

# THE ACTION OF ULTRA-VIOLET RAYS UPON THE FROG'S EGG

## I. THE ARTIFICIAL PRODUCTION OF SPINA BIFIDA

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SIXTEEN FIGURES

While the method of the experimental embryological investigation detailed in the succeeding pages is of importance because of the constancy with which the condition of spina bifida may be produced in the embryo, the real value of the method and of its results lies in the interpretation which is thereby rendered possible of the physiological value of the several component portions of the fertilized ovum. The really fundamental problems to be determined by investigations of this nature are, first, whether the fertilized ovum is to be regarded as a composite structure made up of various system- or organ-anlagen, or the chemical progenitors or 'ferments' of such, distributed in definite and, perhaps, constant positions throughout the cytoplasm. Or, second, is the ovum to be considered in the sense of a unicellular organism, differing in no great respect from the physiological structure of unicellular organisms in general but possessing that specific potential to elaborate 'ferments' and pro-anlagen at successive genetic stages, and, ultimately the anlagen of the later developmental stages? In the former instance it is presumed that the embryonic parts are pre-localized in the cytoplasm of the ovum and make their appearance, in the words of Lankester, as "a sequel of a differentiation already established and not visible." In the second assumption the embryonic parts are unrepresented in the ovum, the regions of the cytoplasm being

then, so far as the future embryo is concerned, of equipotential value. It appeared to the author that a means by which a limited area of cytoplasm could be destroyed and yet left in its original relations to surrounding parts would afford a solution to this question. Accordingly, recourse was made to ultra-violet rays of such a degree of intensity as to cause the disorganization of the cytoplasm in from one to thirty seconds and of such a degree of concentration as to influence limited surface areas. Acting upon the suggestions made by Prof. E. H. Merritt, an apparatus was constructed which met these requirements fully.<sup>1</sup>

This apparatus consisted of a large induction coil actuated by a 110-volt direct current reduced by an unknown resistance. The potential, moreover, was raised by means of several Leyden jars shunted between the electrode wires. The terminals were made of iron, and were spaced about 5.0 mm. The eggs used for the purpose were those of the various forms of frogs occurring in the neighborhood of Ithaca, New York. These were obtained early in the morning, as soon after laying as was possible. At the time at which they were influenced they were in the undivided stage. Development was allowed to progress in the laboratory in some instances, and the eggs influenced at several later developmental stages, but no egg further along in its cycle than about the 64-cell stage was used. Furthermore, care was taken to reject such eggs as were collected late in the laying season for that particular species of frog, and particularly those located near the center of the egg bunches, specifically to avoid dealing with those possessing a tendency towards abnormal development. In preparation for exposure to the rays, the eggs were freed from their jelly, which had been found impervious to the light, and placed under a perforated tinfoil diaphragm. The perforations differed in size in different experiments. After the egg had been rotated so that a predetermined part had been brought directly under the center of a circular perforation in the dia-

<sup>1</sup> At this point I desire to express my indebtedness to Professor Merritt for helpful suggestions and to the Department of Physics of the University for the use of the apparatus with which the experiments mentioned in this paper were conducted.

phragm, both were then brought under the electrodes of the apparatus and the circuit closed.

While in the numerous experiments conducted, the various portions of the white and of the black hemisphere and of the equator of the frog's eggs were influenced in order, the author decided to limit the scope of this present communication to those effects produced by the rays when influencing the white hemisphere and the equator of the egg. Indeed, in addition to the significance of the findings of the investigation in the interpretation of the larger problem of ovum structure, the immediate purpose in presenting this paper is to establish the fact that the condition of spina bifida may be produced at will by this method.

