THE EFFECT OF THE DESTRUCTION OF PERIPHERAL AREAS ON THE DIFFERENTIATION OF THE NEUROBLASTS.

M. L. SHOREY

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I INTRODUCTION

Observation of surgical cases in which the amputation of a limb has been followed by the degeneration of the nerve fibers innervating it, and finally of the nerve cells from which they arise, and cases of paralysis accompanied by the atrophy of the muscles

concerned, have demonstrated that an intimate relation exists between the life of a muscle and that of its motor nerves. And at least since 1851, when Weber described an abnormal calf embryo with the nervous system entirely wanting below the second dorsal vertebra and an accompanying loss of the corresponding muscles, there has been a tendency to believe that there is likewise a dependence in development. Other teratological cases in which a defect in the nervous system has been accompanied by a defect in the organs normally innervated by the missing nerves have been recorded, and the effect of the absence of the nerves in experiments in regeneration has been adduced as evidence in favor of such a view. It is obvious, however, that neither of these conditions can furnish conclusive evidence regarding developmental processes, for, in the case of monstrosities it is impossible to know the nature of the original lesion, and in regeneration, while there are doubtless similarities between it and normal development, it is evident that neither the conditions nor the processes can be identical.

Bardeen, in 1900, made careful observations on the relations between the myotomes and nerves at early stages of embryonic development, and from the fact that there is no apparent contact between the two until a considerable degree of differentiation has been reached concludes that early development is independent. To quote, "The early development of the nerves is one of passive independence, without any immediate relations to the myotomes." But it seems to me that such evidence must be regarded as inconclusive, for the fact that two organs are not in direct contact does not exclude the possibility that the one may influence the other.

To reach decisive conclusions regarding the interdependence of two organs or tissues in development it is necessary to study the behavior of one in the absence of all possible effects from the other. This can be done only by destroying some portions of the developing organism, or by removing the part to be studied to a controlled environment. Both these methods have been used in studying the relation of the nervous system to peripheral organs, but in most previous studies the object of the investigation was to discover the effect of the destruction of nervous tissue on the organ inner-

vated. A few experiments dealing with the effect of an abnormal environment on the developing nerve have, however, been made.

Harrison ('06), experimenting on frog larvæ for the purpose of ascertaining whether other cells than those of the nerve center enter into the formation of the nerve fiber, obtained results which bear on the question under consideration. The spinal cord of these embryos was extirpated, and small pieces of it transplanted under the skin of the abdominal walls. Small nerve trunks arise from these and run for some distance in various directions. From portions of the ganglionic crest thus transplanted small ganglia and nerves may arise. His own conclusions are as follows: "The nerve center (ganglion cells) is shown to be the one necessary factor in the formation of the peripheral nerve. When it is transplanted to abnormal positions in the body of the embryo it then gives rise to nerves which may follow paths where normally no nerves run, and likewise when the tissues surrounding the center are changed entirely nerves proceeding from that center may develop as normally." In another experiment fragments of the medullary tube, isolated before visible differentiation, were placed in a drop of lymph, and it was found that these develop short fibers. From this experiment he concludes that the outgrowth of the fiber is largely independent of external stimuli, although its direction is doubtless influenced by a number of factors. That the direction is influenced is shown by a number of experiments in which a limb bud transplanted to some other region of the body receives normal innervation from the nerves of the region to which it is transplanted.

Braus ('06) removed one of the anterior limb buds of several toad larvæ, and ten days later found that the brachial plexus was as well developed on the injured as on the uninjured side, although by far the greater part of the musculature had been destroyed, and at the time of the operation no nerve fibers running to the limbs were visible. Older operated embryos showed the brachial plexus present but diminished in size. From these experiments Braus concludes that "*Der Befund am Plexus brachialis ist eine sehr deutliche Illustration dafür, dass die Entwicklung der peripheren Nervenfasern unabhängig ist von den Endorganen.*"

The work of the two last investigators has been published since, or about the time that I began to investigate the relation of nerve and muscle tissue in the chick embryo, and did not reach my hands until a later date. The experiments to be described were undertaken for the purpose of studying the behavior of the developing nervous system, when it is itself left quite intact and with all its relations normal except that a peripheral area to which nerves should be distributed is destroyed.

The work was begun at the suggestion of Professor Frank R. Lillie, and I wish to express my indebtedness to him for proposing a problem which has proved to be of intense interest, and for suggesting the first methods by which it was to be attacked. I wish also to express my appreciation of his continued interest and helpful criticism. Acknowledgments are also due to Professors C. O. Whitman, C. M. Child, and W. L. Tower for suggestions in regard to illustrations, photography and material, and to Miss Sabella Randolph, Mr. Kenkichi Hayashi and Mr. C. E. Brues for aid in the preparation of drawings and photographs. Figures 36 and 37 are copied from Lillie's *Embryology of the Chick*.

II EXPERIMENTS ON THE CHICK

A Choice of Material

The chick embryo was chosen as furnishing a form favorable for these experiments; the work of Lillie ('04) in destroying various portions of the embryo had shown that no regulation or regeneration was to be expected and my own work has entirely confirmed these results. After two days of incubation, the earliest period at which I have operated, and in regions where no differentiation is apparent, the primordium for each organ is so definitely laid down that the destruction of it, or any considerable portion of it, results in a corresponding loss in the fully formed animal. Lillie's work had also shown that comparatively large areas may be destroyed without interfering with the normal development of the rest. It would therefore be possible to destroy the primordia of definite muscles, or of sensory areas, before they had been penetrated by nerve fibers, and then determine the course of the nerves which

should innervate them. The rapidity of development, and the availability of material at practically all times of the year were also points in favor of the chick.

B Character of the Operation

It was decided that the first experiments should be the removal of the wing and after some preliminary tests the period at which the wing bud is just apparent to the naked eye as a small elevation on the body wall was chosen as the most advantageous for the operation. The period of incubation before this stage is reached varies from 68 to 88 hours according to the time of year, the development of the embryo when the egg is laid, and fluctuations in the temperature of the incubator, so that it is impossible to tell in exactly what condition the embryo will be found when the egg is opened, but Fig. 1, a photograph taken in this case after 74 hours of incubation, gives approximately the external appearance at the time when most of these operations were performed. The wing, was never larger than this, and in the majority of cases it was smaller. Fig. 2, a photograph of a cross-section in the region of the wing, shows the relation and development of different parts at this time. Even if the wing bud is removed close to the body, and no part of the wing proper develops, some of the muscles, or portions of them, extending dorsally from it to the vertebræ, and ventrally to the sternum, always appear. It, therefore, seems evident that the primordium for these has not yet grown into the wing, but is still contained in the muscle plate. Parts of the bones of the shoulder girdle will also form, so that only a portion of their primordium can be located in the wing bud at this period.

Lillie has determined that the wing develops from the 17th, 18th, and 19th somites, and that it, with the other structures arising from these somites, is innervated by the 14th, 15th, and 16th nerves, the sensory root arising from the 12th, 13th and 14th ganglia. Later in development these three nerve trunks unite to form a plexus before their distribution to the muscles and sensory areas of the arm, but at this stage they have not yet reached the place of union and each appears as a distinct trunk extending toward the base of the wing but not yet reaching it. Neither is it in contact with the myotome.

The neural tube exhibits but little differentiation; no definite cell areas are marked off within it, and only a thin layer of nerve fibers or axones has formed at the periphery. There is a small ventral root, but the cells from which these axones arise have not yet become localized as the ventral horn.

By operating at this stage of development it was possible to remove the greater part of the wing without any direct injury to the nervous system. As stated above, some structures, or parts of them, belonging to the wing may still develop, and the intrinsic muscles of the vertebral column are entirely uninjured; still the peripheral area, both sensory and motor, is much decreased, and as the greater part of the peripheral nervous system is still to be developed, and most of the differentiation of the spinal cord still to occur, it is possible to study the effect of the loss of the end organ on the size and distribution of the peripheral nerves, as well as its effect on the cells of the central system with which they are connected, either directly or indirectly.

C Methods

During the period of incubation preceding the operation the eggs were turned every 12 or 24 hours to prevent the blastoderm from adhering to the shell membrane, but to insure the accurate location of the embryo a number of hours must elapse between the last turning and the operation. The method of opening and closing the egg was essentially the same as that described by Lillie ('03) and earlier by Miss Peebles ('98), but instead of placing strips of egg membrane about the edge of the shell used as a cover for the opening in the operated egg I found it more effective to leave a margin of membrane attached to this cover. If then the curvature of the cover is the same as that of the egg to which it is applied, and there is no break in the membrane, a perfect closure is effected.

