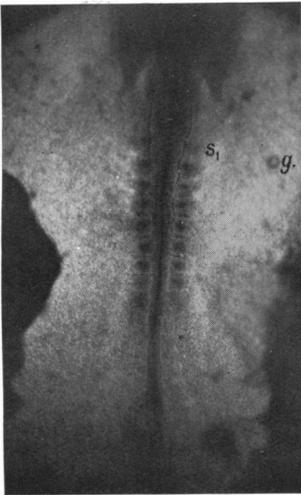


Fig. 1.

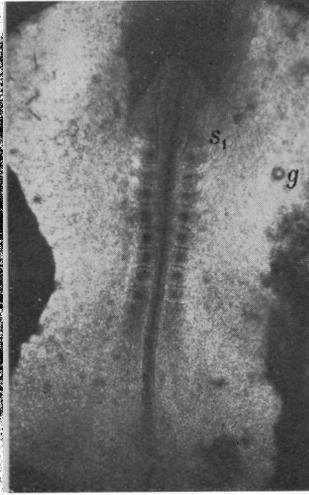


Fig. 2.

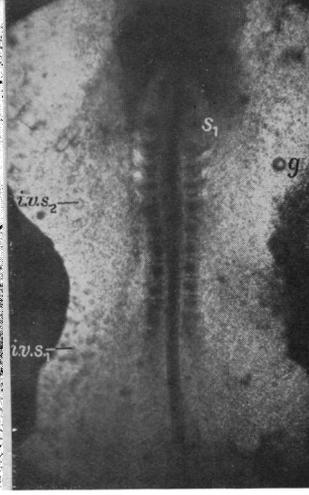


Fig. 3.

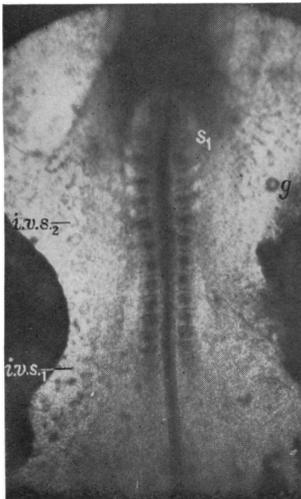


Fig. 4.



Fig. 5.

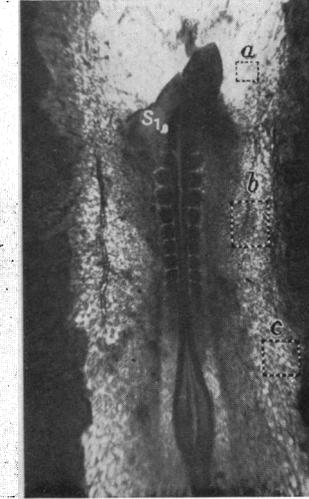


Fig. 6.

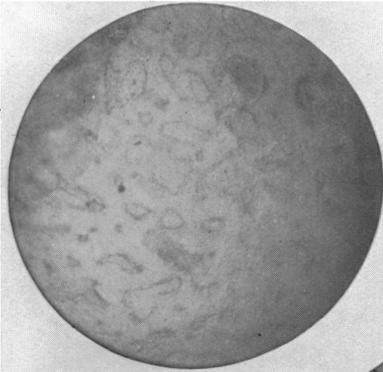


Fig. 1.

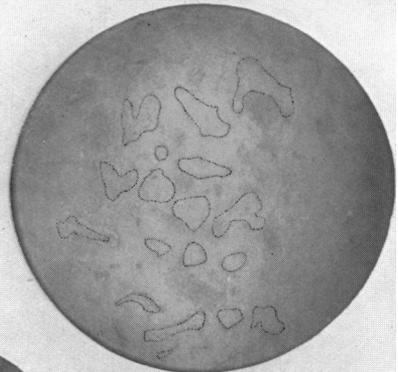


Fig. 2.

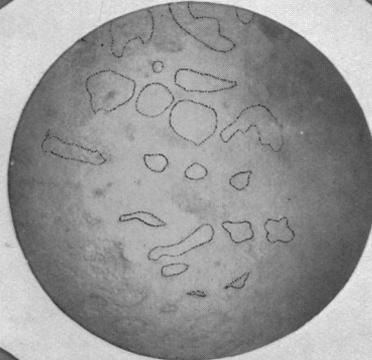


Fig. 3.

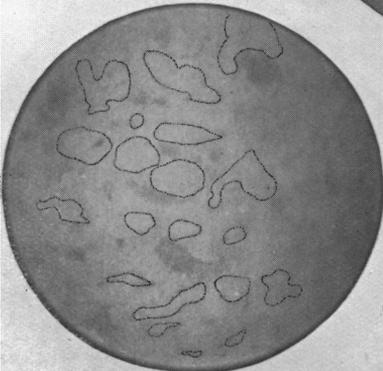


Fig. 4.

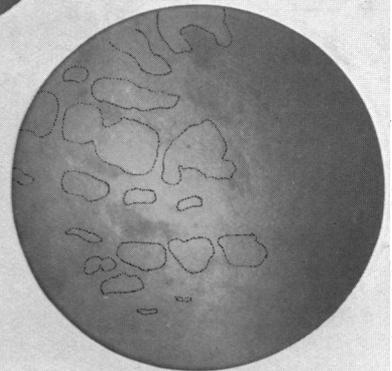


Fig. 5.

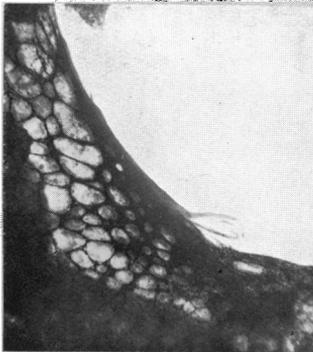


Fig. 6.

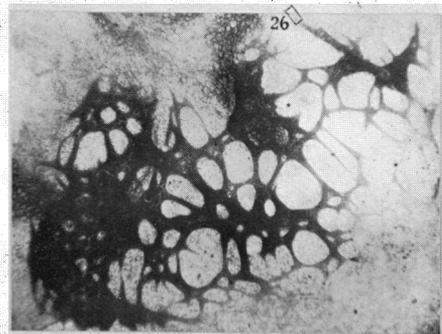


Fig. 7.