The nature of the earliest spontaneous activity of the chick embryo

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WITH ONE PLATE

INTRODUCTION

The chick embryo begins to be active during the 3rd day of development. The activity is first confined to the cervical somites and gradually spreads to the posterior somites as the maturation of the embryo continues. In 3- to 8-day-old chick embryos this activity is periodic with a pattern of brief activity phases alternating with longer inactivity phases. On the 9th day, the activity begins to increase, and at 13 days it becomes almost continuous. For a complete description of this developing activity see Hamburger & Balaban (1963) and Hamburger (1963). As Hamburger points out, one of the aspects of embryonic motility which requires further experimentation is the investigation of the source of the earliest activity: is the periodicity generated from within the nervous tissue, i.e. neurogenic; or from within the muscle, i.e. myogenic? At 7½ days the activity of the chick embryo is definitely dependent upon the nervous system. Hamburger (1963) has reported that isolation of the anterior and posterior parts of the spinal cord by the ablation of a central segment effectively divides the uninterrupted band of somites into anterior and posterior segments which contract with independent rhythms at 7½ days. The results of experiments with the drug curare suggest that the activity prior to 7 days may also be initiated by the nerve (Levi-Montalcini & Visintini, 1938; Kuo, 1939; Hamburger, 1963). However, the curare experiments are open to criticism, as curare has not been tested on isolated early embryonic muscles.

As Hamburger (1963) points out, the origin of this earliest motility will not be known until the crucial experiment of complete removal of the nervous system has been performed. In the dogfish embryo this experiment provided conclusive evidence that the earliest motility of this embryo is myogenic (Wintrebert, 1918a, b; Harris & Whiting, 1954). This phenomenon may be restricted to the dogfish where overlapping of somitic myoblasts and the presence of muscle pacemakers

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can account for such a co-ordinated and regular series of contractions (Harris & Whiting, 1954). However, the possibility that a myogenic phase of activity also exists in the chick must be considered. It has been known since the classical work of Lewis (1915) that isolated muscle fibers can contract in tissue culture, and this observation has been confirmed by other workers (see Murray, 1960). However, the question arises whether this contraction is comparable to that observed in muscle of chick embryos of corresponding age in vivo. The rhythms reported for this myogenic activity are not at all analogous to the activity described by Hamburger & Balaban (1963); however, this may be due to the conditions prevailing in tissue culture or to the amount of tissue involved. Szepesenwol (1946) found that an explant of several muscle fibers from a single muscle was able to contract in unison; however, in myotomes isolated from 1- to 3-day chick embryos, this uniform activity was not observed until the explants had reached a total age of at least 7 days. On the other hand, somites isolated together with a segment of spinal cord became active at 4 days of total age; this is the same time at which activity is first observed in vivo.

Muscle isolates have been studied in other environments, such as intra-coelomic grafts (Hamburger, 1939; Eastlick, 1943) and chorio-allantoic grafts (Hoadley, 1925; Hunt, 1932); but no data on motility were reported. The chorioallantoic membrane would seem to provide an ideal site for a motility study, being physiologically adequate and allowing frequent direct observation of the grafts. It was therefore decided to study the development of motility in innervated and in non-innervated somite tissue on the chorioallantoic membrane.

MATERIALS AND METHODS

The procedure for chorioallantoic grafting outlined in Hamburger (1960) was followed in these experiments. White Leghorn chick embryos were used as both donors and hosts. The donors were operated on between stages 15-18, 23-33 somites (Hamburger & Hamilton, 1951) but stages 17-18, 29-36 somites, were used most often; the hosts were 9 days old in all cases. The host implantation sites were always prepared before the operation on the donors in order to facilitate the speed of the transplantation.

