STUDIES ON THE BLOOD VESSELS IN THE MEMBRANES OF CHICK EMBRYOS

PART I. ABSENCE OF NERVES IN THE VASCULAR MEMBRANE

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PLATE 5

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Experiments on the behavior of the blood vessels have with few exceptions been confined until now to tissue in which there are nerves. In all the organs of the body in adult human beings or in animals that have attained full growth, in which such investigations have taken place, the presence of vasomotor nerves has been demonstrated either anatomically or physiologically (1).

It is to be expected that in investigations of the reaction of blood vessels in tissues or an organ which is not innervated, the part usually played by the nervous factor can be ascertained. Experiments carried on in such preparations might make possible in a new way the elucidation of questions having clinical interest, as for instance, those arising in arterial hypertension (2) in which hypersensitivity of all vessels is present as well as in arteriosclerosis (3) in which the level of irritability is reduced and finally, in cases of inflammation (4, 5) in which the part played by the nervous system is still the subject of discussion.

On the Absence of Nerves in the Yolk-Sack of Chick Embryos

Concerning the occurrence of nerves in the membranes of chick embryos there exist in the literature, so far as we know, no references (6). There are, however, numerous investigations on the innervation of membranes of human beings and other mammals to which we intend to refer by way of comparison. The question whether the umbilical cord and the placenta contain nerves dates according to Schott (7) from the time of Galen. Reports of more recent investigations, to the

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literature of which Schmitt (8) has referred, and which are based in part on histological investigation carried out in tissues studied mainly by the method of impregnation with silver, and in part on pharmacological methods, contain the conclusion that the umbilical cord contains nerves of its own but only in that portion close to the body, and that its greater distal portion as well as the placenta are free. Ikeda (9) recently, at the suggestion of Aschoff, has reinvestigated this matter and has confirmed the statements of earlier authors.

We have attempted to solve the question as to whether the membranes of chick embryos contain nerves in part by physiological, in part by histological investigations.

Physiological Experiments

Technique.—The experiments were conducted in a constant temperature chamber at 38°C. Sources of error due to fluctuation of temperature are therefore avoided. Eggs in all stages of development, but mostly those 3 to 4 days old, were studied. In older eggs the separation of the membranes is more difficult but can, after practice, be conveniently managed. Eggs were opened at the air-chamber end without turning or jarring them, as much of the shell as was necessary to expose the vascular region being removed by means of forceps. The embryo surrounded by sufficient of its membranes could by this means be exposed. The loss of albumen was, of course, prevented. The manipulation of the egg on opening it must be gentle; otherwise the blood vessels will be found even at the beginning of the experiment to be contracted, so that weak stimuli would lead, while they are in this state, to erroneous inferences.

For electrical stimulation the usual form of a reliable inductorium was used. The source of current was an Edison storage battery. When drugs were injected the point of the needle was inserted laterally until it came to lie under the membranes, since preliminary experiment showed that better and more uniform absorption took place than if these substances were applied drop-wise to the surface of the preparation. The experiments were brief, never lasting longer than 10 minutes so that errors due to desiccation might be avoided. The observations were made with a Zeiss dissecting microscope furnished both with binoculars and bin-objectives. The embryos were protected from excess heat by placing a water filter between the source of light and themselves.

We observed the behavior of blood vessels of the yolk membrane in some 1600 eggs at various ages. The experiments which we report now, selected from the great number which were performed, permit our making the following statements concerning the innervation of these vessels.

1. One locus of the membrane was touched several times with a fine

glass rod. Depending on the number of contacts and their strength, either constriction or dilatation took place. These stimuli brought about this effect both in capillaries and in small arteries. Even the strongest stimulus of this mechanical sort brought about no change in the immediate environment. The effect of the stimulus was strictly confined, wherever it was exercised, to the site which was stimulated.

2. A faradic current was employed to stimulate a given locus of the vascular layer, electrodes of varying diameters being used. Both the smallest arteries and the larger arterial branches were stimulated. One electrode consisted of cotton wool moistened with Ringer's solution which was placed on the membranes at a distance from the vascular zone, that is to say, beyond the marginal vein. The other electrode had the diameter of a hair. As the result of the stimulus, dilatation occurs either in a capillary or at one point of the artery, but is so small in extent as to be recognized only microscopically. If an electrode having a larger diameter is used the effect is apparent in a larger area. In all cases the effect of stimulation, whether it be contraction or dilatation, is to be recognized only at the point at which the stimulus is applied or, when the strongest current in our scale was used, in the immediate neighborhood. Involvement of neighboring regions or a propagation from the capillaries to larger arteries, or propagation in the reverse direction was never noticed.

