Motility in Chick Embryos with Substitution of Lumbosacral by Brachial and Brachial by Lumbosacral Spinal Cord Segments

C. H. NARAYANAN AND VIKTOR HAMBURGER
Department of Biology, Washington University, Saint Louis, Missouri 63130

ABSTRACT In two and one-half-day chick embryos, brachial segments were substituted for lumbosacral segments (Bl series) and vice versa (Lb series). Recordings were made of spontaneous (type I) motility in 9- to 17-day embryos at daily intervals. Total embryonic motility (per cent time spent in activity in 10-minute periods) and total wing and leg motility did not differ significantly from normal in both series. Furthermore, frequency of movements (per minute) was recorded for wings and legs separately and for combined wing/leg movements. In both experimental series, combined wing/leg movements were significantly more frequent and isolated wing or leg movements less frequent than in normal embryos. This means that two brachial or two lumbosacral segments in the experimental embryos are more tightly coupled than the brachial and lumbosacral segments in a normal embryo. Regional differences between brachial and lumbosacral segments are thus demonstrable as early as nine days. The increased frequency of combined wing/leg movements persists after hatching. In hatched chicks of the Bl series, legs are incapable of making alternating stepping movements; they are always abducted and adducted simultaneously as in wing flapping. This is another expression of regional specificity of the brachial cord. We assume that regional specifications of spinal cord segments are built into the cord at the stage of operation.

The experiment of interchange of different regions of the embryonic spinal cord was initiated by Detwiler ('23), using early tail bud stages of the salamander, *Ambystoma punctatum*. The experiment was designed to explore several significant problems of neuroembryology, such as the determination of regional differences in the spinal cord; the formation of nerve plexuses and intrinsic nerve patterns in limbs innervated by heteronymous spinal cord segments, and the capacity of foreign nerves to activate limb movements. Detwiler and his students (see '36) as well as Holtzer ('50), Piatt ('57), Székely ('63) and Straznicky and Székely ('67) have extended this work in urodeles. B. Wenger ('51) and Straznicky ('63, '67) have performed corresponding experiments in chick embryos, with similar objectives. The experiments reported below are based on these previous investigations and related limb transplantations.

It has been established that foreign nerves grow into limb buds and form typical sensory and motor patterns although, in urodeles, the nerve distribution is never completely normal (Piatt, '57). In chick embryos, only the distribution of major nerves was traced and found to be normal. Obviously, the limb structures play a major role in the determination of nerve patterns. As far as *function* is concerned, Detwiler ('20) has shown by means of transplantation of limb primordia to the flank, in *Ambystoma*, that coordinated forelimb movements can be performed only if at least one segmental nerve is derived from the brachial spinal cord (segments 3–5). Transplants innervated exclusively by thoracic nerves showed poor motility and no coordinated movements. Holtzer ('50) and Székely ('63) have confirmed these findings. This was the first evidence for the basic principle, amply documented later by Weiss ('41, '55) that, from early stages on, specific neuronal connectivities for coordinated limb movements are built into the brachial and lumbosacral regions of the spinal cord sectors.

In salamander embryos, the prospective thoracic segments (7–9) can acquire the "brachial" properties, including support of coordinated movements, if the transplantation to the brachial position is done in early tail bud stages (Detwiler, '20; Straznicky and Székely, '67). In chick embryos, no such adjustment was found (Wenger, '51; Straznicky, '67).

Of particular interest are experiments in which the brachial and lumbosacral sectors are interchanged, since normally they both produce limb movements, though of different rhythms. In salamander gait, similar fore- and hindlimb movements alternate in a characteristic sequence; but, of course, profound differences exist in posture and in the anatomical structure of the fore- and hindlimbs. Székely ('63) observed in larvae of *Pleurodeles waltlii* and *Triturus vulgaris* after transplantation of lumbosacral segments to the brachial level, and in the reverse experiment, that either completely normal alternating coordination developed, or a forelimb moved in parallel with the hindlimb of the same side, "so that during smooth locomotion, the camel-gait walking was obvious" (p. 437). There are thus indications of regional-specific differences between the two limb-innervating spinal cord levels in urodeles, from early larval stages on.

The analysis of such differences should be greatly facilitated in chicks, where the posthatching performance of the wings and legs is strikingly different; the wings perform synchronous, parallel movements while the legs show alternating stepping movements. Straznicky ('63) substituted lumbosacral for brachial segments in two and one-half-day chick embryos (stages 15 and 16) and made the interesting observation that after hatching the movement of a wing was tightly linked with that of the leg of the same side. Apparently, wing flapping movements were never observed. Movements of the wing were restricted to the shoulder joint. The author suggests "that the grafted lumbosacral segments can somehow select from impulses descending from higher centers to answer only to those which are addressed to the normal lumbosacral segments" (p. 151), though other explanations are possible (see p. 428).

Our own experiments on chick embryos have extended the analysis in two directions: In addition to repeating the experiment of Straznicky ('63) we have performed a large series of transplantations of the brachial to the position of the lumbosacral segments. The hatched chicks of this series showed the same close coupling of wing and leg movements as was observed by Straznicky. But the legs, rather than performing alternating stepping movements were ab- and adducted synchronously and in parallel, as in wing flapping, thus demonstrating the fixity of the brachial neural apparatus. Our main effort was directed toward an analysis of *embryonic* motility in the two series of experiments. We had established previously (Hamburger et al., '65; Hamburger, '68, '70) that limb movements, up to 17 days are spontaneous (non-reflexogenic), periodic, convulsive in type, rather jerky and with no obvious coordination of left and right wings or legs, except for occasional wing flapping, toward the end of the period. It is in the nature of these movements that region-specific differences between the brachial and lumbosacral neural action systems are difficult to detect. However, by recording certain parameters of motility, we were able to trace certain differential patterns back to the nine-day stage, which is the earliest stage we have studied.

MATERIALS AND METHODS

The majority of the embryos used in the current investigation were from the Kimber strain of white Leghorns (Flock no. 137.H); less than a third came from the Babcock strain of white Leghorns. All the eggs were incubated for about 52–60 hours in a commercial force-draft incubator at 37.5° ± 1° C and 70–80% relative humidity.

Surgical procedure

All the operations were carried out aseptically. Two series of operations, designated *Bl* and *Lb*, respectively, were performed in embryos of stages 15–16 (Hamburger and Hamilton, '51), i.e., at the 24- to 28-somite stage, or 52–60 hours of incubation (fig. 1). In the *Bl* group the lumbosacral spinal cord segments 23–29

were replaced by a corresponding length of four brachial spinal cord segments plus the equivalent of three thoracic spinal cord segments, which were excised from a donor embryo. Similarly, in the *Lb* group the brachial spinal cord segments 13–16 plus three prospective thoracic spinal cord segments were replaced by seven lumbosacral spinal cord segments excised from a donor embryo.