Since the embryo lies on the left side (I found only two or three exceptions to this in the fifteen hundred or two thousand eggs used in these experiments) the right wing was always the one removed. For this purpose small spear-headed scalpels were first used and this method may be used with success if the wing bud

has attained considerable size, but for the earlier stages, at which it was finally decided to operate, destruction by electrolysis is much more satisfactory. Number 12 sewing needles, one of them bent so as to penetrate the amnion and lie along the whole extent of the wing bud with as little tearing of the membrane as possible, were used for the operation. These were held in electric handles connected with dry battery cells furnishing a current of about two volts. The destruction is almost instantaneous, so that the current need be applied for but a very brief period.

There existed the possibility that by this method of operation the electric current might penetrate beyond the region with which the needle was in contact, and thus lead to the direct destruction of cells within the spinal cord. Such a possibility is eliminated by the following facts:—in a number of embryos in which the wing was removed by scalpels the same defects appear as in those of the same age operated on by electricity. Moreover the defects always appear in the same region, and follow the same order, and it would scarcely be possible to suppose that an electric current, free to extend in any direction, should always follow exactly the same path and destroy the same cells, leaving those immediately surrounding them quite normal. And a third reason is found in the fact that until twenty-four hours after the operation the path of the current is well defined by means of the presence of the injured tissue, and in specimens killed at this period, and in which it is possible to determine by this means that the injury did not extend into or even close to the spinal cord, the nervous system is still defective and the defects are always of the same nature.

I had at first many infected eggs, and occasional ones later, but as I have at other times operated every day for months with only one or two cases of infection, I believe that only sufficient care is necessary to avoid it entirely. The surface of the egg was disinfected with mercuric chloride, and all instruments sterilized. There continued, however, to be a large percentage of loss from tears in the blastoderm, puncturing of bloodvessels, extensive injury to the amnion, and other causes whose nature was not determined. Probably most cases of death within a short time after the operation are traceable to injury to the circulatory sys-

tem, but when it occurs 5 or 6 days later, as it did in some experiments which I attempted to carry beyond that period, it seems to be in some way connected with defects in the amnion, for in all these cases which were examined carefully an aperture was found in the region of the operation. This would agree with the work of other observers who have shown that anamniote embryos may develop normally for a time, but that later this defect is fatal. That the amnion can, however, regenerate if not too extensively injured and form a completely closed sac was demonstrated by other experiments in which I knew it to have been injured by the operation, so that an improved technique would doubtless obviate this difficulty.

Killing and fixing in sublimate æctic, and staining with Delafield's hæmatoxylin and Orange G was found to be entirely satisfactory for the end desired, and was the method used in the majority of cases.

As the effects would naturally be most pronounced after a considerable interval of time I began with a study of the oldest embryos, those killed between 5 and 6 days after the operation. When the conditions in these had been ascertained, specimens killed respectively 4 days, 3 days, 2 days, and 1 day after the injury was made were examined, and the results will be given in this order.

Experiments on Embryos After Three Days of Incubation

Specimens Preserved Five or Six Days After the Operation

Experiment 538. This egg was incubated 84 hours and was approximately in the stage shown in Fig. 1 at the time of the operation. The right wing was removed and the egg returned to the incubator for 5 days 5 hours, so that when preserved the age of the embryo was 8 days 19 hours. The only evidence of the right wing was a small rounded elevation scarcely noticeable on a casual examination. The wound was completely healed and the embryo vigorous and entirely normal externally except for the missing limb.

It was killed, sectioned, and stained as given above, and the operated and unoperated sides carefully compared.

The bones and muscles were well defined and easily identified and the course of the nerves traced without difficulty. Fig. 3 shows the location of the chief wing muscles and Fig. 33, *A*, the peripheral distribution of the brachial plexus on the normal side. Since the branches of the 14th, 15th and 16th nerves which innervate the vertebral musculature are not affected, these muscles being quite intact, and the branches arising from the separate trunks before the formation of the brachial plexus, their distribution has been neglected.

On the operated side the bones of the shoulder girdle are present but defective; the clavicle and scapula are, however, only slightly abnormal. Of the muscles the trapezius, rhomboideus, and subclavius are not noticeably affected; the pectoralis major, pectoralis secundus, coraco-brachialis, latissimus dorsi, teres et infraspinatus and subscapularis are present but defective, while the deltoides supraspinatus, biceps, pectoralis tertius, coraco-humeralis, scapulo-humeralis, and triceps are entirely wanting. Fig. 33, *B*, shows the peripheral distribution of the brachial plexus on this side. It will be seen by comparing this with *A* that wherever a muscle is missing the corresponding nerve is also missing; otherwise the distribution is the same as that of the opposite side.

As *A* and *B* are drawn on the same scale they show something of the quantitative loss, but measurements give this more exactly. It is difficult to arrive at the exact dimensions of the nerve trunks from sections without making reconstructions, but as a means of comparison each of the three nerves involved was measured directly below the union of the ventral root with the ganglionic root, and the number of sections in which each appears at this place counted.

From the data thus obtained the following percentages of loss for the nerve trunks were calculated:

	WIDTH PER CENT	THICKNESS PER CENT
14th nerve.....	34.1	25
15th nerve.....	17.6	36.3
16th nerve.....	35.8	30

Measurements of the ventral roots show this loss:

	WIDTH PER CENT	THICKNESS PER CENT
14th nerve.....	31.9	45.4
15th nerve.....	8.1	33.3
16th nerve.....	36.2	33.3

Measurements of the dorsal roots, which are more difficult to take accurately, because in some sections the boundary between them and the vertebræ is obscure, show a less, but still a constant loss:

	WIDTH PER CENT	THICKNESS PER CENT
14th nerve.....	36	0
15th nerve.....	1	22
16th nerve.....	38	11

The ganglia of the operated side were also smaller, and this loss is expressed quantitatively in the following table:

(In this it must be remembered that the 12th, 13th and 14th ganglia correspond to the 14th, 15th and 16th nerves.)

	LATERAL DIAMETER PER CENT	DORSO-VENTRAL DIAMETER PER CENT	THICKNESS PER CENT
12th ganglion.....	24.3	16.7	20
13th ganglion.....	22.7	22.7	33.3
14th ganglion.....	20.3	30.2	20

The loss in the spinal cord will be best shown by a photograph of a typical section (Fig. 14). This was taken in the region of the 15th nerve. The most evident difference here is in the ventral horn, this being decidedly smaller throughout the operated region. It is also noticeable that the abnormality is practically confined to the region of the 14th, 15th and 16th nerves, the first observable difference appearing three sections anterior to the beginning of ganglion 12, and although very careful measurements show slight differences to the eleventh section beyond the 14th ganglion it is markedly less immediately beyond that ganglion. This loss in the ventral horn might conceivably be due to either of two reasons: the number of cells might be less, or the individual cells or part of them, might be smaller, or the loss might be due in part to both of these causes. Two methods of deciding this

question were adopted. First the cells of the antero-lateral part of the ventral horn, this being the region affected, beginning with the section on which the 12th ganglion appears, and going to the section on which the 15th appears, were counted on each side. There is evidently much chance for error in this, particularly as the sections were not cut with this in view, and a change of focus brought different cells into the visual field, but the cells of each section were always counted a number of times, and the probability of error is equally great for each side. While, therefore, the figures obtained probably do not represent the actual number of cells, I think they may be regarded as giving a fairly accurate comparison of the two sides. The total loss for the whole area was 50 per cent, and this was distributed to the three nerves as follows:

	PER CENT
14th nerve.....	47.4
15th nerve.....	53.2
16th nerve.....	48.8

Many cells of successive sections, both in this and other specimens, were then measured with the ocular micrometer, and camera lucida drawings were also made and the dimensions taken, and while it was possible to demonstrate a difference in the size of the cells, the larger were found to be as often on the operated as on the unoperated side, and no abnormally small or otherwise defective cells could be demonstrated. Both methods of estimation, therefore, seem to indicate conclusively that the loss is in the number of cells.

The loss in the posterior horn is less, but still evident. Measurements showed that the distance from *A* to *B* (Fig. 14) is always less on the injured side and also that the distance from the center to the periphery in the dorsal part of the cord is slightly decreased. A quantitative estimate of this difference seemed impracticable.

Experiment 206. The period of incubation before operation was 3 days 1 hour. The wing, a small elevation as in experiment 538. The right wing was again removed, and the embryo preserved five days later, so that when examined it was 8 days 1 hour old. A slight thickening only was visible in the region of the

right wing, but aside from this defect the embryo was normal externally.

Sectioning showed that the scapula was entirely lacking, the clavicle nearly normal, and the coracoid and head of the humerus present but somewhat defective. On the uninjured side the same muscles were to be distinguished as in the last experiment, but on the operated side only the merest remnants are present, and these are so abnormal in shape and position, as well as in size, that it is difficult to identify them. Probably they are the pectoralis major, trapezius, latissimus dorsi, triceps, subclavius and deltoides. Fig. 4 is a photograph of a cross-section through the injured region.