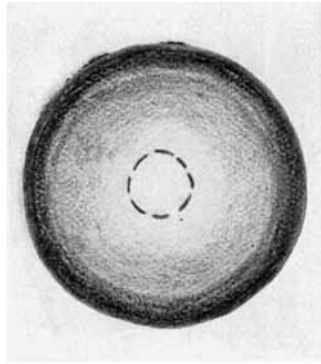


Figure 1

The aperture in the diaphragm used for this experiment was 0.4 mm. in diameter. The eggs averaged 1.7 mm. in diameter. Consequently but a small surface area proportionately of the total area of the egg was influenced. The latter amounted to 425.0 sq. mm. whereas but about 5.0 sq. mm. of this surface could be influenced by the rays. The relative sizes of these areas is brought out more clearly by reference to figure 1, which represents by a broken-line circle the portion of the surface area of the egg sphere illuminated. Further, it was found that the depth of penetration of the 0.4 mm. pencil of rays depended upon the length of exposure to the light. Uniform exposures of 30

seconds were employed in this series. A section through an egg so influenced is shown in figure 2, in which the depth to which the rays had penetrated is represented by the shaded portion on the right of the sketch. In this instance the rays passed in the plane of the section and at right angles to a tangent at the center of the surface of the affected area. The direct results of the illumination were corroborative of those previously observed by other investigators using violet rays, such as granulation of the chromatin and certain degenerative changes noted in the cytoplasm. No attempt was made, however, to study this aspect of the influence of the rays. It was noted in extremely long exposures of from 1 to 10 minutes, that masses of protoplasm

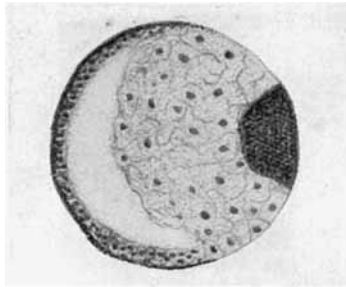


Figure 2

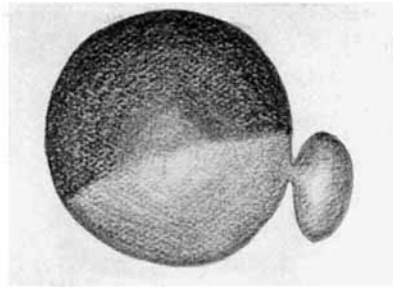


Figure 3

were in some instances extruded upon the egg surface, retaining, however, a slender connection with the main mass of the egg. In instances of such exovation, the egg died early after having made but little developmental progress. Such an exovate is shown in figure 3. It is of importance to note the fact demonstrated by the sketch that the mass of the exovate was approximately equal to that of the influenced area of the egg (compare with figure 2). The most plausible inference to be drawn from this phenomenon, in the terms of the interpretation of the ovum as an organism, seems to be that of an effort on the part of the ovum to rid itself of the chemically altered or dead protoplasm which can only act as a hindrance to its further developmental progress.

Reference to various series of experiments selected at random bring out the value of the method in the constancy of production of the condition of spina bifida. In one series of thirty-one 16-cell eggs used at the beginning of the experiments and exposed to the 0.4 mm. ray for 30 seconds each—various regions of the equator and of the white hemisphere being influenced—twenty-one developed abnormally, and but ten normally. Of the abnormal embryos, eight presented the condition of spina bifida. In another and later series of fifteen eggs in the 4-cell stage, influenced in the same manner, none developed normally. Most of these died during the early stages. Four, however, lived to swimming forms with two tails. Later in the spring, after the technique had been still further perfected and the eggs of the green frog were available, from which it was possible to remove the enveloping jelly more readily and more completely, the percentage of spina bifida embryos rose. In one set of five undivided eggs influenced in a similar manner, one died about twelve hours after the experiment, having made no developmental progress, and the four others grew to swimming forms presenting the condition of spina bifida, each having two tails. This last instance is merely representative of the high percentage of these forms of malformation obtained when the white hemisphere is influenced by the ultra-violet rays.