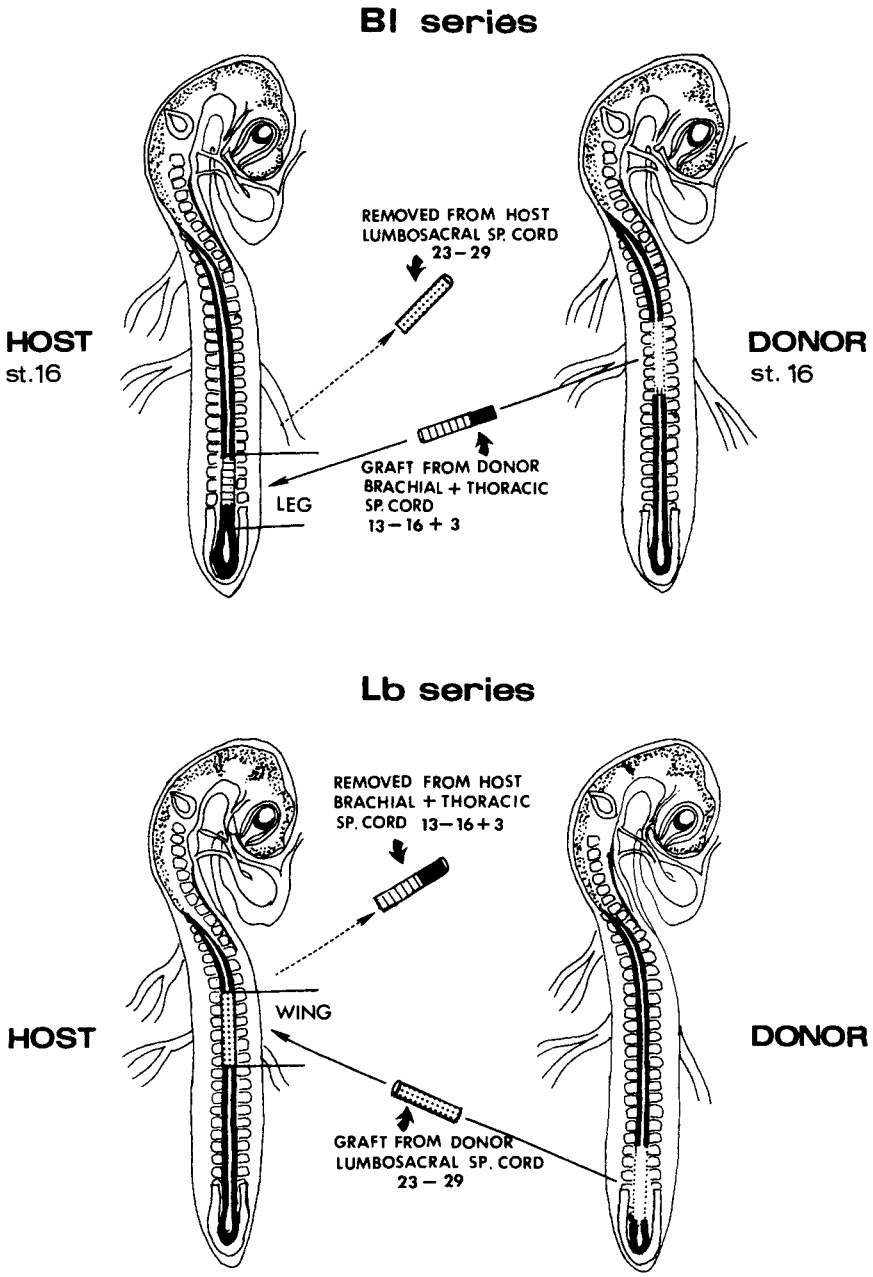

Fig. 1 Schema of two types of operation, stages 15–16. Top: Transplantation of brachial to position of lumbosacral spinal cord (*Bl* series). Bottom: Transplantation of lumbosacral to position of brachial spinal cord (*Lb* series).

The method of opening the egg and the preparation of the embryos for microsurgery have been described in previous publications (e.g., Narayanan, '70). The vitelline membrane and the portion of the neural tube to be manipulated in the donor embryos of either series were lightly stained with Nile blue sulfate impregnated in agar. The region of the neural tube to be removed in the host embryos of either series was stained with neutral red impregnated in agar. This differential staining of graft tissue and host tissue helped greatly in the exact positioning of the graft while being maneuvered into the gap in the host embryo.

The excision of the spinal cord segments from the donor and host embryos of both series was carried out with the vibrating needle (Wenger, '68). Utmost care was taken to avoid injury to the adjoining somites while preparing the gap to receive the graft tissue, and also, while transferring the graft tissue with a Spemann micropipette from donor to host embryo. In the majority of cases the notochord was included in the grafted spinal cord segments in order to prevent the graft tissue from collapsing or from becoming curved.

Controls for the experiments were obtained by excising the lumbosacral or the brachial level of the spinal cord, respectively, and then refitting them at the same level. All operated embryos and controls were raised as long as possible, and in some cases up to and beyond hatching. In all, 297 embryos have been used in this study. Of these 192 embryos belonged to the *Bl* group; 105 were of the *Lb* group.

Recordings and observations

Experimental and control embryos which were used for recording were prepared in the following way: after being removed from the incubator the embryos were kept in a temperature- and humidity-controlled observation box made of plexiglas, as described by Hamburger et al. ('65). Each embryo was allowed to acclimatize in this box for approximately 15 minutes. Observations of the embryo were made through a window in the shell covered with a coverglass and sealed with paraffin. Light from a Universal AO microscope illuminator filtered through a glass heat filter was directed on the embryo. The spontaneous activity of the embryo was observed under a binocular stereomicroscope and recorded mechanically by pressing recording keys connected to a Sanborn Polygraph. Quantitative aspects of motility for both the control and experimental embryos were recorded. All recordings described below were made at one-day intervals between incubation days 9 and 17.

The actual amount of time spent in activity and inactivity was recorded for each embryo for one ten-minute period. Two recordings were made simultaneously during this interval: One was based on any or all movements that could be observed; this is referred to as *total activity.* The second recording was based on the activity specifically of the wing movements in the case of the *Lb* group, and of the leg movements in the case of the *Bl* group. The latter recordings included movements as part of the total activity as well as independent activity of either the wing or the leg alone in the absence of any other movement. This second set we designate as *total wing activity* or *total leg activity* respectively. The recordings represent the duration of each activity and inactivity phase performed during a ten-minute observation period. The per cent of time spent in activity during the ten-minute observation period was calculated. Even very short phases of activity and inactivity were included. The figures for table 1 were obtained by pooling all data for all recordings of all embryos at a given stage.

After the completion of the recordings of spontaneous activity, each embryo was observed and recorded for an additional period of five minutes. During this time the *frequencies* of three categories of movements were recorded: wing movements in the absence of other movements; combined wing and leg movements; and leg movements in the absence of other movements (table 2). The latter recordings were made with a stenograph recording device as described by Hamburger and Oppenheim ('67).

Differences between means are assumed significant at $P < 0.05$ or lower P-values. Statistical comparison of experimental and control means were made

using the nonparametric Mann-Whitney "U" test (2 tailed) for all quantitative data, except for the last mentioned relations of wing and leg movements. For the latter the Students "t" test was used (Sokal and Rohlf, '69).