The loss in the peripheral nervous system may be seen by comparing *A* and *C* of Fig. 33. The abnormality is much greater than in the last experiment, but this is to be expected because of the changed positions of the muscles as well as the increased loss in size.

Again it will be seen that wherever a muscle has been destroyed the nerve is also absent.

Measurements in this specimen show the following losses:

		WIDTH	THICKNESS	
NERVE TRUNKS		PER CENT	PER CENT	
14th.....		34.2	66.3	
15th.....		21.4	20	
16th.....		30	12†	
VENTRAL ROOTS		WIDTH	THICKNESS	
14th.....		20	33½	
15th.....		24	0	
16th.....		20	11.1	
		LATERAL DIAMETER	DORSO-VENTRAL DIAMETER	THICKNESS
GANGLIA		PER CENT	PER CENT	PER CENT
12th.....	21.3	16.7	12.½	
13th.....	22.3	28.9	11.1	
14th.....	16.7	14.9	35	

The cells of the ventral horn were counted as before, showing losses as follows:

	PER CENT
14th nerve.....	53.1
15th nerve.....	49.7
16th nerve.....	71.8

It will be observed that, while the percentage of loss in the size of the ganglia is not increased, is in fact slightly less than in the previous experiment, the loss in the ventral horn is greater, and this is exactly what would be expected, for the destruction of the muscles is much more complete, despite the fact that a slight projection of the wing remains, while the loss of sensory areas is not correspondingly great. The loss in the posterior horn is of the same nature as in experiment 538, and no attempt was made to determine this quantitatively. Fig. 15 is a photograph of a cross-section of the spinal cord in the region of the operation.

Experiment 225. This embryo was operated on after a period of incubation lasting 74 hours, and was returned to the incubator for 5 days 1 hour, making its age at the time of preservation 8 days 3 hours. A small portion of the wing was left, but the ventral portion of the body wall from the wing to the leg was not developed, and sections showed some distortion of the pelvic region. Detailed observations were, however, confined to the part anterior to the leg. Figs. 7 and 8 are photographs of this embryo.

The scapula was affected little if any, and a large part of the humerus was present; the muscles dorsal to these bones are normally developed. Only the most anterior portions of the coracoid and sternum are present and the muscles are as follows:—the subscapularis, deltoides, latissimus dorsi, triceps and pectoralis secundus are all present, but more or less abnormal; only remnants of the biceps and pectoralis major are to be found, while the teres, subclavius, pectoralis secundus and tertius are entirely wanting. Fig. 5 shows the chief bones and muscles.

The distribution of the peripheral nerves is shown in Fig. 34. Branches innervating the pectoralis and biceps are smaller on the operated side, and no trace of those normally reaching the missing muscles is found. Quantitative estimates of the loss throughout the injured region were not made for this specimen, but measurements were taken here and there and the results agreed with those obtained in the two previous cases studied. The defects in the spinal cord were also of the same nature, and are shown in Fig. 16.

The effect of the injury in the region between the wing and the

leg presents some new features. This area is developed from 6 somites, the 20th to the 25th inclusive, and each of the 6 nerve trunks innervating it has practically the same peripheral distribution. The course of these nerves is shown in Fig. 37, from which it will be seen that the nerve divides at once into a dorsal, a lateral, and a ventral branch. In this specimen the parts innervated by the dorsal and ventral branches are normal, but there are only remnants of the area to which the lateral branch is normally distributed, and in some cases it is entirely absent. From the conditions found in the brachial region it would be expected that the lateral nerve would be smaller or wanting, and this was found to be true. But the nervous system is further affected, for instead of 6 ganglia and nerve trunks, there are only 4. It is impossible to say which ganglia are missing, for this result is brought about partly by an increase in the thickness of those present, and partly by a greater distance intervening between successive ganglia. While the loss of the 2 ganglia is compensated for in some degree by the increased thickness, the area of the cross-sections of any ganglion is always less, and the nerves are decreased in size. As the beginning of both the ganglia and the peripheral nerves must have been established before the operation, it seems difficult to make it account for this condition. But as it occurred again in another ectopic embryo and only in this, it can hardly be regarded as a mere coincidence. Moreover, a younger embryo, to be described later, gives a clue to its meaning.

Another noticeable feature is the effect on the spinal cord (Fig. 17). There is no antero-lateral projection of the ventral horn in this region, and no definitely localized area of loss can be demonstrated, but the whole half is decreased in size while retaining its normal shape and proportions.

Examination of these three cases demonstrate clearly that as early as 5 days after the extirpation of peripheral areas well defined defects appear in the nervous system, but consideration of this period alone can give no conclusive evidence in regard to the process by which this condition was reached. Logically any one of three reasons might be assigned:

- 1 The injury inflicted has caused this portion of the embryo to

develop more slowly, and the condition of the operated side is merely an earlier stage of development.

2 The missing nerve elements have formed, and then degenerated.

3 The neuroblasts are in some way dependent on the surrounding tissue, particularly their normal end organs, for development, and when these are wanting they do not differentiate.

The first hypothesis may be disposed of at once by comparing the defective cases with earlier stages of development (figured on the normal side of the experiments which follow), from which it will be seen that the condition in the operated embryos is not found at any time in the normal process of growth. Considering the stage of development that had been reached at the time of the operation, it is evident that if the second hypothesis is correct, the missing nerve elements must have both formed and degenerated within five days. And the degeneration must have been complete, for no cells in the process of destruction could be found. The rapidity of development in the chick makes this possible, perhaps, and the question could be decided only by an examination of earlier stages.

Specimens Preserved from Four Days to One Day After the Operation

Three specimens were preserved on the fourth day after the operation. In one of these the injury was so slight that it was of practically no value. In another the spinal cord was apparently directly injured, and this was also discarded. In the third the operation and results were as follows:

Experiment 179. The egg was incubated for 66 hours, and at the time of the operation the wing bud was scarcely distinguishable. As a result of operating at this early stage it was found on examining the embryo 4 days 1 hour later that a small portion of the wing had not been removed, and that the body wall for a considerable distance posterior to the wing was entirely absent.

Only the merest remnant of the scapula was left and this was much out of position; the coracoid was present but abnormal in position, shape and size. The humerus was also abnormal, and

probably the sternum, although this was not yet outlined with sufficient definiteness to make it certain. On the unoperated side all the muscles given in the previous experiments, though smaller and in some cases less definite, could be identified. On the operated side only the pectoralis major and the subclavius could be distinguished with certainty, but it is probable that two other muscles, small and out of position, are the trapezius and pectoralis secundus. For these points examine Fig. 6. Besides these, small masses of muscular tissue, entirely unidentifiable, are developed here and there, and mingled with them are masses of glandular tissue, which are apparently portions of the Wolfian body, for a connection can be traced to the main mass of this organ. Portions of other organs are also out of position, and the general confusion that results makes the tracing out of the peripheral nervous system on this side extremely difficult, for any muscular tissue, no matter how small the amount, nor how much out of position, attracts nerve fibers, but Fig. 35, *A*, represents the chief points. *B* shows the distribution on the normal side.

Measurements of the nerve trunks taken as described above show the following loss in width:

	PER CENT
14th nerve.....	22
15th nerve.....	30.3
16th nerve.....	28.5

The loss in the ganglia is shown by this table:

	LATERAL PER CENT	DORSO-VENTRAL PER CENT
Ganglion 12.....	20	14.8
Ganglion 13.....	39	32.9
Ganglion 14.....	12.7	14.1

The cells of the ventral horn were not counted, but after a careful comparison which showed that there was no appreciable difference in their size on the two sides of the spinal cord, the greatest width of the antero-lateral portion was taken, beginning at the end of the 11th ganglion and extending to the end of the 14th. A loss of 39.8 per cent in width was found. Through the greater part of this region, however, the distance from the central canal

to the inner border of the motor nucleus was greater on the operated than on the unoperated side. This is shown in Fig. 18, a photograph of a section of the spinal cord at the level of the 15th nerve. An explanation of this may perhaps be found by assuming that the normal number of neuroblasts were formed, but part of these were arrested in their development, and never reached their normal location in the ventral horn. As before, while the loss in the dorsal horn is evident, it is impossible to arrive at any satisfactory quantitative estimation of it.

As mentioned above this embryo was also ectopic, and showed the same peculiarity as Experiment 225 in the loss of ganglia and nerve roots between the wing and the leg. Unfortunately the posterior portion of this specimen was destroyed, and the number lost is therefore uncertain, but as far as the series goes there are 7 post-brachial ganglia on the normal side and 5 on the abnormal. As in Experiment 225 there is an increase both in the thickness of the ganglia, and in the number of intervening sections, but the dorso-ventral and lateral diameters were taken throughout the course of ganglion 16, and from these the average loss in area for each section was estimated to be about 25 per cent. Measurements of the succeeding ganglia were taken at intervals, and showed a constant loss; percentages were not reckoned. The peripheral distribution of these nerves on the normal side agrees with that shown for Experiment 225, while on the injured side the lateral branch is missing or defective according as the lateral musculature is missing or defective.