The following experiment was made with the view to extending these observations and to detecting the possible existence of conduction over nerve paths:

The central end of an artery which led to a vascular area which we wished to stimulate was tied off and the whole vascular region was isolated by a circular incision so that every connection with the embryo was severed. In this isolated vascular region electrical stimuli were made just as before. The result of stimulation was the same as when the region was still in continuity with the embryo. On account of the absence of a circulation, that is to say of additional blood, the column of blood in the vessel when it was dilated, the degree of dilatation depending, of course, on the strength of the stimulus, appeared to be paler than before. Measurement of the diameter microscopically indicated, however, the same degree of dilatation as when the connection with the embryo was intact.

3. A fine hollow needle was inserted outside the vascular area and then pushed forward so that its opening came to lie beneath it.

Sodium iodide solution 6 per cent was injected in the amount of 0.1 cc. Dilatation of the vessels occurred at once but only at the point of injection. If larger amounts were injected a larger region was affected. If the same amount of saturated solution of sodium iodide is injected, stasis in the region of the drop immediately occurs. On account of the stagnation of blood which results, blood accumulates in the portion of the vessel central to this point. This appearance quite obviously represents a secondary phenomenon. The effect of the stimulus itself remains confined to the site of the stimulus. It appears to be unnecessary to report our experiments with other chemicals. The result was always the same—the stimulus affected merely the locus at which it was applied. If larger amounts were injected there was always the possibility that after absorption of the substance, and distribution in the circulation, a result would occur at points far removed from the point of stimulation and would there give the impression that a stimulus had been applied. But when small amounts are used and are confined to a small area the effect of the stimulus takes place only at the point of stimulation.

These observations differ from those of blood vessels which are known to be innervated. In the skin there occurs with every stimulus of low intensity an easily demonstrated erythema, an effect of the stimulus which can be seen far beyond the point of stimulation (Ebbecke (10), Lewis (11)). Reactions like this can be understood only as the result of an action in which the nervous pathways are involved. In the pancreas of rabbits for instance, Ricker and Regendanz (4) found that the effect of strong stimuli involved a larger region than the point stimulated in contrast to that of weaker ones, the effect of which was merely local. In such cases the result is brought about by a reflex (nervous) action. In our experiments we could never bring about an effect beyond the region of stimulation even if strong currents were used. The restriction of the reaction of the blood vessels in the vascular areas in our preparation to the site of the stimulus suggests strongly that there are no nerves in the vascular layer of these embryos.

Among the drugs that have been tested which have an effect upon the blood vessels adrenalin is the only one which has an action conspicuously different from the ordinary on the vascular area of these membranes.

Adrenalin, in a solution of 1 per mille, was injected through a needle at a point just under the vascular area since it had been learned that adrenalin applied to the surface had no effect even when large amounts were used. 0.1 cc. of a 1 per mille solution brought about no visible contraction. It was not until an amount of 0.4 cc. or more had been injected that a contraction of the blood vessels took place, but even then the effect was never immediate. Indeed the earliest evidence of action was observed only after ½ minute. In most experiments contraction did not begin until the end of 4 to 5 minutes.

But even large doses like those just mentioned were not effectual in all cases. Of 38 observations, constriction took place in 25, in 4 the effect was uncertain, and in 9 there was no effect at all. Because of the weak action of the drug we were obliged to use relatively speaking unusually large amounts of fluid, 0.5 cc. or more. Control experiments with equally large doses with physiological salt solution of about the same hydrogen ion concentration, had as a matter of fact a very similar result. We are inclined to attribute the effect to the solvent therefore rather than to adrenalin. Even if we were to attribute the effect observed to adrenalin, the extent of it was remarkably small.

The contrast between this result and that in tissues which are normally innervated is striking. For in the latter it is easy to obtain a reaction everywhere by the local application of 0.1 cc. of a 1 per cent solution. The small effect in the vascular area of our preparations is accordingly altogether unusual.

Results similar to ours have been reported by Schmitt (8) in the case of blood vessels of the placenta which were treated by a perfusion method. If into the blood stream of the perfused placenta 1 cc. of suprarenin in the concentration of 1 to 1000 was injected, no effect was observed on the rate of flow in the vessels. Since it is generally recognized that the action of adrenalin takes place by virtue of its effect on nerves or on mechanisms of a nervous nature, the small effect of adrenalin on the vessels of the vascular membrane of chick embryos may be taken to represent further evidence that a nervous mechanism is wanting in these structures.