Detailed notes were taken of the prehatching and hatching movements during the period between 17 days and hatching, but no quantitative recordings were made. In order to compare the behavior of the experimental embryos with those of the control embryos, careful observations were made of the following behavioral events as described by Hamburger and Oppenheim ('67): (1) pre-tucking, (2) tucking, movements involved in getting the right side of the head under the right wing, (3) membrane penetration movements associated with puncturing the chorio-allantoic and overlying shell membrane, (4) pipping movements; that is, the first cracking of the shell preceding hatching, and (5) climax, the actual hatching process when the embryo rotates and cracks the shell around the contour of the air space and emerges from the shell. Posthatching behavior was also studied and recorded cinematographically.

Dissection and preparation for histological study

Only the embryos which attained hatching age or survived artificial hatching, and controls were sacrificed. They were fixed in neutral 10% Formalin or in the Chloral hydrate/alcohol/nitric acid formula recommended for the Cajal-DeCastro silver technique (Levi-Montalcini, '49), or in Trichloroacetic acid alcohol fixative of Huber ('27). For optimum preservation the fixing fluid was injected into the body and the embryos were then immersed in the fixing fluid. In order to prepare the legs for cross sections in the *Bl* group, the nitric acid content in the DeCastro fixing fluid was increased to 5% as suggested by Mayer (quoted by Lee-Microtomists Vademecum), to ensure decalcification of the bones. The lumbosacral level of the chicks of the *Bl* group and the brachial level of the chicks of the *Lb* group were dissected while still in the fixing fluid. The plexus nerves in both experimental series were carefully dissected under a binocular microscope down to the elbow or thigh level respectively. Thereafter, the grafted spinal cord segments of representative cases were embedded in paraffin and sectioned at 12 μ. The sections were stained with toluidine blue or Heidenhain's hematoxylin.

Spinal cord, plexus, nerve patterns

In order to evaluate the effects of the spinal cord substitutions on behavior, it was necessary to ascertain the structural condition of the spinal nerves. The dissections showed that in all cases the grafts had healed in perfectly and were continuous with the adjacent cord segments of the host. Further evidence for spinal cord continuity in the hatched individuals was provided by the unitary response of the animal to visual and acoustic stimuli. Further details will be given for the two series separately.

Bl-series. The emergence of nerves from the grafted spinal cord and the nerve patterns in the lumbosacral plexus and leg were ascertained through careful anatomical dissections in 25 cases. The grafted part was readily identified by the lateral swelling which however was less pronounced than in controls (see figs. 6, 7). The glycogen body characteristic of the lumbosacral segment was absent.

The plexuses formed by the transplanted four brachial plus three thoracic segments were exact replicas of the normal lumbosacral pattern; typical crural and ischiatic nerves emerged from the plexuses. The former normally consists of three (or 4) roots and the latter of four roots, though variations occur in rare instances. In 19 of the 25 experimental cases (i.e., 75%), the plexus nerves of both sides were organized in the typical fashion, the crural division comprising invariably three roots and the ischiatic division four roots; that is, the thoracic segments participated in the plexus formation. The perfect replication is also evidenced by the formation of the obturator nerve (Ob in figs. 6, 7) which follows its normal course. In the remaining six cases, there were variable deficiencies in the number of segmental nerves, and differences between left and right plexuses. For instance, in Bl56 (fig. 7), the ischiatic plexus of the left side is represented by only one nerve, but that on the right side

is complete. It should be mentioned that in all cases, the diameter of all crural and ischiatic nerves was considerably smaller than in the controls.

Lb-series. Seven cases were dissected. The lateral enlargement of the transplanted cord was very conspicuous, and the glycogen body was fairly well developed. In four of seven cases, the plexus was deficient, but the first two segmental nerves were always present. Some of the posterior transplant segments apparently formed thoracic nerves. All nerves in the experimental cases were considerably smaller in diameter than normal.

RESULTS

Survey of the material

Thirty embryos of the *Bl* group were used for recording of spontaneous motility (type 1, Hamburger and Oppenheim, '67) and frequency of movements, up to 17 days of incubation (tables 1, 2); 13 embryos were artificially hatched (i.e., pulled out of the shell). The posthatching behavior was observed in two cases, Bl 32 and Bl 56, for nearly a week.

For the *Lb* group, 25 embryos were used for recording of spontaneous motility (type 1) and frequency of movements (tables 1, 2); seven embryos were artificially hatched and studied for 6 to 12 hours after the time at which they would have normally hatched.

Spontaneous motility (type 1), 9 to 17 days — Bl series

Figure 2 shows that total activity increases steadily to a peak at day 13, and then declines to almost the level of activity observed on day 9. The curve for the experimental embryos runs parallel to the curve for the control embryos, but

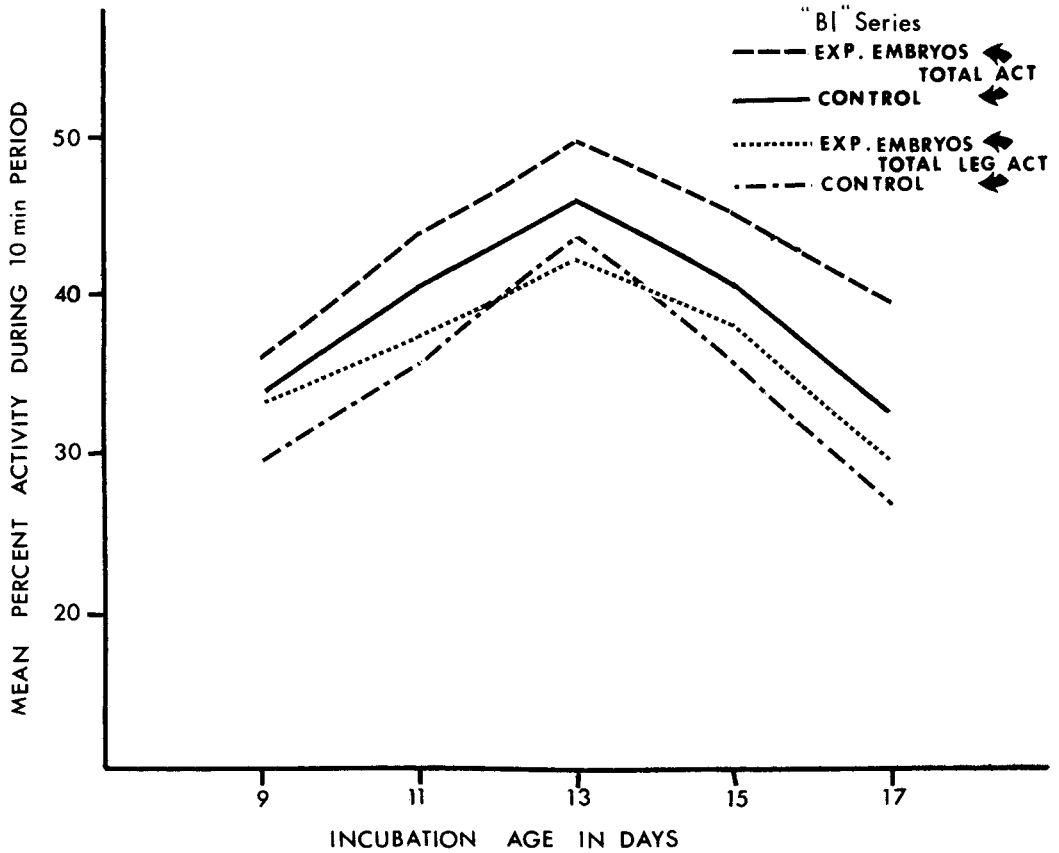

Fig. 2 Comparison of mean per cent of time spent in (a) total activity and (b) total leg activity, between control and experimental embryos of the *Bl* series.