Using a finely sharpened tungsten needle and watchmaker's forceps, two types of transplants were excised in situ from the donor: (a) somites 14-20 of one side, (b) somites 14-20 of one side with adjacent spinal cord. In one surviving case in group (b), the somites of both sides were explanted. In about 50 per cent. of the cases wing tissue was included in the transplants. In two cases (a) and (b) were from the same donor. The transplants were transferred in saline to the host embryo using a Spemann pipette. The total time for the extirpation and transplantation was 10 min.

After 24 hr., the vascularization of the graft was checked. The successful grafts were observed two to three times daily in a temperature-controlled plastic
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box (37°C.) for periods of 10 min. The spontaneous activity was timed carefully with a stopwatch and recorded. At the end of each observation period, the piece was stimulated by applying light pressure to all areas of the graft with a tungsten loop, and the ability to respond to this stimulus was recorded. Observation of the graft was continued until fixation was necessary. The variation in the time of fixation (Tables 1 & 2) is due to several factors, namely, excessive hemorrhage in the graft, cessation of twitching, infection of the chorioallantoic membrane, or poor health of the host embryo as indicated by disturbance of the chorioallantoic circulation. In some cases, even though the graft and the host were still in good condition, the pieces were fixed after 3 days of growth on the membrane. The tissue was fixed in Bouin's (CH 16-35; 104-279) or Zenker's (CH 38-60) fixative, embedded in paraffin, sectioned at 8 μ, and stained with Heidenhain's hematoxylin and with Mallory's connective tissue stain.

RESULTS

The somite tissue, with and without adjacent spinal cord, grew well on the chorioallantoic membrane of the host embryo. The grafts remained compact, but they were usually irregular in shape. When a limb bud was included in the graft, it grew out, and often differentiated with digits. The size of the grafts varied with the time of growth on the membrane; the average size was 3 × 4 × 2 mm. The tissue was grown on the membrane for periods of time ranging from 2–7 days.

Histology of the grafts

A complete histological study revealed the normal differentiation of muscle, cartilage, loose connective tissue, and nephric tubules in both innervated and non-innervated grafts. These tissues were healthy in appearance and developed in the proper relation to one another. Intervertebral muscles, vertebrae, and limb cartilage with adjacent muscle, could be clearly identified. In both groups some small hemorrhages could be found in the tissues, and the epithelium was occasionally thicker than normal epithelium.

Grafts of six somites without spinal cord

Twenty-nine grafts of somite tissue transplanted with no spinal cord grew well on the chorioallantoic membrane. In fourteen of these grafts, no muscle or very few fibers were found. These grafts are not included in the results. Histological examination of the other fifteen grafts revealed good muscle development. These muscles contain typical myofibrils and show little sign of degeneration (Plate, Fig. F).

The grafts were observed for activity two to three times daily under a binocular
dissecting microscope. During these 10-min. observation periods, no spontaneous twitching and no twitching in response to mechanical stimulation was observed in any graft (Table 1).

**Table 1**

_Grafts of six isolated somites grown on the chorioallantoic membrane of a 9-day chick_

<table>
<thead>
<tr>
<th>CH No.</th>
<th>Total age of graft (days) when fixed</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>6</td>
<td>1 2 3 4 5 6 7</td>
</tr>
<tr>
<td>24</td>
<td>8</td>
<td>1 2 3 4 5 6 7</td>
</tr>
<tr>
<td>25</td>
<td>8</td>
<td>1 2 3 4 5 6 7</td>
</tr>
<tr>
<td>28</td>
<td>10</td>
<td>1 2 3 4 5 6 7</td>
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<tr>
<td>30</td>
<td>7½</td>
<td>1 2 3 4 5 6 7</td>
</tr>
<tr>
<td>32</td>
<td>6½</td>
<td>1 2 3 4 5 6 7</td>
</tr>
<tr>
<td>35</td>
<td>6</td>
<td>1 2 3 4 5 6 7</td>
</tr>
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<td>38</td>
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<td>40</td>
<td>5½</td>
<td>1 2 3 4 5 6 7</td>
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<tr>
<td>41</td>
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<td>1 2 3 4 5 6 7</td>
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<tr>
<td>105</td>
<td>5½</td>
<td>1 2 3 4 5 6 7</td>
</tr>
<tr>
<td>213</td>
<td>5½</td>
<td>1 2 3 4 5 6 7</td>
</tr>
</tbody>
</table>