As has been mentioned above, the most effectual barrier to the penetration of the rays was the investing jelly. When this was completely removed an exposure of 10 seconds was sufficient to influence the egg. The presence of a very thin layer, however, completely blocked the passage of the rays even during an exposure of as much as 10 minutes. The author attributes most, if not all, of the irregularities in percentage production of spina bifida embryos to the presence of this jelly. The later results of the experiments were sufficiently assuring to warrant the conclusion that, when it could be positively known that the rays under the above conditions had actually penetrated the ovum in the regions above mentioned, the condition of spina bifida could be invariably brought about in the developing embryo. Taking these difficulties into consideration, however, the per-

centage production of the condition ranged between 85 and 90 per cent of the total number of eggs used.

An observation that recurred repeatedly was to the effect that the developmental period required by the eggs was lengthened as a result of the rays' influence. Under laboratory conditions ordinarily from 3 to 4 days were sufficient for the appearance

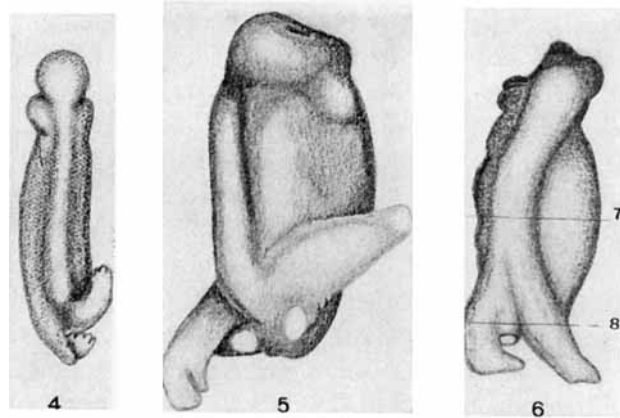


Fig. 4 A specimen of cauda bifida demonstrating the asymmetry of the tails. In this egg the rays had struck a portion of yolk farthest removed from the equator. The tails are provided, as is shown, with peculiar toe-like processes on their free extremities.

Fig. 5 In this tadpole the right tail encountered the body axis at an acute angle directed anteriorly. Two yolk plugs are to be seen, and the same splitting of the extremity of the left tail as was noted in figure 4.

Fig. 6 The lines 7 and 8 on this cauda bifida tadpole indicate the level of the cross-sections shown in the respective figures. In figure 7 the notochord lies ventral to the well differentiated neural tube. In figure 8 the asymmetry of the halved neural tube is shown, more particularly on the right side. Ventral to this lies the notochord, all traces of which are absent from the left half of the sketch. This right neural tube-half lay in the more actively used tail.

of the free-swimming tadpole-forms of the green frog; in the case of the experimented eggs, however, 5 or 6 days were required and in some instances 8 days. Furthermore, it was noted that during the 12 hours immediately ensuing upon the experiment the eggs seemed to have entered into a condition of temporary suspension of development, later resuming that process but with greatly lengthened tempo.

The further observation was made that free-swimming forms, such as are represented in figures 4, 5, and 6, seemed to be able to move about by the use of either tail, but that the swimming movements were more vigorous in one than in the other. This is an interesting fact in connection with the results obtained by study of the microscopical sections of the same specimens. In these it was learned that in the more favored of the two tails the neural tube was greater in diameter and extended a longer distance towards the tip of the tail. In some of the specimens,