TABLE 1

Activity data for control and experimental embryos

		9d		11d		13d		15d		17d	
	Age in days	t [2]	l [3]	t	l	t	l	t	l	t	l
		9 to 17 days — Bl series									
No. embryos/No. records	Control	17/17	17/17	13/13	13/13	13/13	13/13	14/14	14/14	12/12	12/12
	Experimental	13/13	13/13	9/9	9/9	8/8	8/8	8/8	8/8	9/9	9/9
Mean per cent activity [1]	Control	33.7	29.6	40.3	35.4	45.8	43.8	40.3	35.5	32.2	26.4
	Experimental	36.3	33.5	44.0	37.7	49.4	41.9	45.3	38.0	39.7	29.3
										$P < 0.05$)	
		9 to 17 days — Lb series									
		9d		11d		13d		15d		17d	
	Age in days	t [2]	l [4]	t	l	t	l	t	l	t	l
No. embryos/No. records	Control	10/10	10/10	12/12	12/12	9/9	9/9	9/9	9/9	11/11	11/11
	Experimental	11/11	11/11	7/7	7/7	6/6	6/6	5/5	5/5	6/6	6/6
Mean per cent activity [1]	Control	34.3	24.9	40.6	34.0	42.5	35.0	36.6	28.4	29.9	23.8
	Experimental	40.5	30.6	41.6	33.7	43.0	33.9	36.6	33.2	27.2	24.4

[1] Total activity during ten minute observation period.
[2] t, total activity.
[3] l, total leg activity.
[4] l, total wing activity.
All tests of significance on Mann–Whitney U (2 tailed).

on a slightly higher level. However, the difference for any of the incubation days 9 to 15 is not statistically significant (see table 1 and fig. 2), but the difference between the means on day 17 is significant ($P < 0.05$).

Ordinarily, observations and recordings were made through a window and, since the position of the embryos was not disturbed, overall motility was recorded for whichever part was clearly exposed. We have considered the possibility that the transplantation of the brachial spinal cord segments in the place of the lumbosacral cord might affect particularly the movements of the legs. Therefore, in a special set of recordings, we have obtained data for leg movements performed in the absence of any other body movements or in conjunction with other body movements. These recordings were made only for the right leg since it was usually the one in view through the window. The data are plotted in figure 2. The trend of leg activity in both control and experimental embryos is remarkably similar. The difference of means between control and experimental embryos is not statistically significant for any of the days 9 through 17.

Spontaneous motility (type 1), 9 to 17 days — Lb series

The mean per cent of time spent in activity during a ten-minute observation period is shown graphically in figure 3. As in the *Bl* series, activity increases steadily to a peak value at day 13 and declines thereafter to slightly below the level of activity observed during day 9. Although the curve for the experimental embryos is slightly higher than that of the control embryos the curves follow the same general trend very closely. The activity levels for all of the days of incubation are practically the same except for day 9. The differences on day 9, however, are not statistically significant (table 1, fig. 3).

We have obtained data from a special set of recordings for wing movements alone in the absence of any other movement or in conjunction with movements of other parts of the body. These recordings were only of the right wing since it was most frequently under the window. The data are represented in figure 3. The trend of wing activity in both control and experimental embryos is similar in that a peak value is attained on day 13 and a declining trend follows from then on. The total wing activity in the experimental cases is at a slightly higher level than that of the control embryos for day 9 and day 15; however, the differences are not statistically significant. It is of interest to note that the values for wing and leg activity in both experimental and control series are approximately the same on all days, except day 13, when the leg activity is considerably higher than that of the wing.

Frequency of movements, 9 to 17 days — Bl series

As outlined earlier, (p. 418) three different categories of movements were recorded for a period of five minutes for each embryo. The results are summarized in table 2; the means are presented for each category for alternate days between days 9 and 17 (fig. 4a,b,c). The individual wing movements (fig. 4a) are maintained at frequencies varying from six per minute to nine per minute. Whereas the frequency of wing movements in the control embryos shows an increase during day 17, that for the experimental embryos shows a decrease. However, the difference is not statistically significant.

The individual leg movements (fig. 4b) in the experimental embryos are at a much lower level than the controls on days 9, 11, 13 and 15 and higher on day 17. The differences are statistically significant on days 9, 11 and 17 but not for days 13 and 15. Interestingly enough, a decrease in the frequency of leg movements between days 15 and 17 is observed in the control embryos but not in the experimental embryos. The situation is the reverse for experimental and control wing movements (fig. 4a).

The simultaneous movements of wing and leg are considerably greater than those for the control embryos for all days recorded (fig. 4c). The difference is statistically significant for the entire period. By comparing the three categories of movement at the different stages between days 9 and 17 it becomes quite clear that for each stage there are more simultane-