Although the spinal cord changes its shape in different parts of its course between the wing and the leg, the condition represented by Fig. 19 may be taken as typical of the character of the loss. This is more difficult to localize than in the brachial region, for the motor nucleus is not so clearly defined, but examination with a high-power lens shows that less neuroblasts have differentiated into the characteristic motor cells. The dorsal horn is also smaller, and all the dimensions of the spinal cord decreased.

Experiment 324. This embryo was preserved 3 days 6 hours after the operation and was then 6 days 19 hours old. The amnion was grown to the embryo in the region of the wound, and in

attempting to detach it the surrounding tissues were somewhat torn. Still it was possible to identify the muscles and trace the course of the nerves.

Not all the muscles are yet clearly defined, but of those which are, the pectoralis secundus, the subscapularis and latissimus dorsi were entirely extirpated by the operation. The humerus and coracoid are abnormal and the scapula completely destroyed. The peripheral distribution of the nerves is shown in Fig. 36. Measurements of the nerve trunks show an average loss of $31\frac{1}{2}$ per cent in width. The ganglia are not noticeably affected. The dorso-ventral diameter of the spinal cord and the lateral diameter of the ventral portion are less on the operated side, but the posterior horn exhibits no change. The boundaries of the ventral horn on the operated side are, in many sections, less sharply outlined, and exact measurements are difficult to obtain, but a very conservative estimate of the average loss in width is 18 per cent. As in Experiment 179 the distance from the neural canal to the motor nucleus is sometimes greater on the operated side, and as this is the case where the boundaries are least definite, I again assume it to be possible that certain neuroblasts were formed which did not reach their normal location in the ventral horn. A section of the spinal cord at the level of the 15th nerve is shown in Fig. 20.

Experiment 404. Of specimens preserved when five days old, or two days after the removal of the wing bud, I shall again confine myself to a detailed description of a single embryo, since others of the same age differ only in the extent of the injury. At this period the bones and muscles are not yet sufficiently differentiated to be identified, so that it is impossible to say exactly what structures have been destroyed, but there is only the slightest prominence in the region of the wing on the operated side. Fig. 9 will show the stage of development as well as the extent of the injury.

Fig. 38 shows the peripheral distribution of the nerve fibers. The branching on the operated side is decidedly less extensive, yet I am unable to demonstrate more than 4 per cent of loss in the average width of the nerve trunks, and I do not feel certain that this is not within the limits of error in measuring these with the

ocular micrometer. The effect on the ganglia is more evident, and the loss is distributed as follows:

	DORSO-VENTRAL DIAMETER PER CENT	LATERAL DIAMETER PER CENT
Ganglion 12.....	1	9
Ganglion 13.....	16.4	11
Ganglion 14.....	3+	3+

The areas within the spinal cord are not sufficiently differentiated to make measurements practical, but Fig. 22, a photograph of the spinal cord in the region of ganglion 13, shows a loss in the ventral horn. The posterior horn seems to be entirely unaffected.

Fig. 23 is a cross-section of the spinal cord in Experiment 79, which was preserved, at the same age as Experiment 404, and agreed with it in all essentials. Other specimens at this stage of development showed the same conditions.

In Experiment 142, killed 24 hours after the operation, in which when examined there was no external evidence of the wing except a slight thickening of the body wall, I am able to demonstrate but a very slight difference in the development of the nervous system on the two sides, and this is found in a decrease in the extent of the peripheral branching and a very slight decrease in the size of the ventral horn. At this period the beginning of the differentiation of bones and muscles is evidenced by aggregations of cells into more dense masses, but no individual structures can be identified. The nerve trunks have reached the inner margin of the wing, and are beginning to branch within it. These conditions are illustrated by Figs. 13, 39 and 24.

Other embryos killed at this period always show a decrease in the extent of the branching of the nerves, but in some cases there is no appreciable effect on the spinal cord.

E. Experiments at Later Stages of Development

The next experiments were designed to test the effect of removing the wing at different stages of its development. I am indebted to Professor Lillie for materials for Experiments 5 and 10, the

operations having been performed by him in the investigation of another problem.

1. After Six Days of Incubation

Experiment 10. This embryo was 6 days old when the wing bud was amputated, and when preserved 4 days later it was found that only the distal part of the limb had been removed, a stump extending nearly to the elbow being left. Although there were some abnormalities in position, shape and size of other parts, it will be seen by consulting Fig. 33, *A*, that the principal nerves involved would be the branches lettered *fw* and *fw*.¹ As the loss would necessarily be small in amount this embryo was not studied with much detail, but there was a slight but evident defect in the ventral horn in the region of the 14th nerve.

2. After Five Days of Incubation

Experiment 5. The period of incubation before the operation in this case was 5 days, and when examined 2 days later it was found that a small stump was left in the region of the right wing. By referring to the figures for the normal side of Experiment 404, which was approximately in the same stage of development as this embryo at the time of the operation, it will be seen that the branches of the peripheral nerves must have been sectioned by the operation. The chief point to be gained from this embryo is that there is evident degeneration of the fibers of the pectoralis, biceps, and triceps nerves. The degeneration extends for only a short distance toward the center and no abnormal cells are found in the motor nucleus.

This is, however, smaller; so that there was evidently a lack of development as well, and this would be expected, for the motor nucleus does not appear to become complete and separated as a distinct unit from the rest of the spinal cord before the 6th day. Cajal states that it is complete by the 5th day, but as the stage of development which he has figured for the 5th day differs but little from that which I have regarded as typical for the 6, the discrepancy probably arises from the difficulty in timing the actual period after fertilization. Moreover the defect in the motor nu-

cleus is less than it is in other specimens where the peripheral areas destroyed were no greater, so that both degeneration and lack of development must doubtless be taken into account.

3. After Four Days of Incubation

Experiment 19. This egg was incubated 4 days before the wing was removed, and preserved one day later. Only a slight elevation was left in the region of the right wing, otherwise the embryo was normal. There was no demonstrable effect on the nervous system except that the branching of the peripheral nerves was less extensive and in some sections the ventral horn was probably slightly decreased, a condition similar to that found when 3-day-old embryos were operated on. There was no degeneration of fibers or cells, although the ends of the nerve trunks were probably sectioned by the operation. This condition I shall attempt to explain later.

F. Removal of Somites

I then decided to study the effects of operating at an earlier stage by removing somites. By choosing a period when the primordium for all the muscles of a given somite is still to be found in the muscle segment, and destroying this, all the motor organs normally innervated by a given nerve would be extirpated. (The only possible exception being areas reached by the sympathetic system.) As nearly as could be determined, this condition is found in the wing region of the chick from about the 45th to the 60th hour of incubation. At this period there is no visible differentiation within the medullary tube, and no outgrowth of peripheral fibers. It would therefore be possible to study the differentiation of the motor nucleus and motor root from the very beginning of their formation in the entire absence of all muscles which they normally innervate.

There are many technical difficulties to be overcome in experiments of this kind, but with suitable apparatus, and some experience, it was found possible to remove the primordium of all the musculature of a given somite without injury to the spinal cord.

A Zeiss binocular microscope was used in order to magnify the somites so that they could be counted, and their extent determined without inverting the object. In order to get sufficient light for this within the egg-shell it is necessary to have either very bright sunlight or an arc lamp with a condenser. The medullary tube and neural crest are very close to the myotomes at this period and the dorsal aortæ are nearly beneath, so that it requires the utmost care not to injure the one or the other. A mechanical method of removing the somite intact would be desirable, but I was unable to devise any means of doing this without injuring the adjacent parts, and I again resorted to electricity. To insure the exact location of the needle an apparatus for holding the electric handles, devised at the University of Chicago, and described by Patterson in the *Journal of Morphology* for April, 1909, was used.

Experiment 60. This egg had been incubated 53 hours before the operation, in which I attempted to destroy the three brachial somites, and was preserved 50 hours after it. As my arrangements for lighting were at that time very inadequate the extent of the injury had to be judged by its effect: About the same areas were destroyed as when the wing bud itself is removed at its first appearance (Fig. 10) and the effect on the nervous system is practically the same. The peripheral branching is less extensive on the injured side (compare *A* and *B* in Fig. 41) and the ventral horn is slightly smaller, both of which conditions obtain in embryos operated on at 3 days and preserved 2 days later.