_Grafts of six somites with spinal cord_

Thirty-three grafts of somites with adjacent spinal cord tissue grew well after transplantation to the chorioallantoic membrane of 9-day chick embryos. No twitching was observed in thirteen of these grafts. Histological examination of these thirteen cases revealed that in some cases the muscle was not yet well developed; and in the other cases with good muscle differentiation, distinct nerve fiber bundles could not be found. None of these grafts were grown more than 4 days on the chorioallantoic membrane.

Twitching was observed in the other twenty grafts. Histological examination of these active grafts revealed a healthy condition of all tissues. Small hemorrhages were rare in transplants with regular activity, and more frequent in the transplants in which the activity consisted of isolated twitches. The muscle tissue varied in mass; the transplants with the best activity showed conspicuous groups of muscle bundles (Plate, Figs. A & B). Muscle fibrils were discernible, and normal attachment to cartilage was observed in a number of instances. The spinal cord was found either in the center or at the periphery of the graft; it was of variable length, and showed good differentiation in a number of cases. The excellent condition of the spinal cord shown in Fig. A (Plate) is due to the fact that in this case the somites of both sides were included in the graft. The
EXPLANATION OF PLATE

Abbreviations: c, cartilage; g, ganglion; m, muscle; n, nerve; spc, spinal cord.

Fig. A. A section through chorioallantoic graft CH 147. Left and right somites were included in this graft.

Fig. B. A section through CH 12 showing spinal cord, nerve, spinal ganglion, and muscle adjacent to cartilage.

Fig. C. A section through CH 11. The extent of the spinal cord and nerve fibers penetrating muscle bundles are shown.

Fig. D. An enlargement of Fig. C showing the intimate association between the nerve and the muscle bundle.

Fig. E. A section of CH 12. A large nerve can be seen proximal to the muscle bundle; smaller branches extend from this nerve to the muscle fibers.

Fig. F. Muscle in non-innervated graft CH 30. Notice myofibrils in the muscle fibers. Magnification for each figure is shown by a line representing 100 μ.

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spinal cord shown in Fig. B (Plate) is more characteristic of grafts with unilateral somites. However, in all cases normal differentiation of spinal cord with central canal and ependymal lining, neural epithelium, and marginal layer was observed (Plate, Figs. A, B and C). Spinal ganglia were observed in several cases (Plate, Figs. A & B). Although they were found in the normal position, they were often irregular in size and shape. In every one of the transplants of this group which showed motility, nerve fiber bundles of different sizes were seen to enter into the muscle tissue and to distribute themselves in it (Plate, Figs. C, D & E). No such fibers were observed in any of the muscles of this series which did not show contractility. In some of these inactive grafts, nerve fibers emerged from the spinal cord but they could not be traced to muscle bundles.

Spontaneous contractions were observed in nineteen of the twenty innervated grafts listed in Table 2. The grafts did not all begin to contract spontaneously during the first day after grafting; however, by the second day thirteen of these cases were active. CH 138 and CH 226 showed a great delay of spontaneous activity which began on the 4th and 5th day after grafting respectively. Case CH 104 showed no spontaneous activity during its 2 days of growth on the
membrane, but this graft did respond with a definite twitch when it was stimulated with a tungsten loop during its 2nd day of growth. In all cases in which periodic spontaneous twitching was observed, the contraction occurred in a specific area which remained the same in all observation periods. This was established by comparing sketches made at the end of each observation period. A gradual increase in the contracting area was observed in CH 11, 17, 133 and 147.