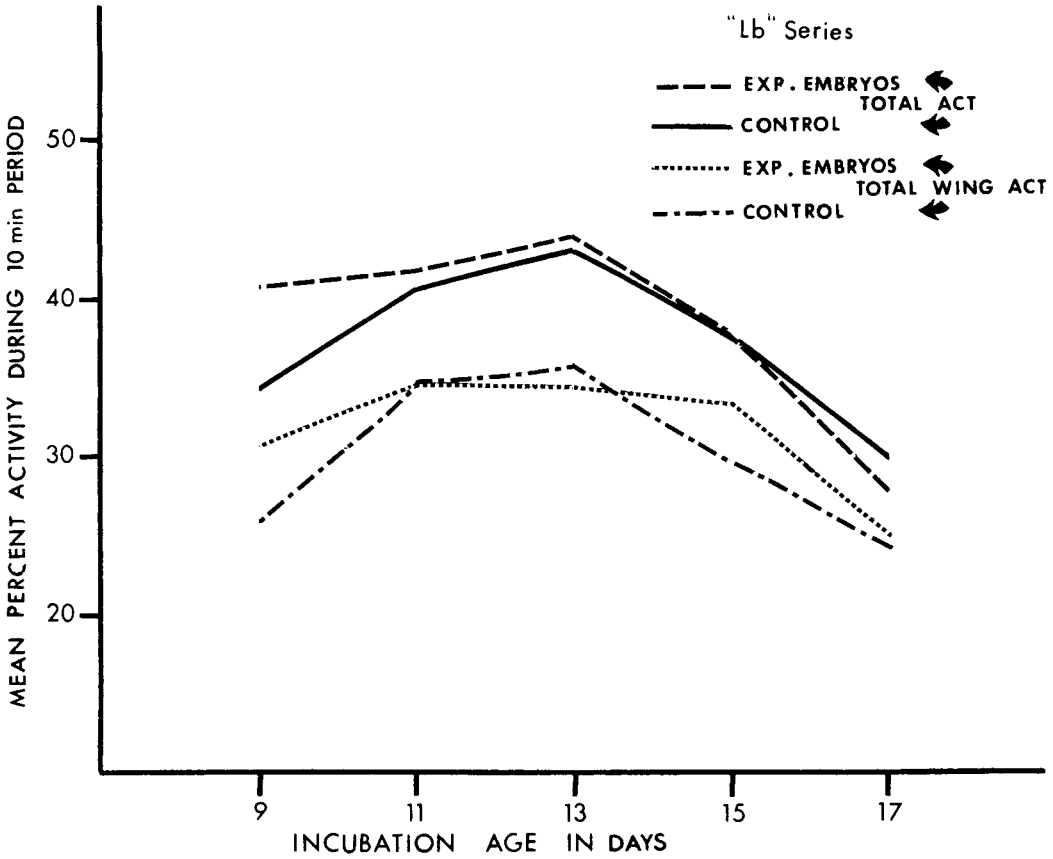

Fig. 3 Comparison of mean per cent of time spent in (a) total activity and (b) total wing activity, between control and experimental embryos of the *Lb* series.

ous movements of wing and leg than either wing or leg movements alone.

Frequency of movements, 9 to 17 days — Lb series

Figures 5a,b,c and table 2 depict the results of the recordings between 9 and 17 days of incubation for the *Lb* series. The individual wing movements (fig. 5a) are consistently at a lower level in the experimental embryos throughout the observational period. The differences are statistically significant on days 9, 11, 13 and 17 (table 2). Concerning leg movements alone (fig. 5b), no significant differences were found between the control and experimental embryos. Simultaneous wing and leg movements were performed more frequently by the experimental embryos than by the control embryos. The differences are statistically significant on days 9, 13, 15 and 17 (table 2). It is of great significance that the simultaneous wing and leg movements are at a higher level than in controls in both the *Bl* and *Lb* series, and the differences of means between experimental and control embryos are statistically significant throughout, except for day 11 in the *Lb* series (see discussion).

Prehatching and hatching behavior — BL series

With the exception of four experimental embryos that were in malposition II (head at the pointed end of the shell), the majority was in a position typical of day 17: the head was near the round end of the shell, with the neck bent on itself, while the rest of the body was oriented lengthwise with respect to the shell (see Hamburger and Oppenheim, '67, fig. 9). The

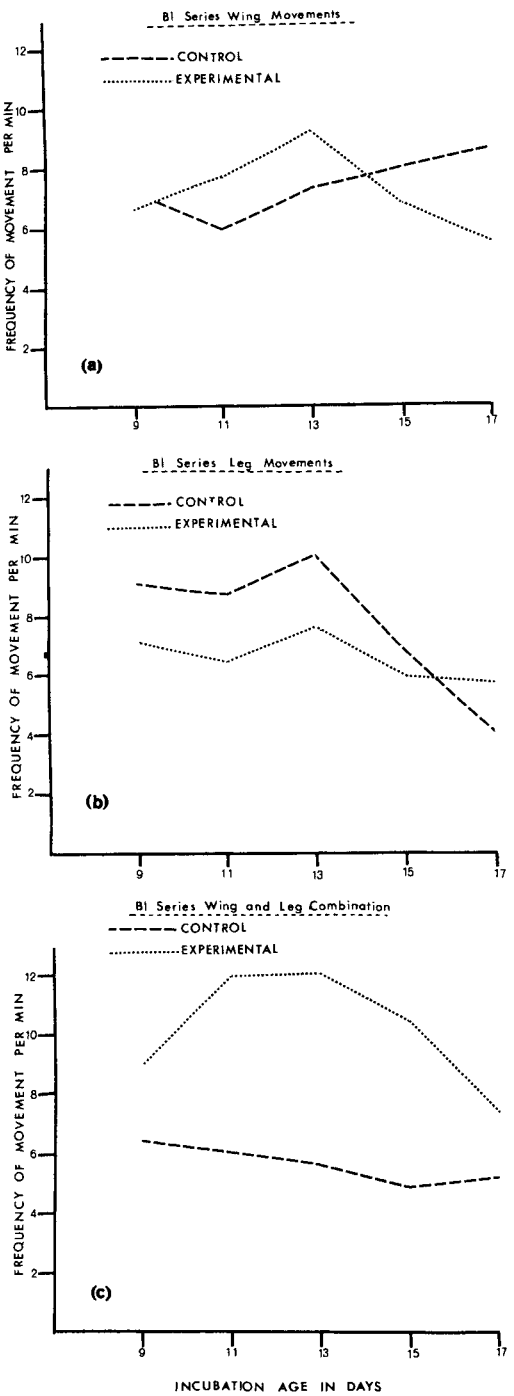

Fig. 4 Comparison of stenograph recordings of frequencies of three categories of movements based on incubation age of control and experimental embryos of the *Bl* series between 9 and 17 days. (For further explanation see text and table 2).

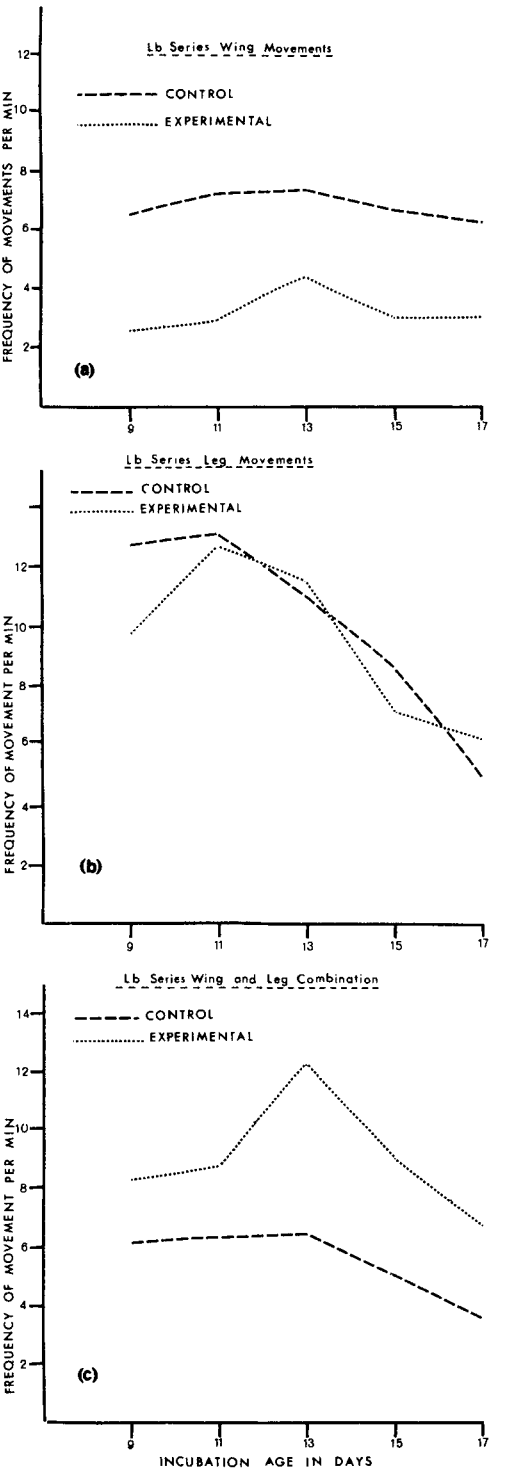

Fig. 5 Comparison of stenograph recordings of frequencies of three categories of movements based on incubation age of control and experimental embryos of the *Lb* series between 9 and 17 days. (For further explanation see text and table 2).