Experiment 84. In this embryo the operation was performed after 45 hours of incubation, and it is evident that all the musculature of the second brachial somite was destroyed. It was preserved 24 hours later. There is a slight injury to the most dorsal portion of the spinal cord, but as the evidence that the direct injury was confined to this region is so unmistakable, I have not hesitated to regard this specimen as furnishing conclusive evidence concerning the behavior of motor neuroblasts in the absence of all muscles which they normally innervate. Moreover the ganglia are wanting on both sides throughout the brachial region, and as, at the time of operation, the ganglionic crest would lie in close contact with the dorsal portion of the spinal cord, it was doubtless

directly destroyed. This embryo, therefore, offered a favorable opportunity to study the behavior of the motor nerves alone.

On the normal side the nerve trunk, though smaller than when the sensory root is also present, has reached the inner margin of the wing, the typical condition at this stage of development. (Fig. 2.) The fibers on this side follow a straight course and form a compact mass as usual. On the operated side, the ventral horn is much smaller but some fibers are formed; these, instead of following a straight path through the musculature to the region where the inner margin of the wing would normally be found, end in an irregular mass a short distance from the medullary tube. (The same condition was found in Experiment 69 and is represented in Fig. 40.)

Experiment 69. In this specimen, preserved 24 hours after an attempt to remove the three brachial somites, there were two elevations in the region of the wing with a depression between them, but these were together much smaller than the normal wing. On sectioning I found that the myotome of the first brachial somite was entirely missing, but there was some lateral outgrowth of tissue to form the wing. In the second somite there was no myotome, and practically no thickening of the wall in the wing region. In the last somite both the myotome and the wing bud were nearly normal. There had again been a very slight injury to the spinal cord in the most dorsal portion and as the brachial ganglia were entirely wanting it is probable that the neural crest was destroyed by the operation.

The conditions in the second brachial somite were practically identical with those described in the last experiment with the possible exception that a slight thickening of the body wall may contain a very small amount of muscle tissue, not yet sufficiently differentiated to be identified. But as there is no evidence of the myotome, which should be prominent at this period, and the nerve fibers behave exactly as in Experiment 84, it seems probable that absolutely all of the primordium of the muscle cells was destroyed. The ventral portion of the spinal cord and the path of the nerves on the operated side are represented in Fig. 40. The nerve trunk on the operated side follows the normal course in direction and extent, but again lacks the sensory fibers.

From these two experiments, and others which are not so conclusive, but which furnish evidence corroborating these results, I think it is safe to conclude that even when the primordium of all the musculature of a single somite is destroyed, some motor cells and motor fibers will develop within the medullary tube of this segment. They will be fewer in number than on the normal side, they will extend a shorter distance from the spinal cord, and instead of forming a nerve trunk with well-defined boundaries, in the initial stages, they will run freely into the mesenchyme, forming an irregular mass. Their behavior in later stages of development is shown by the experiment which follows.

Experiment 706. The period of incubation was 50 hours, and an attempt was again made to destroy the 17th, 18th and 19th somites. The embryo was examined and preserved 92 hours later. Figs. 11 and 12 give the external appearance. There is a slight elevation in the anterior part of the wing region on the operated side, and examination of sections shows that a portion of the 17th myotome is left, the 18th is entirely wanting, and only a fragment of the 19th is present. No brachial plexus was formed on the operated side, and each nerve must therefore be treated separately. The 12th ganglion is smaller than on the normal side, as is also the corresponding ventral horn, and the course of this nerve, the first brachial, is shown in Fig. 42, *A*. There is no distinct ganglion for the somite following this, but in the place where it should appear there is a clear space much the shape of a ganglion (Fig. 21), containing occasional cells which appear to be of a ganglionic character. These become more numerous posteriorly and finally blend with the 14th ganglion, which is abnormal in shape. The interpretation of this would seem to be that the cells of the 13th ganglion, which would probably have been partially formed at the time of the operation, have been attracted to the region of the 14th, and have united with it. There is no distinct nerve root in this somite, but there is a motor nucleus and most of the fibers arising from these cells pass out at various levels of the spinal cord to join the third brachial nerve. A few fibers end freely in the mesenchyme, as in Experiment 69, a short distance from the spinal cord. The ventral horn of the third somite is smaller on

the operated side, as is also the ganglion, but the nerve is not noticeably smaller, due presumably to the addition of fibers from the somite above. Most of this nerve passes down to the next somite as the sympathetic branch, which is much larger on this side than it is on the normal side. Its course is shown in Fig. 42, *B*. The effect on the spinal cord is illustrated by Fig. 21, a cross-section in the region of the 15th nerve.

In this process of the union of two ganglia and nerve roots is to be found an explanation of the condition described in Experiments 225 and 179, where, in the injured region, there were but four ganglia and nerves instead of six, while no one region of the spinal cord was more defective than another. As was shown in experiments 69 and 84, some nerve fibers do develop in every somite even when all the end organs of the motor cells are destroyed and the sensory area much decreased. (It is impossible to have a region in which there may not be some sensory tissue.) These fibers at first wander freely in the mesenchyme without forming a compact nerve, but are later presumably attracted in the direction of the end organs above or below, and finally the ganglia and nerve roots unite. This process had been completed in Experiments 225 and 179, without leaving any direct evidence of its having occurred.

G. Summary

1 The complete destruction of the primordium of any muscle before its innervation results in the complete suppression of the branch of the peripheral nerve leading to it.

2 Complete destruction of all the muscles of a given somite does not, however, lead to the complete suppression of all the motor cells and motor fibers at this level of the spinal cord, but the number is very greatly reduced, and these present innervate muscles of the somite above or below.

3 The nerve leading to a defective muscle resulting from the destruction of a portion of its primordium before innervation is always decreased in size, but it is usually larger in proportion to the size of the muscle than the normal nerve is in proportion to the size of the normal muscle. This holds true even if the most minute fragment of the muscle is left, and in general it may be

said that the smaller the muscle, the greater is the proportionate size of the nerve.

4 Muscle fibers in abnormal positions without any apparent attachment to the skeleton, always receive innervation.

5 Except in the early stages of development no nerve fibers are ever found wandering free in the mesenchyme, but they follow a definite path leading to a muscle or other end organ.

6 Decrease in the number of the peripheral motor nerve fibers is always accompanied by a decrease in the size of the ventral root. In some cases, an extensive destruction of musculature leads to the union of two ventral roots, and a consequent decrease in the number of nerve trunks.

7 Defects in the size of the motor nerves are always accompanied by a corresponding defect in the ventral horn of the spinal cord, but in no case is the latter entirely lacking, even when the musculature of the somite in which it is located has been completely extirpated.

8 In some specimens the distance from the central canal of the spinal cord to the inner edge of the ventral horn is greater on the operated than on the unoperated side, and the line of demarcation between the ventral horn and the surrounding cells is less definite.

9 Extirpation of sensory areas has the same effect on the spinal ganglia and dorsal roots that the extirpation of motor organs has on the ventral roots and motor nucleus. In case the ventral roots are united the ganglia and sensory roots are also united.

10 A decrease in the size of the ganglia is accompanied by a decrease in the size of the dorsal horn, but this effect is not observable until a somewhat later period.

11 In regions where the ganglia and nerve roots of two successive somites unite, the entire half of the spinal cord is slightly decreased in size, but there is no definitely localized area of loss.

12 Effect on the spinal cord is mostly confined to the region from which the nerves innervating the extirpated parts arise, but slight defects both anterior and posterior to this level are usually observable.

13 The first defect in the nervous system is demonstrable about

24 hours after the operation, its extent and character depending on the extent of injury to the periphery. If only the brachial musculature is removed no effect manifests itself at this time except a slight decrease in the extent of the peripheral branching, and a more doubtful decrease in the size of the ventral horn. If all the musculature is destroyed no definite nerve trunks are formed, but the fibers wander free in the mesenchyme and the ventral horn is more defective.

14 The effect of operations performed on the second or third day differs only in the amount of musculature involved.

15 In operations at a later period, when connection with the end organs has already been made, and therefore involve sectioning a nerve already formed, degeneration may occur. This starts at the peripheral end of the nerve and extends slowly toward the center, so that the spinal cord is affected at a much later stage than it is when the operation causes inhibition of growth.

16 In operations performed on the fourth day, since no degeneration occurs when the destruction of the musculature is not complete, the nerves already formed and sectioned by the operation would seem to be directed to the peripheral organs which are not destroyed.

17 No degenerating fibers can be observed when the extirpated areas are removed before the nerves reach the region of the operation, and in no specimen studied has degeneration reached the nerve cells; so that it may be added as the final demonstrable fact that—

18 Under the conditions of the experiment, the defects which appear in the nervous system are not due to degeneration, but to the failure of the neuroblasts to develop.

H. Discussion

In discussing the bearing of these facts on the problem of the differentiation of the neuroblasts, I shall limit myself, for the sake of clearness, to a consideration of motor neurones, for the extent of an injury to the musculature can be more sharply defined than an injury to sensory areas, and the process of differentiation of the motor neuroblasts can be more easily followed.