The activity which was observed in these grafts usually consisted of single rapid twitchs separated by rest periods of different lengths. In two grafts (CH 60 and CH 147) an occasional double or triple twitch was observed. A survey of the recordings of motility showed that a certain degree of regularity of intervals between twitchs could be detected in seven of the nineteen cases. No such regularity of inactivity phases was detected in six of the remaining cases; and in the other six cases there occurred only occasional single twitchs never amounting to more than six twitchs in a single 10-min. observation period. We shall discuss these three arbitrary categories separately.

**Regular spontaneous twitching**

This was observed in grafts CH 11, 12, 17, 42, 133, 137 and 147 during their 2nd and 3rd days of growth on the membrane (5-6 days total age). The data are not sufficient to permit detailed statistical analysis of patterns; in particular, it is not possible to determine any directional change in individual grafts. Preliminary analysis is possible by collectively analyzing the data from all grafts showing regular activity. To do this, the periods of rest between individual twitchs were grouped according to their length and the percentage of the total number of twitchs falling into each category of time was plotted on a graph (Text-fig.). This graph shows that almost 50 per cent. of the activity of these grafts is in the form of twitchs separated by 10 sec. or less, and that 94 per cent. of the activity consists of individual twitchs separated by 60 sec. or less; furthermore no rest period is longer than 90 sec. Specific examples clearly show this periodicity. CH 11 at 5 days total age shows an activity pattern of single twitchs in which two to four independent twitchs of the muscle occur within 10 sec., and this active period is then followed by a period of rest which is usually 45 to 60 sec. in length. CH 12 shows a slightly different pattern of activity; here one to two twitchs occur within a 10-sec. period; and the rest period usually varies from 35 to 60 sec. These examples represent the greatest amount of regularity achieved by these grafts.

CH 147 is particularly interesting; this is the graft that includes somite tissue from both sides of the spinal cord. Here the activity consisted of two or three consecutive contractions in two separate areas of the graft. The most common sequence of motility was a single contraction on the left followed by a single contraction on the right of the graft; this occurred 70 per cent. of the time. Occasionally there were three consecutive contractions, left–right–left, or there was a single contraction on the right, instead of the left–right contraction sequence.
At no time did simultaneous contraction occur on both sides. It was observed that the contracting area was longer on the right side than on the left side. The periodicity of contraction which was established by this graft was very similar to that found in grafts containing only a single strip of somites; two double contractions (left-right) occurred within 10 sec., and this activity period was followed by a long inactivity period of 40 to 60 sec.

**Irregular twitching**

This was observed in grafts CH 60, 119, 138, 167, 251 and 271. In these cases the inactivity period ranged from less than 10 sec. to 240 sec.; and no pattern could be detected in individual or collective analysis. Only 22 per cent. of the activity consisted of twitches separated by 10 sec. or less; and 75 per cent. of the rest periods were 60 sec. or less. For example, the activity period of CH 60 consisted of a single twitch, and this was followed by a rest period which varied from 12 to 160 sec.

**Isolated twitches**

Isolated twitches were observed in CH 106, 155, 197, 226, 245 and 279. These grafts did not twitch more than six times in any 10-min. observation period. CH 106 twitched four times during the first 4 min. of the observation period during its 2nd day of growth on the membrane. CH 226 was observed to contract only once in seven observation periods; this contraction occurred during the 5th day of growth on the membrane.
There is a certain correlation between the regularity of the twitching and the amount of nerve and muscle tissue in the graft. It would be of interest to examine this correlation further by varying the amount of muscle and nerve tissue in the grafts.

The sensitivity of the grafts to mechanical stimulation was tested by gently pressing on their surface with a tungsten loop. In nine of these cases a single contraction was observed to follow this stimulation. Histological examination revealed the presence of a ganglion with a large nerve fiber bundle emerging from it in only one of these cases.

**DISCUSSION**

The data presented above show that without exception, only somites transplanted with spinal cord contract spontaneously under the conditions of the experiment. Those transplants with spinal cord but not contracting were either devoid of sufficient muscle or lacked innervation of the muscle fibers which were present. These findings are consistent, and suggest that all motility observed is dependent on a nerve-muscle connection. They give further support to the contention that the earliest motility of the somites in the chick embryo is in response to regular nerve discharge. To validate this conclusion, other possibilities have to be ruled out.