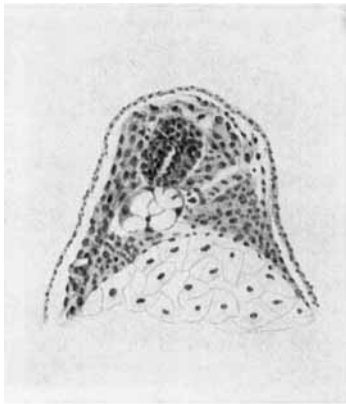


Figure 7

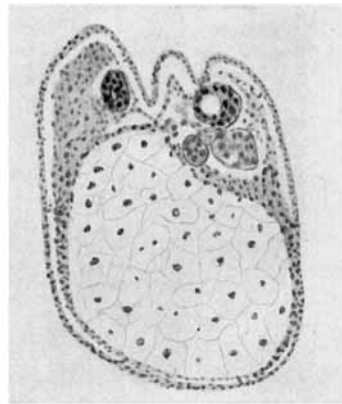


Figure 8

as is to be seen in figure 8, the notochord was limited to one tail. Figures 7 and 8 are cross-sections of the tadpole represented by figure 6. The right was the more active of the two tails during life. In figure 7 the neural tube, cut just cephalic to its bifurcation, is seen to be well differentiated, with the notochord lying ventral and adjacent to it. The section of figure 8 was taken immediately caudal to the bifurcation and shows the notochord confined to one (the right) tail.

Several specimens presented a peculiar relation of one tail to the longitudinal axis of the body; such are figured in 5 and 9. In the latter figure the main axis of one tail joined that of the trunk at almost right angles, whereas the other tail coincided fairly well with the main body axis. In figure 5 the right tail

met the body axis at an acute angle, looking forward. Such tails were, of course, useless from the functional standpoint; but their importance cannot be overestimated in furnishing exaggerated examples of the asymmetry of some of the types of spina bifida, such as were observed above in the cross-sections.

The study of the serial cross-sections demonstrated, furthermore, as these were followed in order caudally, that just posterior to the level of bifurcation of the neural tube each half tube des-