So far as the process of differentiation of a motor neurone can be observed, it consists in the movement of the neuroblast into the region of the ventral horn, increase in its size and change in the shape of the cell itself, and the development of a fiber. Every motor cell which is entirely self-differentiating would, under the conditions of the experiment, reach the ventral horn and develop a fiber, for this region is uninjured and the food supply unaffected. But as in some cases the motor nucleus and motor roots are not more than 25 per cent of their normal size, and as it has been shown that this is not due to degeneration but to failure to develop, it is clear that at least 75 per cent of the motor cells are influenced in their development by the presence or absence of the muscles which they normally innervate. Moreover, as 3 or 4 days after the operation there are more undifferentiated neuroblasts on the injured than on the uninjured side, and the cells which are found in the ventral horn are perfectly normal, it seems equally apparent that even the initial stages of differentiation are wanting, and that these cells are entirely dependent on the presence of their muscular end organs for differentiation.

But in every specimen examined, even when there is no trace of musculature in the somite concerned, some motor cells do differentiate completely. We are then forced to one of two conclusions:—either we have two classes of motor neuroblasts, one of which is entirely dependent on stimulation from the periphery for differentiation, and the other entirely independent of such stimulation; or the neuroblasts which do develop normally are influenced by the musculature of adjacent somites. It seems improbable on purely *a priori* grounds that such a wide difference in the physiological activity of cells having a similar origin, and normally a similar destiny, as the first assumption postulates, can exist. But if it is to be assumed that the muscles of other somites are a source of stimulation, it is perhaps necessary to show that the nature of the stimulus may be such that it would naturally be expected to extend to other regions of the spinal cord than that from which the nerves innervating these muscles normally arise. This I shall attempt to do by considering some of the factors involved in differentiation.

The essential feature in the differentiation of any cell must be a change in its protoplasmic structure, and the possible factors involved in this change may be reduced to three, the protoplasm of the cell itself, the food supply, and chemical substances or physical forces coming either directly or indirectly from the medium immediately surrounding the cell. It is obvious that under the conditions of the experiment the food supply is not affected and is, therefore, the same for both the neuroblasts which differentiate and for those that do not, and that the change in the protoplasmic structure of the cell must be due to the way in which the food is assimilated. The manner in which assimilation takes place is still hypothetical, but the most probable theory yet advanced is that the blood proteids are broken down by enzymes or other chemical substances and the active amino acids or other radicals then recombine after the pattern of the cells of the tissue involved. That they recombine differently in different cells is due to the fact that the blood proteids are broken down at different points, and this is in turn due to the presence of different kinds or amounts of enzymes or other activating chemical substances. When differentiation occurs, unless a vital force is postulated, it is necessary to assume that the kind or amount of enzymes must have been increased or decreased, or some other chemical substance or substances have been added or subtracted. The cell during its whole embryonic history has been repeating the same cycle of processes, namely, assimilating food in a definite way, increasing in size and dividing, and it is impossible to conceive that any tendency to develop in a certain direction, any adaptation to conditions, or any need of the organism can produce a new chemical substance or inhibit the action of one already present. Differentiation of any cell must therefore occur because of a change in the chemical composition or physical properties of the lymph surrounding it.

In case of the neuroblasts, the cells outside the medullary tube are also differentiating and the products of their metabolism must change, either in kind or amount, and these products must enter the lymph. It is therefore evident that the presence or absence of muscles in a given somite must influence the character of the

medium surrounding the neuroblasts in its immediate neighborhood, and thus a change in the chemical inter-reactions may be effected. This would give at least a possible explanation of the influence of the muscles on the nerves by which they are normally innervated. Moreover, although the metabolic products of muscular activity would necessarily be found in greatest amount in the somite in which the muscles were located, with the free exchange of lymph they must also affect, to some extent, the character of the lymph in adjacent somites. This would give a perfectly natural explanation for the differentiation of some motor nerves in somites in which the primordium of all the muscles had been extirpated. And it is very significant that in such cases, when the fibers are sufficiently developed for connection with the end organs to be apparent, they are found to innervate structures in the somite above or below. I therefore regard the development of some motor nerves in regions where the muscles which they normally innervate have been destroyed, as in no way inconsistent with the belief that neuroblasts do not differentiate into motor nerves without the presence of muscular end organs, but rather as a necessary corollary of this condition. And as these experiments clearly demonstrate that the majority of neuroblasts which normally develop into motor nerves show no sign of differentiation in the absence of their end organs, I conclude that in the chick all such neuroblasts are entirely lacking in the power of self-differentiation.

III EXPERIMENTS ON AMPHIBIANS

A Historical and Critical Survey of Previous Work

It is a universally accepted principle in biology that the fundamental properties of protoplasm are essentially the same for all organisms, otherwise it is useless to attempt to formulate any general laws governing the animal world. And whether an organ or a cell attains its adult condition because of powers inherent within itself, or whether it must be acted on by outside forces to reach this condition, must be regarded as fundamental. Yet other investigators, working on different forms, have reached the conclusion that the neuroblasts are entirely self-differentiating, and one

is naturally led to inquire if there is any possibility of placing a different interpretation on these observations.

Harrison's experiments, described above, in which portions of the medullary tube placed in a drop of lymph developed nerve fibers, are open to the objection that the lymph necessarily contained products of the metabolism of various organs of the body, and it is therefore not certain, indeed it is improbable, that the neuroblasts were removed from the influence of end organs whose physiological activities were similar to those which they normally innervated. Such an experiment is, therefore, not crucial, though I believe it furnishes the strongest evidence in favor of self-differentiation that has yet been given. The experiments in which pieces of the medullary tube were transplanted to abnormal positions inside the body of the tadpole are open to the same objection to a still greater degree.

A number of points require further investigation before Braus's experiments in removing the limb of *Bombinator* can be regarded as proving conclusively that the neuroblasts are self-differentiating. His conclusions rest mainly on the fact that ten days after the removal of the limb the brachial plexus is as large on the operated as on the unoperated side of the animal, although some weeks later, after metamorphosis, it is smaller. "Die Verschmächtigung bei dem älteren Objekt," he says, "ist demnach eine sekundäre." But before this can be accepted the degree of differentiation of the nervous system ten days after the operation must be considered, and the impossibility of stimulus from any brachial muscle or other muscles be established.

In regard to the specimens examined near the end of metamorphosis he says, "Derselbe (the brachial plexus) ist allerdings dünner als derjenige der normalen Seite der Larve und auch ventralwärts mit *Muskelanlagen der Bauchwand in Verbindung*." In the specimen killed 10 days after the removal of the limb he states that the trapezius and interscapularis muscles are present, so that in neither of these cases are the neuroblasts removed from all muscular influence, and experiments on the chick have shown that the defect in the size of the nerves is not proportional to the defect in the musculature. A certain degree of development may

thus be accounted for, and it cannot be asserted that the condition found in the older embryos is due to degeneration rather than to lack of development, unless there is evidence of degeneration, or it can be shown that the differentiation of the motor horn is once completed, and the motor roots at some period contain the full number of fibers, for the question of self-differentiation must be reduced to a consideration of individual cells, and under the conditions of Braus's experiments any neuroblast which normally differentiates into a motor nerve, and failed to do so here, could not be self-differentiating.

B Purpose, Methods, and Material

The following experiments were conducted for the purpose of making further observations on this point:

Obviously the method of studying this problem by extirpating limb buds cannot give crucial results with any animal in which the regeneration of these parts occur, and I have not succeeded in getting amphibian material in which the limbs are not regenerated repeatedly. In my experiments I found that *Bufo americanus* regenerated less rapidly, and perhaps less completely, than *Amblystoma tigrinum* or *Rana pipiens*, and probably in no instance were all vestiges of musculature connected with the limb removed. Whatever the cause, I have no specimen which was allowed to live long enough for regeneration to be expected in which it did not occur. Nevertheless I believe that the results obtained indicate that the process of differentiation of the neuroblasts is essentially the same in the amphibians as it is in the chick.

Amblystoma tigrinum, *Rana pipiens*, and *Bufo americanus* were used. With *Amblystoma*, sometimes the fore limb, sometimes the hind limb, and sometimes both were removed, either when the limb bud was just apparent or before it had attained any considerable size, and in many instances the operation was repeated as often as the beginning of the formation of a new limb bud could be detected. The limbs develop slowly, and in the early stages a large proportion of the musculature innervated by the sciatic plexus forms such an intrinsic part of the body of the tadpole that the removal of the projecting portion of the limb leaves many muscles

quite intact. This fact, taken in connection with the constant and apparently immediate regeneration, makes it necessary to remove the limb a number of times before there is any considerable difference in the amount of musculature on the two sides. But the differentiation of the nervous system is equally slow and it is possible to materially decrease the muscular mass before the motor nerves are well developed. The toad was treated in the same way, but regeneration occurs more slowly, and differentiation of the nervous system more rapidly, so that I have no specimen in which the limb was removed more than twice. With the frog, I shall mention only experiments in the removal of somites.