First, is the absence of contraction in non-innervated grafts due to some adverse condition present only in these grafts; for example, excessive hemorrhage or lack of sufficient muscle tissue? This interpretation is very unlikely, for the histological study shows that adverse conditions which might interfere with contraction occurred in both innervated and non-innervated grafts. In fact, some of the contracting innervated grafts showed more hemorrhage and less muscle tissue than the non-innervated grafts of the same age.

Second, can the rôle of the spinal cord be attributed to factors other than transmission of impulses? It is unlikely that the nerve influence is mediated by a general trophic substance supplied by the spinal cord. We have observed several cases in which spinal cord and muscle were present in the same graft, and yet the muscle did not contract. Since large nerve fiber bundles were not found in these pieces, it is concluded that the absence of actual innervation of the muscle is alone responsible for the absence of motility. In further support of this conclusion, it was observed that the grafts which established the most regular activity pattern were the most thoroughly innervated.

Third, is the muscle of non-innervated somites less well developed than that of somites grafted with adjacent spinal cord? Histological examination of these grafts revealed that the muscle fibers which developed in the non-innervated grafts are completely normal in appearance. They contain normal myofibrils, are associated with cartilage, and are in no way distinguishable from innervated muscle fibers. It is true that they are not as yet completely differentiated. Zelena (1957) reports that non-innervated muscle of the chick can never differ-
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entiate normally beyond the myotube stage. Eastlick (1943) has shown that non-innervated muscle in intra-coelomic grafts differentiates normally up to 10 days. This is the time at which innervated muscle develops cholinesterase in its motor end plates (Drachman, 1963). After this initial normal development, macrophages invade and destroy the tissue. However, none of the grafts in our series were grown on the membrane past a total age of 10 days; and, as expected, no mass degeneration of the muscle was observed. The possibility is not excluded that these cells may still vary significantly from normal. Zak & Gutman (1960) have shown that denervation of adult rat muscle results in a disturbance of protein synthesis; however the effect of this disturbance on activity is not discussed. Furthermore, no studies of this type have been made on embryonic tissue.

It is concluded that the only significant observable difference between the motile and non-motile grafts in our experiment is the functional innervation of the muscle of the former, and the absence of this innervation in the latter. This finding is not consistent with the well-known fact that myogenic contraction can, and often does, occur spontaneously in tissue culture. Is the activity observed in vitro due to some factor peculiar to this environment? Muscle tissue is sensitive to ionic changes, and no attempt was made to adjust the concentration of ions such as potassium, sodium, and calcium so that the tissue culture media would exactly duplicate the internal milieu of the embryo of the corresponding age. Furthermore, the surrounding tissue environment varies from normal, for the amount of tissue used is small and usually consists of a single tissue type together with fibroblasts. The grafts growing on the chorioallantoic membrane more closely approximate the in vivo condition in all the above points; they are organ rather than tissue cultures. The muscle in the graft is grown in its normal connective tissue environment with typical cartilage associations. It is true that the host is 6 days older than the donor tissue, and differences in ionic concentration and other factors could possibly be responsible for lack of motility in the non-innervated grafts. The possibility has not yet been examined experimentally, but it is unlikely in view of all the positive arguments that favor the role of innervation in the motility of our grafts.