Anatomical Evidence

We have examined the embryonic membranes of chicks by means of a large variety of histological methods using the mesentery of rabbits as well as other organs, amongst them the skin, heart and lungs, by way of controls. Our best results were obtained by the use of Bielschowsky's method of silver impregnation in block, and also with the rongalite white method.

1. Investigations with Bielschowsky's Method.—We impregnated by Bielschowsky's method, 6 embryos and their membranes aged 3, 4 and 7 days, according to the recommendations of Schmorl (12), and cut them in serial sections. In the case of older embryos only single sections of the membranes were made. As early as 72 hours after the beginning of incubation, small, fine nerves were found in the region of the brain and in the ganglia of the head. At the end of 96 hours nerves could be found everywhere in the brain and spinal cord as well as in the spinal ganglia (Fig. 1). At the end of 7 days nerves were to be found in all parts of the embryo.

Contrary to the situation in the embryo itself, nerves were to be found nowhere in the membranes. In those sections in which the transition from embryo to membranes could be clearly seen it was apparent that the nerves were confined to the structure of the embryo and did not proceed over to the membranes, as for example in a specimen 7 days old (Fig. 2, b). Small single fibrils were seen stretching beyond the region of the lateral limiting sulcus (Fig 2, a) but in no section could they be traced to the membranes themselves. The last of the nerves found in Fig. 2 is shown by means of greater magnification in Fig. 3.

2. Investigations with the Rongalite White Method.—Whereas the silver impregnation method according to Schmorl's description gave always completely satisfactory results, difficulties were encountered at first with the rongalite white method. The only investigators who seem to have used this method have been Kreibich(13) and Glaser (14, 15). They succeeded even in cases where other methods failed in obtaining first-rate results both with intra- and supra-vital staining. Since an intra-vital technique was impossible on account of the small size of the objects, we have used the supra-vital method only. The mesenteries of rabbits were again used as controls. Since both the authors just mentioned have described the method in a cursory way only, we have decided to describe it in detail, according to the procedure which gave us the best results.

Rongalite white, which is methylene blue reduced by means of rongalite, was prepared in the following way: 1 gm. of pure methylene blue was dissolved in 200 gm. of distilled water, was heated, and then 6 gm. of rongalite and 15 drops of hydrochloric acid were added. This solution was boiled until it became clear and was then filtered. Staining was carried out in the following way. Organs were taken from the embryo immediately after death and were bathed in physiological salt solution. Rongalite white 5 per cent to 10 per cent was next added. After pieces of tissue had been stained for 45 minutes they were fixed in ammonium molybdate 5 per cent. After thorough washing they were placed in absolute alcohol and xylol and were then drawn up on to glass slides.

By this method we examined the membranes of 19 embryos of 3, 4, 8, 9, 10, 13, 18 and 20 days' incubation after they had been stained and a control provided for each membrane. Whereas in the mesentery, nerves could be beautifully demonstrated everywhere, none could be found in any of the membranes.

DISCUSSION

It appears then from our histological investigations that we were unable by means of the two best methods available to us to detect nerves in the membranes of the chick embryo. Neither did we find primitive cell processes which might be regarded as undifferentiated nerves. Our results therefore are in complete agreement with those which have been published in the case of the placenta both of man and of other mammals.

Is the conclusion to be drawn that the membranes contain no nerves? The objection may be made that since we are dealing with embryonal tissue we should not expect to find fully differentiated nerves. But the fact is that these tissues pass through a definite cycle of development, the course of which we have been able to follow from beginning to end, and that we have been unable to demonstrate nerves in any stage of their development, although the embryos themselves, from on the 3rd day on, always contained them.

Our anatomical results are in agreement with our physiological investigations. The absence of a reaction in the immediate neighborhood of an area, even when strong stimulation has been applied to the area itself, and the atypical behavior of the blood vessels in adrenalin experiments, can be understood only as resulting from the absence of nerves.

CONCLUSION

The agreement of physiological experiment with anatomical findings justifies our conclusion: the blood vessels of the vascular membrane of chick embryos do not contain nerves.