The methods used to ascertain the extent of the defect in the nervous system were the same as those employed in studying the chick embryo.

C Experiments on Amblystoma

Experiment 25. The left hind-leg bud was first removed May 3 and again successively on May 22 and June 1. The larva was preserved June 5. Some idea of the difference in the two limbs at this time may be obtained from Fig. 25. The bones of the pelvis are present but smaller on the operated side, and a small portion of the femur is distinguishable, but there is no evidence of any of the other bones of the limb. For the purpose of the present problem it will be convenient to divide the muscles innervated by the sciatic plexus into three groups: (1) The proximal muscles of the thigh; (2) the muscles of the leg proper; (3) the muscles of the bladder and its immediate neighborhood. In this experiment, groups (1) and (3) are uninjured. Of group (2), there is a considerable muscular mass, but it is impossible to identify individual muscles, so that it can only be said that the amount of musculature is much decreased. The sensory area is, of course, also decreased.

The sciatic plexus is formed from the union of three nerves as shown in Fig. 43, *A*, drawn from the normal side of this larva. Nerve I innervates mainly the proximal portion of the thigh, and as this region is uninjured, only enough of its course is given to indicate the branch which joins the sciatic plexus. *B* drawn on the

same scale as *A* gives the course of these nerves on the injured side. The sciatic plexus itself, at the point marked (4), is about 75 per cent smaller on the operated side, and branches (1) and (2) are proportionately decreased. The branch marked (3) goes to the bladder and is of normal size. Following backward to the individual nerve trunks which form the plexus, II is found to be 40 per cent and III, 64 per cent smaller on the operated side. Of the two ganglia which correspond to these nerves, a rough but conservative estimate shows one to have lost at least 50 per cent in volume, while the other has lost about $33\frac{1}{3}$ per cent. Fig. 27 was taken from a transverse section of the spinal cord in the region of the second nerve of the sciatic plexus. This is fairly typical of the cord throughout the injured region, although occasionally a section is found in which there is little difference between the two sides.

But while there is a decided defect in the nervous system at this period, 33 days after the first removal of the limb, from this specimen alone it is impossible to determine whether it is due to degeneration or to failure to develop, although there is no evidence of degeneration. As will be seen from the figures the nerve trunks are, at this time, well established, and differentiation within the spinal cord, if not complete, is at least well advanced. It is therefore necessary to examine younger specimens for signs of degeneration, and to determine as nearly as possible the period at which differentiation of the motor neurones is complete.

Younger Embryos. I found that the length of time which must elapse after the operation before a defect in the nervous system appears depends on the degree of differentiation attained, and neither age nor size is an exact criterion of this. Size is a much better measure, but my records were made with time as the standard, with some comparative data in regard to length and the rapidity of growth. In one specimen in which the limb was slightly larger than usual at the time of its removal, and in which the larva continued to grow rapidly, a slight diminution in the size of the motor horn can be demonstrated at the end of ten days. Fig. 26 is typical of the spinal cord in the operated region of this embryo, but there are sections in which no difference in the two

sides can be detected, and even a few in which the motor nucleus is larger on the injured side. On the whole, however, there is no doubt but that the ventral horn of the normal side is larger. The brachial plexus is also somewhat decreased in size. Aside from this specimen, I have no other in which the nervous system is noticeably affected until 14 days after the removal of the limb. The following experiment will serve to illustrate this condition:

Experiment 27. The forelimb, in which the beginning of the formation of two toes could just be detected, was removed May 3, and the specimen was preserved May 17. The animal grew slowly and regeneration was correspondingly slow, but evident. At the time of preservation the nerves have entered the limbs, but are still little developed, the main trunk being but .03 mm. in diameter in the widest part. The motor roots are very minute, and the motor horn is just beginning to be perceptible as a distinct unit. Figs. 28 and 29 are taken from a cross-section in this region. The defect shown in the spinal cord is rather extreme, the difference between the two sides not being quite so great in most cases.

I have no specimen younger than this, except the one just cited, in which the nervous system is not developing normally, and I have older specimens in which it is also normal. But in all of these cases some of the limb muscles are present, and regeneration is going on, so that the amount of loss in the mass of the musculature is comparatively small, and the point I wish to establish is that when the peripheral area is extensively injured a defect appears in the nervous system before differentiation is completed. Comparing the nervous system of the normal side of these larvæ which were killed respectively, 10, 14, and 33 days after the removal of the limb, it is evident that there are less fibers in the nerve trunks and nerve roots, and less cells in the motor horn of the two former. Since a defect has already appeared, there can be no time at which all the neuroblasts which normally differentiate into motor nerves are found together in the motor horn.

Have the missing motor cells been formed and then degenerated? To answer this I shall compare the larva in Experiment 27 with another of the same age, kept under the same conditions, and apparently developing parallel with it, but which was killed nine

days after the removal of the limb. In this specimen the normal nerve roots can be traced but a short distance beyond the spinal cord (Fig. 30), and I cannot demonstrate with certainty the presence of any motor fibers, but there is some evidence of the beginning of the formation of the ventral horn. Only five days elapsed between the time when this larva was killed and the one in Experiment 27. There is no sign of degeneration in the latter case, and when it is remembered that, so far as is known, the atrophy of tissue requires a longer period of time than its formation (compare surgical literature and experiments described by Bethe and others, and Experiment 5 on the chick embryo, for this point) there seems little possibility that, at the rate of development of this animal, these cells could have been formed and completely degenerated. Moreover, as the extent of the defect is continually increasing, if all the motor nerves are self-differentiating, there must be a continual degeneration, and tissue in the process of destruction is so apparent that it could not have escaped detection in some of the specimens studied. I can therefore see no reason for believing that the defects in the nervous system of the specimens studied is due to degeneration.

D Experiments on Bufo lentiginosus Americanus

Little need be said in regard to the experiments on the toad, for they furnish no new points of interest. The presence of some muscles, and the fact that regeneration occurs, makes it impossible to study the behavior of the nerves in the absence of all the organs which they normally innervate. But in one specimen preserved nine days after the removal of a hind limb, there was a defect in the motor horn, although it was not yet completely formed on the normal side. Examination of several specimens showed that the motor horn and ventral root are always smaller if there is a marked loss in the mass of musculature, and no evidence of degeneration could be found. The toad, therefore, confirms the results obtained by experiments on amblystoma.

E Removal of Somites in Rana Pipiens

I thought it possible that the capacity of the frog for regeneration might not extend to the reproduction of a whole somite, and I therefore tried removing these as a means of getting at the initial stages in the development of the nerve fibers. For this purpose larvæ were removed from the egg capsules from 24 to 36 hours before hatching, and from 1 to 3 somites completely removed. It is perfectly easy to do this without injuring other parts, but the animals all died three or four days later; however, by leaving a small amount of tissue close to the spinal cord and notochord they develop as rapidly as the normal larvæ, and after a very few days there is no noticeable external defect.

On sectioning these the loss is evident, but regeneration and probably re-differentiation of adjacent parts is found to occur, for no muscles are missing, but all are decreased in size and this loss extends to somites above and below the injured region. This process is well advanced before there is any well-defined differentiation of the medullary tube, so that again it is impossible to ascertain the effect of the complete lack of the end organs of any nerve. Fig. 31 shows the condition of the musculature in a specimen preserved two months after the removal of the somites, and Fig. 32 shows the spinal cord of this section more highly magnified. The defect in the ventral portion of the spinal cord is evident throughout the affected region, and comparison with older larvæ shows that neither the motor horn nor the ventral roots have attained complete development. These were also examined for signs of degeneration, but none were found, so that these results again confirmed the conclusions reached by experiments on *Amblystoma*.

F Summary of Conditions Observed in Amphibians

1 If a limb bud of *Amblystoma tigrinum* or *Bufo americanus* is removed the following conditions are found:

a If the regeneration is rapid and extensive the development of the nervous system is apparently normal.

b If the regeneration is slight, or if the limb is repeatedly

removed, so that there is a considerable loss in the amount of musculature, the nerve trunks, ganglia, and that portion of the spinal cord in direct communication with the injured area are defective.

c The extent of the defect depends both on the amount of musculature destroyed and the degree of differentiation of the spinal cord at the time when the larva is preserved. In some instances a number of branches of the nerve trunk are entirely wanting.

d There is no appreciable effect on the medullary tube until differentiation is apparent, but the effect always appears before the development of the ventral horn and ventral roots is complete.

e No nerve fibers are found wandering free in the mesenchyme, but they always follow a well-defined path leading to a muscle or other end organ.