The most convincing evidence that the environment of the chorioallantoic membrane comes to the normal environment is the observation that the activity which develops in the best developed grafts is very similar in pattern to the normal activity of the chick embryo as described by Hamburger & Balaban (1963). The myogenic contraction observed in tissue culture shows great variation and bears no resemblance to the normal activity pattern (see Murray, 1960). For instance, Lewis (1915) described the contractions as varying from 120 times a minute to once every 23 sec. In contrast to this the normal pattern of activity in chick embryos less than 9 days of age is as follows: a short activity phase of 10 sec. consisting of two to ten waves of motility separated by short intervals is followed by a longer inactivity phase usually about five times as long as the activity phase,
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i.e. 30–60 sec. (Hamburger & Balaban, 1963). Very similar to this is the pattern of activity of one of the innervated grafts showing regular activity (CH 11), this graft was also less than 9 days of age. Here the periodicity consists of an activity period of 10 sec. consisting of two to four isolated twitches, followed by a longer inactivity period of 45–60 sec. In the other cases showing regular activity, there are differences in the number of twitches within the 10-sec. period, and slight variation in the range of the inactivity periods; however, all these variations fall within the ranges for activity and inactivity found in the normal embryo. This normal periodic contraction was observed by the 2nd day of growth on the membrane, or within a day of the time it normally appears in vivo. This short delay is not surprising considering the time it takes for the graft to get established on the membrane. Altogether, our experimental results give strong and independent support to the contention of previous investigators (see introduction) that the spontaneous motility of the chick embryo is neurogenic from its first beginning.

SUMMARY

1. Two types of grafts from 3-day chick embryos were grown on the chorioallantoic membrane of a 9-day-old chick embryo: six isolated somites, 14–20; and six somites 14–20, with adjacent spinal cord. The activity of these non-innervated and innervated grafts was observed during their growth on the membrane.

2. No activity was observed at any time in the fifteen grafts of six somites grafted without spinal cord; but spontaneous twitching could be observed in twenty out of thirty-three grafts which contained sections of the spinal cord.

3. Histological examination confirmed that the twenty active transplants each contained a segment of spinal cord, and large nerve fibers could be traced to the muscle tissue in the grafts. The failure of the other thirteen grafts to show contraction is accounted for either by the absence of well differentiated muscles or by the absence of nerves invading them.

4. The muscle of the non-innervated, inactive pieces showed the same amount of differentiation as that of the innervated active grafts.

5. The activity observed in the innervated muscle tissue shows three different patterns: regular, with a periodicity similar to that of the intact embryo; irregular and isolated twitches, i.e. less than six twitches in the 10-min. observation period.

6. It is concluded that the spontaneous motility of muscle developing on the chorioallantoic membrane is neurogenic and not myogenic. This finding supports the view that the spontaneous motility observed in vivo is neurogenic from its beginning.

RESUMÉ

1. Deux types de greffons d'embryons de Poulet de 3 jours sont implantés dans la membrane chorioallantoïdienne d'un embryon de Poulet de 9 jours:
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6 somites brachiaux seuls et 6 somites brachiaux garnis du tube nerveux adjacent. La motilité de ces greffons innervés et non innervés a été observée pendant leur développement sur la membrane chorio-allantoïdienne.

2. Aucune motilité n’est observée à aucun moment dans les 15 greffons non innervés ; mais des contractions spontanées ont pu être constatées dans 20 sur 33 greffons comportant le tube nerveux.

3. L’examen histologique a montré que chacun des 20 transplants actifs contenait un segment du tube neural et que d’importantes fibres nerveuses pouvaient être mises en évidence dans le tissu musculaire de ces transplants. L’absence de contractions dans les 13 autres greffons est due, soit à l’absence de muscles bien différenciés, soit à l’absence d’innervation des muscles.

4. La différenciation des muscles était aussi importante dans les greffons non innervés que dans les greffons innervés.

5. Trois types d’activité sont observés dans les muscles innervés : régulière, avec une périodicité similaire à celle de l’embryon intact ; irrégulière et des contractions isolées, c’est-à-dire moins de 6 contractions par 10 minutes.

6. On en conclut que la motilité spontanée de muscles se développant sur la membrane chorio-allantoïdienne est neurogénique et non myogénique. Le résultat fait admettre que la motilité spontanée observée in vivo est neurogénique dès le début de son apparition.

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