TABLE 2

Mean number of movements per minute for control and experimental embryos

	Age in days	9d	11d	13d	15d	17d
		Bl series				
No. embryos/No. records	Control	17/17	13/13	13/13	14/14	12/12
	Experimental	13/13	9/9	8/8	8/8	9/9
Wing movements	Control	7.3	6.0	7.3	8.0	8.6
	Experimental	6.7	7.7	9.3	6.8	5.6
Leg movements	Control	9.1	8.8	10.1	6.8	4.1
	Experimental	7.2 ($P<0.05$)	6.5 ($P<0.05$)	7.7	6.0	5.7 ($P<0.05$)
Wing and leg combination	Control	6.5	6.1	5.7	4.9	5.2
	Experimental	9.0 ($P<0.05$)	12.0 ($P<0.05$)	12.1 ($P<0.05$)	10.5 ($P<0.05$)	7.4 ($P<0.05$)
		Lb series				
No. embryos/No. records	Control	10/10	12/12	9/9	9/9	11/11
	Experimental	11/11	7/7	6/6	5/5	6/6
Wing movements	Control	6.6	7.3	7.4	6.7	6.3
	Experimental	2.5 ($P<0.05$)	2.9 ($P<0.05$)	4.2 ($P<0.05$)	2.9	3.0 ($P<0.05$)
Leg movements	Control	12.8	13.1	10.9	8.2	4.9
	Experimental	9.8	12.8	11.6	7.1	6.2
Wing and leg combination	Control	6.1	6.3	6.4	4.9	3.6
	Experimental	8.3 ($P<0.05$)	8.7	12.2 ($P<0.05$)	9.1 ($P<0.05$)	6.8 ($P<0.05$)

P values according to student's *t* test.
Non-significant differences are not indicated.

beak appeared to be buried in the yolk sac. During day 18, typical pretucking movements were observed, and the embryos managed to lift the head out of the yolk sac. They performed vigorous head movements in the direction of the right wing. The forward thrusts of the head were accompanied by simultaneous wing lifting and wing flutters. Rotatory movements of the head and shoulder characteristic of the tucking event occurred between 18 and 19 days. Most embryos completed the tucking process in the typical fashion.

Between 19 and 20 days the embryos were able to perform movements comparable to the post-tucking behavioral phase, including wriggles of the body and a slight shifting of the entire body forward and closer to the air chamber. However, in the experimental embryos these latter movements were less vigorous. Membrane penetration was accomplished by all experimental embryos. They punctured the chorio-allantoic membrane either close to the air chamber, as normally, or under the window.

Climax movements were not normal in the experimental embryos. None of them was able to perform the climax rotatory movements. They remained essentially in the prehatching position for hours after making the first crack of the shell (pipping). However, the respiratory movements were normal, chirping was heard and the peripheral circulation was clamped off as in normal embryos. These embryos were artificially hatched (pulled out).

Prehatching and hatching behavior — Lb series

The experimental embryos of this series were in typical pretucking position during day 17 and the early part of day 18. During day 18 the majority of the embryos were able to lift the head out of the yolk sac. Forward thrusts of the head toward the right wing were observed. However, the head movements were not accompanied by simultaneous wing lifting and flutters which occur normally at this time. Consequently, none of the experimental embryos of this group were able to tuck the head under the right wing. Instead, the head remained above the right wing. The wing movements were peculiar in that they were weak backward

extensions at the elbow; otherwise wings were motionless for long periods, interrupted occasionally by weak tremors involving the entire wing. Nevertheless, membrane penetration and pipping occurred in the normal sequence, but movements at these behavioral stages appeared to be reduced both in their amplitude and vigor. The climax rotatory movements were performed by the head alone; the shoulder and the rest of the body was not involved in this act. The embryos were able to rotate and crack the shell a little beyond the first crack of the shell, but had to be helped out of the shell in all cases.

Post-hatching behavior — Bl series

As stated earlier, 13 embryos were hatched artificially and seven of these were kept alive for three days to a week. The following description of post-hatching behavior is representative; it pertains to Bl 56 which was kept for a week after hatching and studied in detail; a motion picture documents the description.

The legs were shortened and the muscles distal to the thigh were severely atrophic; the thigh muscles appeared to be nearly normal. In the resting position both legs were extended backward. The tarsometatarsus was stretched completely, in a straight line with the tibiotarsus. The toes were flexed in all joints. The legs moved freely at the hip joints. There was some movement at the knee joint but very little at the tarsometatarsal joint though it could be bent passively. The toes were usually motionless but capable of slight further flexion and relaxation to the flexed resting position. The legs could not support the weight of the body, and the chick was unable to stand up. It lay usually on its left side and occasionally on the right side. When lying on the left side, the left wing was somewhat extended; this position we consider as normal, since tilting of the body to one side usually results in extension of the wing on the tilted side.

The experimental chicks differ from normal chicks in two significant aspects: the two legs never make alternating stepping movements but are always flexed and extended simultaneously in the hip joint; furthermore, in the experimental chicks leg movements are almost invariably linked to wing movements. Observations are best made when the chick is gently held suspended by the neck. In this position, the legs of experimental chicks are extended downward, whereas in normal chicks the tarsometatarsus is usually folded in jack-knife fashion against the tibiotarsus.

Simultaneous wing flapping and leg movements occur in *normal* chicks when they struggle violently to free themselves or in response to strong stimulation. In this situation, one observes occasionally that both legs are adducted and abducted simultaneously; but alternating leg movements with simultaneous wing flapping are the rule. In experimental animals in the same position both legs are always abducted or adducted in parallel; they never alternate. While the wings flapped up and down, both legs moved forward and backward in the pelvic joint, at approximately the same rate as the wings.

We tried to elicit movements in individual limbs by gentle tactile stimulation of one wing or one leg. One can obtain withdrawal of one wing in the experimental chick, without movement of the other wing or of the legs, but this is more difficult to obtain than in controls. Withdrawal reflex of one leg was elicited only after prolonged stroking of the thigh, and then only very rarely, whereas unilateral withdrawal can be elicited readily in normal legs. A particularly interesting response was obtained upon *strong* stroking of the wing surface; it resulted rather regularly in simultaneous forward movement of the wing and leg of the same side. In normal chicks, strong stroking of the wing results in withdrawal of the wing without accompanying leg movement. All these response patterns remained unchanged during the first week after hatching.