2 If the greater portion of a somite or somites is removed from a larva of *Rana pipiens* shortly before hatching, regeneration occurs but the amount of musculature is defective, and the nervous system is affected in the same way as that of the amblystoma or the frog when a limb bud is removed.

3 In any of these cases it is impossible to demonstrate degenerating nerve cells or nerve fibers, and the defects must therefore be due to the failures of these to develop.

G Conclusions

Comparing the data obtained from experiments on amphibians with that obtained from the study of the chick embryo, it is evident that they agree in all essentials; in both, the destruction of a peripheral area leads to a defect in the nervous system, which is due, not to degeneration, but to the failure of certain neuroblasts to differentiate normally. The difference in the extent of the defect and the length of time required for its appearance are obviously due to regeneration, and to the slow rate of differentiation of the nervous system in amphibians. And we are again forced to conclude, either that there are two classes of neuroblasts, one of which is self-differentiating and the other not, or that these which do develop normally are stimulated to do so by the presence of end

organs. In every specimen that I have examined it is certainly possible that the neuroblasts which develop into motor nerves may have been influenced by the regenerating muscles which they normally innervate, or by other muscles in the immediate neighborhood, and in no case cited in the literature which has come under my observation has this possibility been excluded. I believe, therefore, that further evidence is necessary before it can be asserted that any neuroblasts are self-differentiating, and until such evidence is produced, that it is safe to assume that all are alike dependent on stimulation from end organs or the products of the activities of end organs, for differentiation.

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PLATE I

Fig. 1 External view of normal chick embryo after 74 hours of incubation. *w*, wing bud.

Fig. 2 Cross-section through the brachial region of the embryo shown in Fig. 1. *g*, ganglion; *m* myotome; *n*, end of nerve trunk; *w*, wing bud.

Fig. 3 Cross-section through the brachial region of the embryo in Experiment 538.

<i>bi</i>	biceps	<i>ps</i>	pectoralis secundus
<i>cb r</i>	coraco-brachialis	<i>pt</i>	pectoralis tertius
<i>dt</i>	deltoides	<i>ss</i>	supraspinatus
<i>ld</i>	latissimus dorsi	<i>ssp</i>	subscapularis
<i>pmj</i>	pectoralis major	<i>ti</i>	teres et infraspinatus.

Fig. 4 Cross-section through the brachial region of the embryo in Experiment 206.

Fig. 5 Cross-section through the brachial region of the embryo in Experiment 225.

Fig. 6 Cross-section through the posterior part of the wing region in Experiment 179.

FIG. 1

FIG. 2

FIG. 3

FIG. 5

FIG. 4

FIG. 6

PLATE II

Fig. 7 External view of the embryo in Experiment 225. This embryo was ectopic, the body wall as well as the wing having been injured by the operation.

Fig. 8 External view of the same embryo, normal side.

Fig. 9 Cross-section through the brachial region of the embryo in Experiment 404.

Fig. 10 Cross-section through the operated region in Experiment 60.

Fig. 11 External view of the normal side and, Fig. 12, of the operated side in Experiment 706.

Fig. 13 Cross-section through the brachial region in Experiment 142.

FIG. 7

FIG. 8

FIG. 9

FIG. 10

FIG. 11

FIG. 12

FIG. 13

PLATE III

Fig. 14 Cross-section of the spinal cord in the region of the 15th nerve of Experiment 538. The inner margin of the motor nucleus is marked on each side by a cross. The length of the posterior horn from *A* to *B* is also decreased on the operated side.

Fig. 15 Cross-section of the spinal cord in the operated region of Experiment 206. Inner margin of motor nucleus marked by a cross.

FIG. 14

FIG. 15

PLATE IV

Fig. 16 Cross-section of the spinal cord in the region of the 16th nerve, Experiment 225. Inner margin of the motor nucleus marked by a cross. Operated region is represented on the right in this figure.

Fig. 17 Cross-section representing the typical condition of the spinal cord between the wing and the leg in Experiment 225. Operated region on right.

FIG. 16

FIG. 17

PLATE V

Fig. 18 Cross-section of the spinal cord at the level of the 15th nerve in Experiment 179. Inner margin of motor nucleus marked by a cross.

Fig. 19 Cross-section of the spinal cord in Experiment 179, typical of the condition between the wing and the leg of this embryo.

FIG. 18

FIG. 19

PLATE VI

Fig. 20 Cross-section of the spinal cord at the level of the 15th nerve in Experiment 324.

Fig. 21 Cross-section through the brachial region of Experiment 706. The myotome, ganglion and nerve trunk are missing on the operated side. The clear space left apparently by the migration of the ganglion cells to unite with the ganglion below is marked by a +.

FIG. 20

FIG. 21

PLATE VII

Fig. 22, 23 Cross-sections of the spinal cord at the level of the 15th nerve in Experiments 404 and 79, respectively.

Fig. 24 Cross-section of the spinal cord in the operated region of Experiment 142.

FIG. 22

FIG. 23

FIG. 24

PLATE VIII

Fig. 25 Cross-section of *Amblystoma tigrinum*, Experiment 25, 33 days after the first removal of the limb bud. *l*, regenerating limb.

Fig. 26 Cross-section of the spinal cord 10 days after removing the limb in *Amblystoma tigrinum*. *v*, ventral horn in operated side.

Fig. 27 Cross-section of the spinal cord of *Amblystoma tigrinum*, 33 days after the first removal of the limb bud in Experiment 25.

Fig. 28 Cross-section through the operated region in Experiment 27, *Amblystoma tigrinum*. *l*, limb of normal side.

FIG. 25

FIG. 26

FIG. 28

FIG. 27

PLATE IX

Fig. 29 Cross-section of the spinal cord in Experiment 27 more highly magnified. *v*, ventral horn region on operated side.

Fig. 30 Cross-section of the spinal cord nine days after the operation in a specimen developing parallel with Experiment 27.

Fig. 31 Cross-section through the operated region two months after the removal of two somites in *Rana pipiens*.

Fig. 32 Spinal cord of same embryo more highly magnified.

FIG. 29

FIG. 30

FIG. 31

FIG. 32

PLATE X

Fig. 33A Peripheral distribution of the brachial plexus on the normal side of Experiment 538.

<i>bi</i>	biceps	<i>ps</i>	pectoralis secundus
<i>cb</i>	coraco-brachialis	<i>pt</i>	pectoralis tertius
<i>ct, ct', ct''</i>	cutaneous branches	<i>sc</i>	subclavius
<i>dt, dt'</i>	deltoïdes	<i>h</i>	scapulo-humeralis
<i>fw, fw'</i>	fore wing	<i>ss</i>	supraspinatus
<i>ld</i>	Latissimus dorsi	<i>ti</i>	teres et infraspinatus
<i>p mj</i>	pectoralis major	<i>tr, tr'</i>	triceps

B. Peripheral distribution of the brachial nerves on the operated side of Experiment 538. The nerves are labeled as in Fig. 4.

C. Distribution of the brachial nerves on the operated side in Experiment 206. Lettering as in Fig. 4.

The nerves of the normal side were identical both in size and distribution with those in Experiments 538, Fig. 33 A.

Fig. 34 A Peripheral distribution of the brachial nerves of the normal and, B, of the injured side, in Experiment 225. Nerves lettered as in Fig. 4.

FIG. 33

FIG. 34

PLATE XI

Fig. 35A Peripheral distribution of the brachial nerves on the injured side and, B, on the normal side in Experiment 179. Lettering as in Experiment 538. *Un*, muscles unidentified.

Fig. 36A Distribution of the brachial nerves on the normal side and, B, on the operated side of Experiment 324. The nerves which are not lettered innervate areas which are not yet sufficiently differentiated to be identified with certainty.

Fig. 37 Typical distribution of each of the six nerves between the wing and the leg of the chick. *d br*, dorsal branch; *l br*, lateral branch; *v br*, ventral branch.

Fig. 38A Brachial plexus of the normal side and, B, of the injured side in Experiment 404.

Fig. 39A The brachial plexus of the normal and, B, of the injured side in Experiment 142.

FIG. 35

FIG. 36

FIG. 37

FIG. 38

FIG. 39

PLATE XII

Fig. 40 Spinal cord and nerve fibers on the operated side of the second brachial somite in Experiment 69.

Fig. 41A Brachial plexus on the normal side and, B, on the operated side of Experiment 60.

Fig. 42A First brachial nerve and, B, the second brachial nerve on the operated side of Experiment 706.

C. Brachial plexus on the normal side of Experiment 706.

Fig. 43A Sciatic plexus of *Amblystoma tigrinum*, Experiment 25, normal side, 33 days after the first removal of the limb bud.

B Sciatic plexus of the operated side of the same specimen. For explanation, see text.

FIG. 40

A

B

FIG. 41

A

B

C

FIG. 42

A

B

FIG. 43