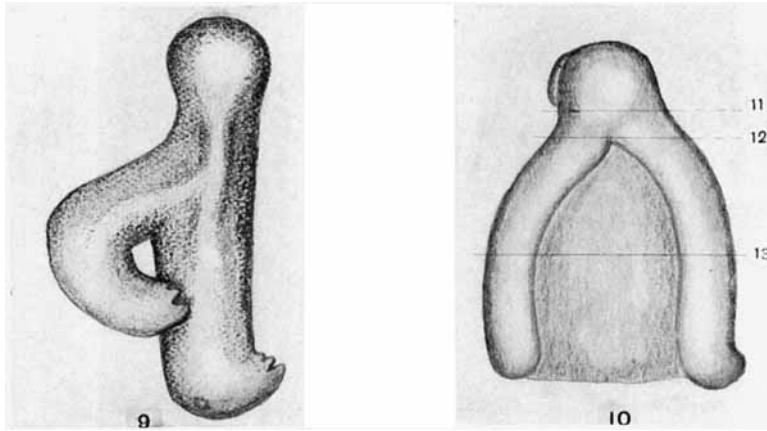


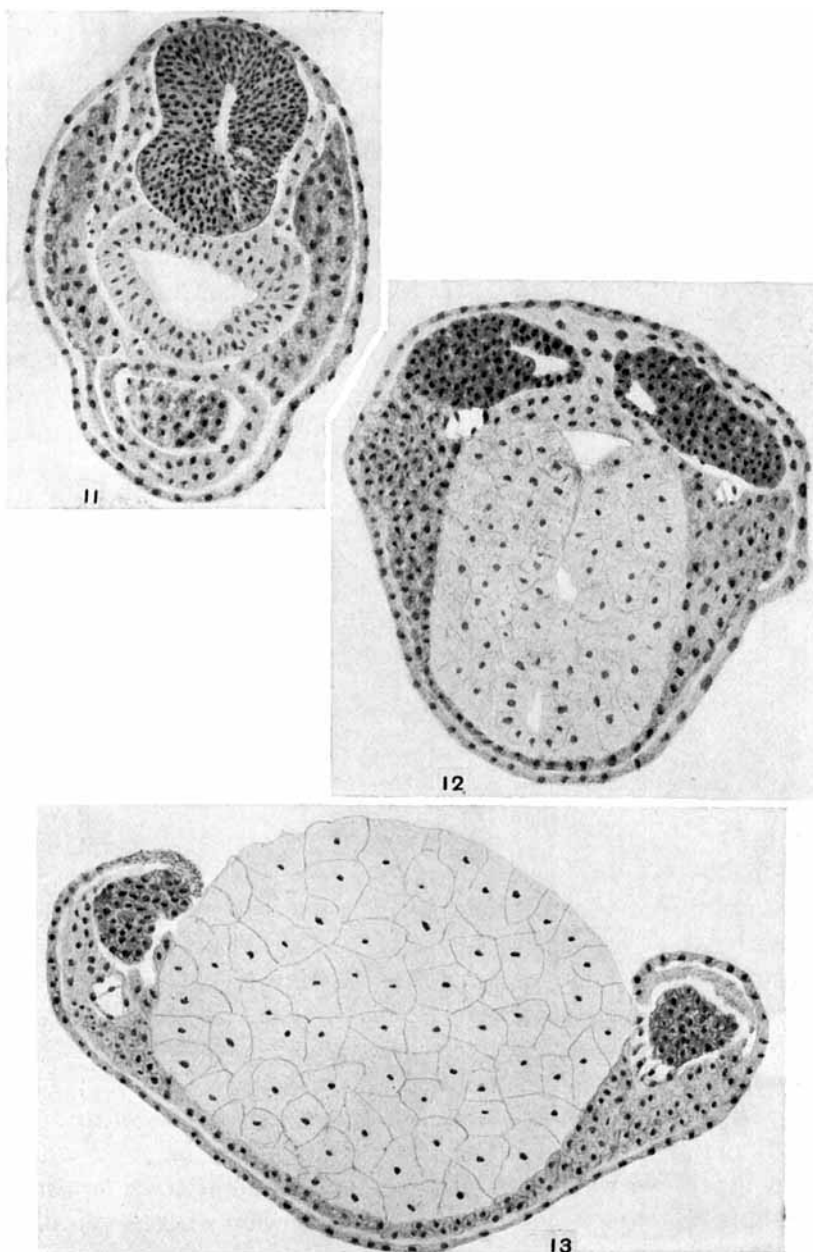
Fig. 9 This figure illustrates a marked instance of asymmetry with a division of the cord well anterior on the embryo. Here again the extremity of each tail is broken up into toe-like processes.

Fig. 10 In this embryo the rays had encountered an area well up on the equator in the median plane; hence, the bifurcation of the neural cord immediately posterior to the optic anlagen. The lines 11, 12 and 13 indicate the levels of the respective cross-sections illustrated by the succeeding figures.

tinued for each tail presented an asymmetrical outline, the lateral wall being considerably thicker than the median. This is shown in part by the right neural tube in figure 8. As the series was followed farther caudally, however, a readjustment of the tube cells was observable, each half now becoming either a solid rod or a tube entirely symmetrical so far as the thickness of its walls was concerned; (see also figures 12 and 13).

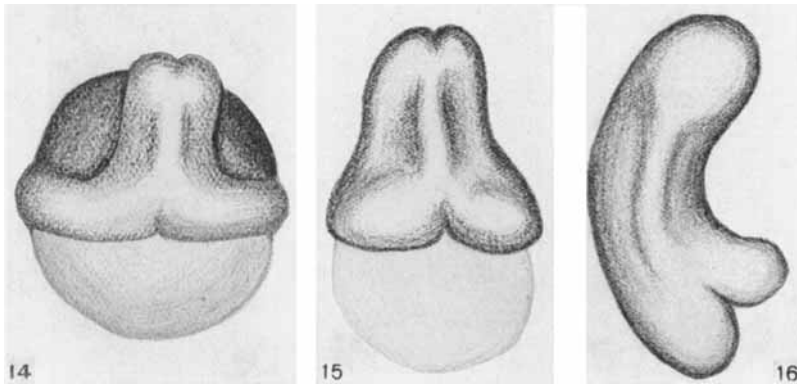
The neural tube was caused to bifurcate at various levels, dependent upon the portion of the hemisphere influenced.