The legs showed tactile sensitivity. As was mentioned, leg withdrawal response was obtained only very rarely; a more frequent response to strong tactile stimulation anywhere on the leg, except toes, was wing flapping and chirping. Stimulation of the leg of one side does often result in movement of the ipsilateral wing. In

controls, the same stimulation elicits strong leg withdrawal only. Acoustic and visual stimulation can arouse leg movements in the experimental chicks which demonstrates the continuity of the longitudinal pathways.

DISCUSSION

As was indicated in the introduction, our experiments are concerned with two interconnected problems: the origin of region-specific differences between the brachial and lumbosacral sectors of the spinal cord; and the pattern of movements elicited in wings innervated from the lumbosacral cord segments and in legs innervated from the brachial segments. Since the patterns of limb movements are fundamentally different in embryos and in chicks after hatching, both periods will be discussed separately.

Motility between days 9 and 17. During this period, embryos exhibit a motility pattern which has been designated as type I. It is characterized by spontaneous (non-reflexogenic), irregular movements, convulsive in type and performed intermittently by all parts of the body that are capable of motility at a given stage of development. The movements of right and left wings and right and left legs are not coordinated, except for occasional wing flapping at the end of the period (for details see Hamburger a.o. '65, Hamburger, '68, '70). "Startles" are designated as type II. At 17 days, coordinated movements are initiated by which the embryo attains the so-called hatching position; they continue through the hatching act; they are designated as type III (Hamburger and Oppenheim, '67). We have suggested that type I motility results from autonomous discharges of neurons in the ventral half of the cord and in higher centers which spread indiscriminately through the cord. Electrophysiological studies have revealed unit-activity and burst firing in normal spinal cords (Provine a.o., '70) and in completely deafferented lumbosacral cord segments (Sharma a.o., '70).

Since in type I motility the four limbs make jerky uncoordinated movements, with no obvious regularity or distinction between wing and leg activity, difficulties were to be expected in finding a quantitative parameter that would discriminate between the neural action systems in the brachial and lumbosacral sectors of the cord. In fact, if the hypothesis of a general, uninhibited and unorganized sweep of electrical discharges through the entire spinal cord during an activity phase is correct without qualification, then one would predict that quantitative parameters of limb movements would not differ in our experimental and normal embryos. This prediction is actually fulfilled with respect to two measures which we have recorded: For one, *total activity* which is defined as the percentage of time spent in activity during a ten-minute observation period, was found to be not significantly different in normal and experimental embryos of both series (figs. 2, 3). The only exception is at day 17 in the *Bl* series; but we do not attribute any significance to this discrepancy, since the general trend of the curves for normal and experimental embryos is the same throughout the entire observation period, including day 17. Obviously the substitution of one spinal cord sector for another, and the presence of two brachial or two lumbosacral segments in the same embryo does not influence total activity. It should be mentioned that the durations of activity and inactivity phases (in seconds) were also calculated from the raw data, and again no significant differences were found between normal and experimental embryos, throughout the entire observation period.

We then thought that by focusing on wing and leg movements specifically, differences between wing and leg motility might be revealed which might have been overshadowed in the total activity. But, except for day 13, total wing and total leg activity, as defined on p. 418 were found to be the same in *normal* embryos, hence there was no basis for discrimination of the two regions. As might have been expected, the values for normal and experimental embryos of both series did not differ significantly. The situation on day 13 is of interest. In normal embryos, the total leg motility on this day only, is significantly higher than the total wing motility (43% as against 35% ; $p < 0.05$). This, then, offers an opportunity for a

test of our theoretical premise. It turned out that the experimental embryos showed exactly the same difference as the normal embryos. In other words, on day 13, legs innervated by brachial nerves (Bl) perform at the high activity level characteristic of normal legs; and the wings innervated by lumbosacral nerves perform at the low level characteristic of normal wings. This means that regional differences in the spinal cord, if they exist, do not manifest themselves in this particular parameter.

So far then, our hypothesis of indiscriminate discharges is borne out by the data; but they have left open the question of whether specific regional differences between brachial and lumbosacral cord sectors do exist in embryonic stages.

We proceeded to apply another measure of limb activity which we thought might be more sensitive than the others, that is, *frequency of movements*, per minute. The frequencies were recorded for three categories: wing movements in the absence of any other movements, leg movements in the absence of any other movements, and combination of wing and leg movements. If we focus attention first to the *normally* innervated limbs of the experimental embryos, that is the wings in the Bl series and the legs in the Lb series (figs. 4a, 5b), we find that the frequencies of movements in these limbs are at the normal levels. *In this respect*, the transplanted and normal brachial levels in the same embryos (Bl series) do not influence each other; and the same holds for the two lumbosacral sectors in the Lb series.

We turn now to the limbs innervated by the heteronymous spinal cord segments (figs. 4b, 5a). We found that the frequencies of movements in such limbs are uniformly lower than those in normal limbs, in both series (with the exception of day 17 in the Bl series and day 15 in the Lb series; but both points are in line with the general trends of the curves). It would seem that the innervation by foreign nerves impairs the independent movements of such a limb. However, it becomes apparent that this explanation is incorrect, if the data on independent limb movements are considered in conjunction with the data for *combined* wing and leg movements. The latter data reveal a new and very remarkable phenomenon: The occurrence of simultaneous movements of wings and legs in both experimental series is very much higher than in controls (figs. 4c, 5c). All differences are significant except for day 11 in the Lb series, but this point is in line with the general trend; it is considerably higher than the corresponding control point. The low frequencies of independent movements now find a simple explanation: wings and legs in experimental embryos move less frequently alone and more frequently together, than in controls; the deficit in the former values is compensated by excess in the latter. If one adds the averages of wing movements alone and combined wing/leg movements, and the averages for leg movements alone and combined wing/leg movements, for different stages, then one arrives at a fairly good agreement with the corresponding figures for the controls.

The pronounced increase in the combination wing/leg movements in experimental embryos of both series is of great interest. The implication is that if there are two brachial sectors, or two lumbosacral sectors, respectively, in the same embryo, they are functionally more tightly linked together than the brachial and the lumbosacral region in the normal embryo. One can assume that a message addressed to the brachial sector is picked up simultaneously by the two brachial sectors in the Bl series, and a message addressed to the lumbosacral level is picked up by the two lumbosacral sectors in the Lb series, whereas a "brachial" message is not picked up as readily, or not at all, by the lumbosacral cord, and vice versa. Alternatively, one can assume that longitudinal fibers destined for termination in the brachial neurons, synapse specifically in both brachial sectors in the Bl series, although the latter are separated by the thoracic segments, thus facilitating simultaneous discharge; and longitudinal fibers destined for lumbosacral neurons facilitate the simultaneous discharge in the two lumbosacral regions of Lb. It should be remembered that we are dealing with a purely quantitative parameter of type I motility, i.e., frequency of movements per time unit, and not with complex

qualitative differences between the action systems for wing and leg motility such as wing flapping or alternate stepping movements in walking. Whatever the explanation of this phenomenon may turn out to be: the increase in combination wing/leg movements must be based on some differential between the brachial and lumbosacral cord. The experiments thus demonstrate unequivocally that regional differences between the brachial and lumbosacral segments of the spinal cord are manifested in type I motility at least from day 9 on.