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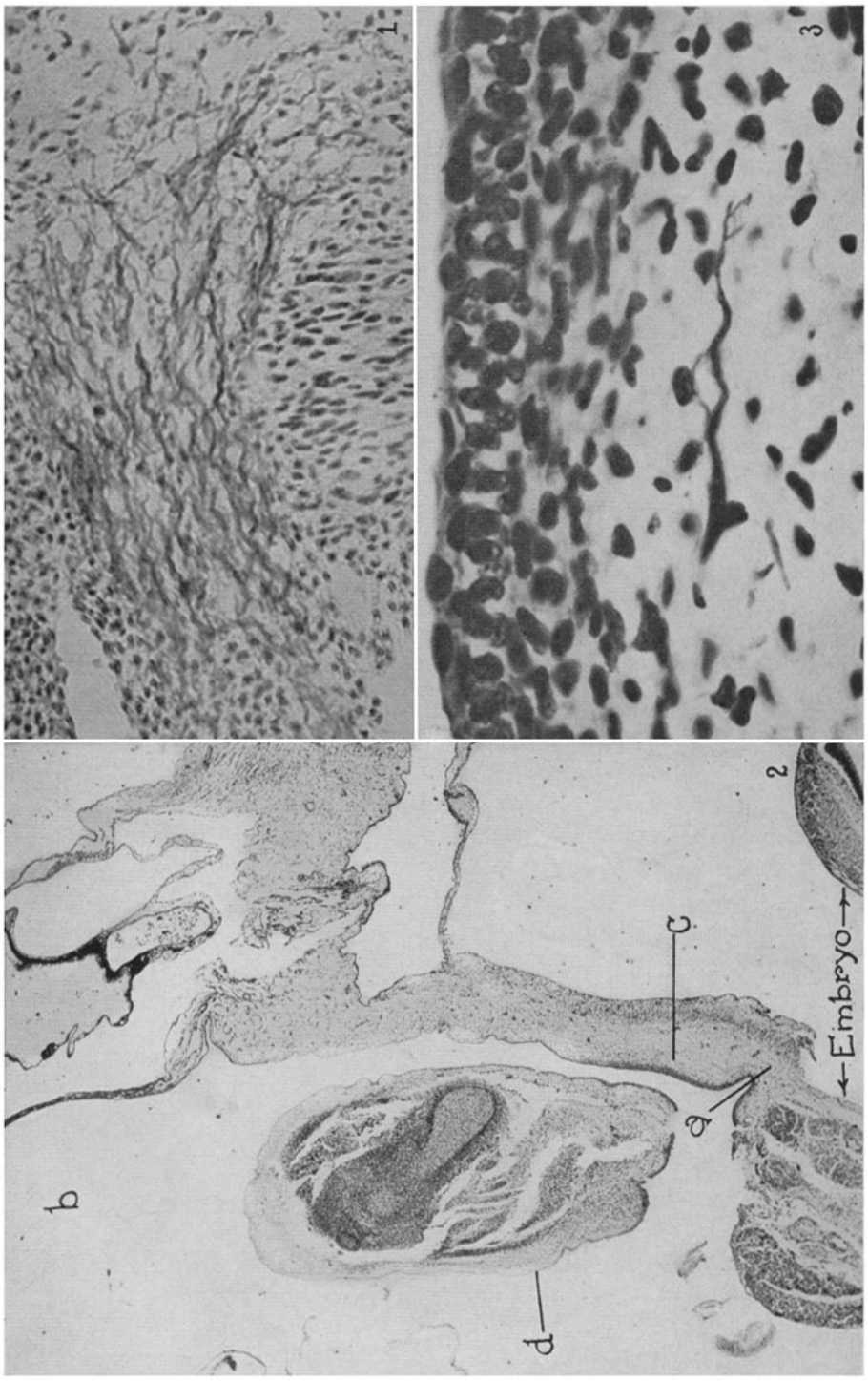
EXPLANATION OF PLATE 5

FIG. 1. Chick embryo No. 177. Incubation period 4 days. Neurofibrils in a ganglion-anlage of the body. Silver impregnation. × 450.

FIG. 2. Chick embryo No. 173. Incubation period 7 days. A cross section through the body is shown at the point of transition from the embryo to the membranes. At (a) the lateral limiting sulcus appears, and at (b) the amnion and chorion. The neurofibrils farthest away from the body (Fig. 3) were found at (c). At (d) there is a cross section of an extremity. Silver impregnation. × 40.

FIG. 3. The region (c) indicated in Fig. 2 is shown. × 1000. It exhibits the most distant peripheral nerve found in this section.



(Lange *et al.*: Blood vessels in chick embryos. I)