Figs. 11, 12, 13 In figure 12 the marked asymmetry of the neural tube halves immediately posterior to the level of bifurcation of the cord is shown. The neuroblasts become adjusted farther posteriorly, however (fig. 13) to form a more symmetrical tube or column of cells.

Where the rays struck close to or on the equator in the median plane of the egg the subsequent bifurcation was noted well forward towards the head region; figure 10 well demonstrates this fact. In figure 9 the point encountered was much lower down on the hemisphere, while in figures 6 and 4 it was still farther posterior. Such are really instances of cauda bifida. Another fact of the utmost significance was gained from studies of the histological sections. Since it was definitely ascertained that the rays had killed the portion of the egg illuminated, in no specimen was there to be observed, however, a deficiency or falling out of a portion of the neural tube, as one would expect



if the proanlagen or anlagen had been encountered by the rays. Notwithstanding the possibility of post-generation, with a replacement of the anlagen thus rendered inactive, the conclusion might be justified, tentatively at least, that the proanlagen are not located in the early stages of division of the ovum either in the yolk hemisphere or along the equator, but are confined wholly to a region well up on the black hemisphere. Before referring in detail to the results of other investigations dealing with spina bifida it will be well to emphasize some general considerations which must be taken into account in connection with the production of abnormalities.

It is taken for granted that there are many linking factors associating the developmental processes concerned in the pro-

duction of spina bifida with those of other forms of malformation occurring in nature and produced experimentally. Accordingly, one cannot well study the one without taking into consideration those general fundamental physico-chemical factors of development underlying both, upon the disturbance of which the production of anomalous conditions is dependent. The fact is well supported that the processes of differentiation in the tadpole, as in some other forms, appears to be dependent upon a series of complex and progressive chemical reactions which are of the nature of oxidations. In the later stages of development we are dealing with an organism whose chemical constitution differs considerably if not completely from that of the undivided ovum. The results of such reactions and chemical changes become apparent in the differentiation of the various anlagen. Eggs placed in pure water free from chemical compounds which might enter into the chemical structure of the organism, but supplied with a liberal amount of oxygen, can and do undergo the several processes of differentiation, finally hatching and swimming about. From that time on, however, when growth of the differentiated parts is initiated the organism requires a supply of various chemical substances for its appropriation. In this connection, it is significant that the great majority of defects produced experimentally are referable to changes in the differentiative stages of the embryo and only secondarily to defective growth phenomena.

Bearing in mind, then, the chemical modifications occurring during the differentiating cycle of the embryo, it is fair to assume that at certain specific times in the genesis of the anlagen their chemical composition is such as to render them more ready participants in chemical reaction with the chemical agent employed. Results so obtained cannot be cited without reservation as applying to the state of the undivided ovum, consequently much of the chemical work conducted along this line is subject to considerable qualification and limited in the insight which it furnishes into the constitution, chemical or physiological, of the ovum. Before the full truth can be established on this point, it must be demonstrated that the toxic action of the chemicals employed was exerted upon the egg during the undivided state

and not afterwards; or, in other words, upon the chemical 'ferments,' or proanlagen, and not upon the anlagen cells when they have attained what might be termed a condition of chemical completeness. Only by this means can we decide between the two conceptions of the ovum; as an organism elaborating its organ anlagen at succeeding developmental periods, or as a composite, mosaic-like structure.

While the artificial production of defects has been known for a long while to be possible, but very few have succeeded in advancing any plausible explanations covering the instance of spina bifida embryos. There are two aspects to the problem of the artificial production of spina bifida which are brought out in a review of the literature dealing with this subject. The first consideration is whether we are dealing with the action of an external agent upon some specific substance in the egg; and the second, whether the nature of this reaction is specific, referable to the agent alone, which possibly reacts upon the egg as a whole.