There is no discrepancy between the previous finding that regional differences do not manifest themselves in total embryonic motility and in total wing and leg motility but that they are demonstrable in terms of number of movements per unit time; this may well be a matter of the sensitivity of the method employed. Nor is the finding of a very high incidence of wing/leg combination movements incompatible with our contention that spontaneous embryonic motility results from a sweep of generalized uninhibited discharges through the entire central nervous system. Generalized discharges cannot be equated with unorganized nerve tissue. We know through histogenetic studies and stimulation experiments that the structural and functional organization of the spinal cord increases steadily and that the specific pathways and interconnections of centers which subserve postnatal adaptive behavior are being constructed during the embryonic phase. Sufficiently sensitive electrophysiological techniques would probably detect preferential discharge patterns that are ordinarily obscured by the general noise level, and brachial-brachial or lumbosacral-lumbosacral interconnections in the experimental embryos may represent such preferentially activated pathways. If our interpretation is correct, then it implies an important qualification of our contention that type I activity is caused by *indiscriminate* simultaneous firing of numerous spinal cord neurons. In this respect it is worth noting that in early stages of teleost and salamander embryos, spontaneous motility is integrated from the beginning (Hamburger, '71).

Posthatching behavior

Two remarkable phenomena were observed in the hatched chicks of the Bl series: First, the close coupling of wing and leg movements which was described for type I motility persists in the posthatching period. Almost invariably, wings and legs move simultaneously. Independent spontaneous wing or leg movements were rarely seen, and it was very difficult to elicit local wing or leg movements by tactile stimulation (see p. 426). Our observations on hatched chicks of the Lb series are very limited; but Straznicky ('63) did experiments identical with our Lb series and made observations on a number of hatched experimental chicks. He found that the wing and leg of the same side always moved together, in a particular fashion. "When walking, the animals raised and adduced the wing to the shoulder joint, exactly in time with the step of the leg on the same side" (p. 147). His interpretation of this synchronization (p. 416) is similar to the one we gave above (p. 428) as explanation of combined wing/leg motility in embryos. We have also confirmed his finding that wing motility is almost completely restricted to the shoulder joint; likewise, in the Bl series, motility is foremost in the pelvic joint; distal parts show only limited movements and distal muscles are atrophic.

The second phenomenon to be commented on is the complete absence of alternating stepping movements in the legs of the Bl series. Instead, the legs were flexed and extended simultaneously in the fashion of wing flapping. No change in this behavior occurred during the week following hatching. However, the flexions did not include distal parts which, as was mentioned, showed very limited motility. Undoubtedly, this motility pattern is the expression of structural and functional characteristics of the *brachial* action system, which supplies innervation to leg muscles. This phenomenon, together with the demonstration of regional differences in embryonic type I motility justifies the conclusion that regional-specific differences between the brachial and lumbosacral segments are already irreversibly fixed in the spinal cord at the stage of

operation, i.e., stage 15–16 (52–60 hours of incubation).

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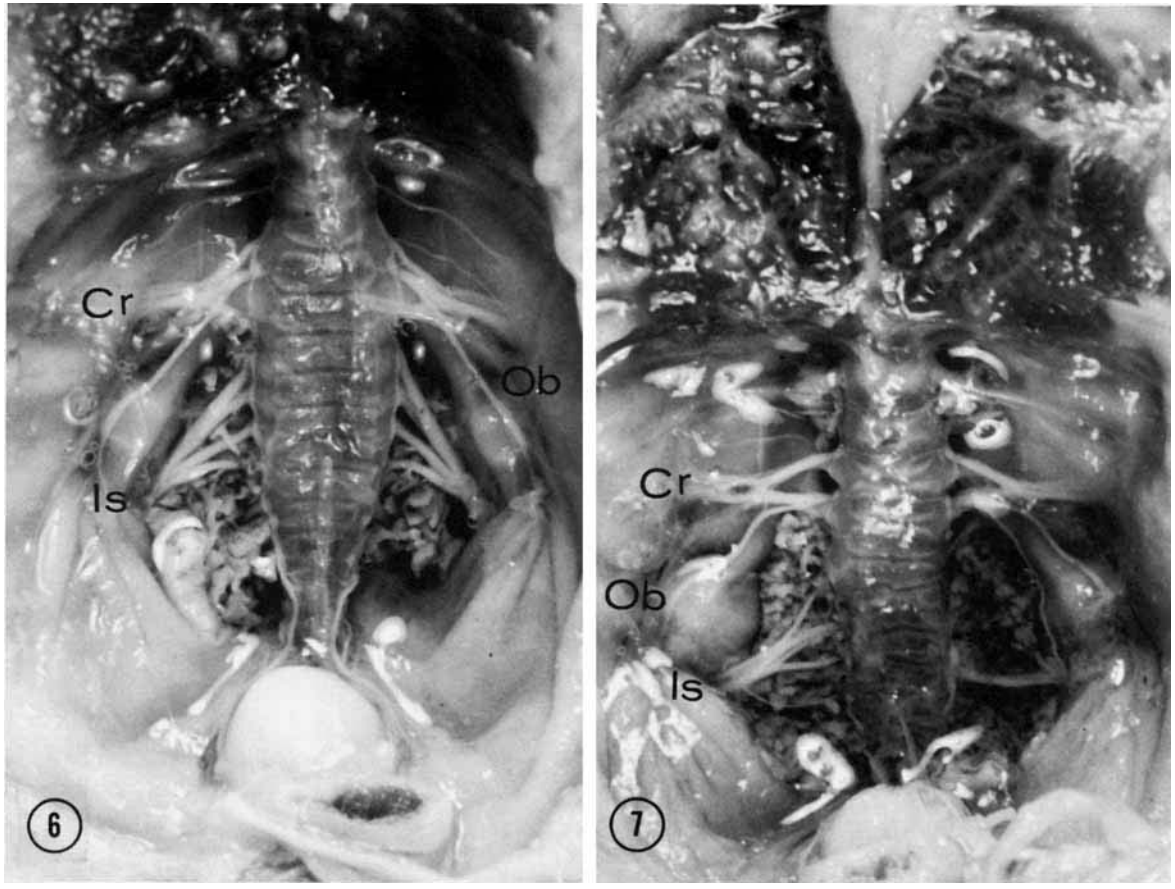

EXPLANATION OF FIGURES

6 Photograph of dissection of control embryo of the *Bl* series seven days after hatching, showing spinal cord, the lumbosacral crural (Cr) and ischiatic (Is) plexuses, and the proximal distribution of spinal nerves, obturator nerve (Ob).

7 Photograph of dissection of chick *Bl* 56, seven days after hatching with brachial spinal cord and three thoracid spinal cord segments transplanted to the lumbosacral level. Note the typical organization of the nerves to form the crural and ischiatic plexuses. The ischiatic plexus on the left is incomplete while that on the right is normal in its composition. Note the differences in size of the individual nerves forming the plexus compared to the control embryo of figure 6.