Morgan in 1894 and O. Hertwig in the year following were both successful in the production by chemical means of a large percentage of embryos showing the defect of spina bifida. That 0.625 per cent solutions of sodium chloride should produce so high as 50 per cent of this form of malformation was an argument in favor of a definite specific chemical or physical property of the compound. Prior to this date observers had recorded only occasional instances of this defect and had failed to give a convincing indication of the nature of the upset in the physico-chemical factors concerned. Roux was the first to call attention to the occurrence of spina bifida among frog's eggs, owing, apparently, to conditions found in nature. Panum recorded 38 instances of spina bifida in chicks, among 404 monsters produced. He, with Dareste and Féré, obtained monsters of various kinds by the employment of variations in temperature (as did Hertwig), by varnishing the egg shells, by shifting the long axis of the egg to the vertical, by traumatic injuries, shaking, magnetism, electrical means, various gases, vapors of lavender and by injecting different toxines and chemicals, such as turpentine, an-

iseed, absinthe, and cloves into the white of the egg. Their inability to associate any given deformity with a known and controllable cause led to a failure in the analysis of the normal developmental factors of the embryo. Richter found three instances of spina bifida among several hundred hen's eggs upon which he had experimented. Spemann, however, produced two-tailed embryos by simply tying a ligature between the two blastomeres, demonstrating the bilaterality of the anlagen but throwing no light on the nature or antero-posterior extent of the organ-building substances. Fol, Rauber, Born, and O. Hertwig attributed the duplicity to double fertilization. This explanation was too compromising regarding the anterior portion of the embryo, and later was found to be unnecessary. Godlewski's experiments with reduced pressure, and Herbst's with lithium salts, Morgan's with the centrifuge, Samossa's with atmospheres of nitrogen and of hydrogen, and Wilson's with Ringer's solution and with sodium chloride, furnish additional evidence of the diversity of ways by which this abnormal condition may be produced.

In this connection, it is interesting to note that Mall has reported 12 instances of spina bifida among 163 pathological human embryos, attributing as a possible cause of the condition, faulty implantation of the embryo. Analysed still further, however, by analogy to the conditions found among lower vertebrates, it seems possible that the human ovum, too, requires but little else than a good supply of oxygen for its differentiation during the early stages of development. At this time the causal forces are operative for the production of spina bifida. Undoubtedly, a deficiency in the supply of oxygen could be brought about by the imperfect imbedding of the ovum in the uterine mucosa. Bearing this fact in mind in connection with the features of differentiation of the ovum given on page 375, it seems superfluous to seek an explanation of the condition in man through the action of chemical substances or of altered temperature. Though the possibility of the direct or indirect dependence of the processes of oxidation upon the action of the latter agents must be

admitted, reasoning from conditions as we find them in the frog, a sufficient and probably a more primal cause, at least, is referable to faulty oxidation.

Guthrie produced these defects by the use of strychnine, caffeine, and nicotine, as had Hertwig, but with concentration far below that of 0.625 sodium chloride. Jenkinson, however, tested out this question of the osmotic pressure of the salt solutions by employing a great variety of isotonic solutions of various salts, such as chlorides, bromides, iodides, nitrates, and sulphates of ammonium, lithium, sodium, potassium, calcium, barium, strontium, and magnesium, and, in addition, solutions of cane sugar, dextrose, urea, and gum arabic. He obtained spina bifida with especial success in his sodium chloride and sodium nitrate solutions. His conclusions are best given in his own words: "There is very little room for doubt, that the malformations in question may be due to some property of a salt other than its osmotic pressure." Bataillon had previously come to the conclusion that malformations were not specific to the means employed. Gurwitsch's belief was that halogens affected the position and development of the blastopore and of the brain, sodium chloride acting upon both, and sodium bromide upon the brain alone, whereas lithium chloride seemed selective on archenteron and blastopore.

It would appear, considering the production of this malformation by the diverse methods outlined above in connection with that detailed by this paper, that we were justified in concluding that in the question of specificity of reaction in the production of spina bifida the weight of argument at present refers the causative forces more particularly to an upset of a specific substance in the egg, rather than a specific action of the agent. It is in the conception of the changes which occur in the chemical composition of the ovum during its differentiation, as previously outlined, that we find support for this statement. It follows that the composition of any particular proanlage or anlage may be such at different stages of its chemical elaboration as to possess a marked affinity for widely varying chemical reagents. The

developmental end-product of the reactions so brought about would be the same, e.g., spina bifida, notwithstanding the wide diversity of character of the chemical reagents employed.

Furthermore, since we cannot deny, we must take into account the possibility of a two-fold manner of production of spina bifida; the one, owing to an upset in the contents of the cells of the unpigmented hemisphere whose yolk is intended for the nutrition or elaboration of the other component, viz., the proanlagen or chemical ferments restricted to the cells of the pigmented hemisphere. For the other, we can assume the possibility of an interference in the function of these proanlagen as the result of the chemical reactions experimentally induced, apart from an upset referable to the composition of the nutritive yolk particles. The author's work, however, points out clearly that in the white hemisphere alone are resident sufficient causes for the production of the malformation, so that, while the possibility of an involvement of the proanlagen exists, the weight of experimental evidence points to the yolk hemisphere as the more vulnerable of the two. Jenkinson observed, for instance, that as the result of chemical action the yolk cells were primarily affected, and Godlewski, employing reduced pressure, came to the same conclusion. The disturbing influences of insufficient aeration and cold, as ascertained by Morgan and others, were noted first in the yolk cells, and to this same region Morgan attributes the causative factors in the results of the centrifuge, while Hertwig drew the same conclusions from studies of overripe eggs.

The production of an area of altered protoplasm, which serves as a mechanical check to the approximation of the lips of the blastopore during the backward progression of the latter, emphasizes very naturally the importance of synchronized tempo in the two processes directly concerned with the elaboration of the neural cord. Under normal conditions the differentiation of the neural anlagen (consequent upon the backward migration of the proanlagen) occurs apparently synchronously with the backward migration of the blastopore and fusion of its dorsal lips. These two processes are approximately coöperative in point of

time, i.e., the anlagen of each half-tube become progressively differentiated in a backward direction at about the time when the half of the dorsal lip in which it is localized meets and fuses with its corresponding fellow of the opposite side. The two processes are not causally dependent upon each other, however, since differentiation takes place in the experimented eggs at about its former rate but now along the equator and not, as usual, parallel to the median plane.

The absence of the proanagen and anlagen of the egg along the equator in the earliest stages of development of the ovum is sufficiently attested for by the ultra-violet method. Incidentally, it should be remarked that the restriction of these proanagen at all times to the pigmented hemisphere seems to the author's mind a very significant fact. In this connection, it should be stated that ultimately the yolk mass is wholly drawn into the body of the embryo. Even though by this later process the neural tube halves may be approximated, subsequent fusion does not take place, however, since each half tube has postgenerated into a whole tube.

The conclusions reached by this method of experimentation upon the fertilized ovum, are, therefore; first, that the killing of a small localized area of the yolk hemisphere or of the region of the equator of the frog's egg produces invariably the condition of spina bifida in the embryo; and second, that the neural tube proanagen, or formative substances, do not lie either in the yolk hemisphere or along the equator of the frog's egg, but are wholly restricted to the pigmented half of the egg. These proanagen attain their definitive positions by a process of backward migration, the rate of which is synchronous with that of the backward progression of the dorsal lip of the blastopore. The action of the ultra-violet rays in destroying a small localized area of the yolk hemisphere or equator results from mechanical causes in an upset of the synchronism of the two factors, i.e., differentiation of the neural anlagen and approximation of the lips. The former proceeds at its normal tempo, while the latter is retarded. Consequently, the former, always restricted to the pigmented hemi-



sphere, come to lie along the equator and are later carried towards the median plane by the subsequent approximation of the lips, but the half tubes, having already differentiated into whole tubes, do not subsequently fuse.

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