Melanoblast-tissue interactions and the development of pigment pattern in *Xenopus* larvae

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SUMMARY

The melanophores of larval *Xenopus laevis* are disparately distributed on the hypomere in that the upper region (UHT) is densely pigmented, the median region (MHT) is moderately pigmented, and the lower region (LHT) is unpigmented. The roles of the melanoblasts and their tissue environment in determining the melanophore pattern was investigated by heterotopic transplantation of hypomorphic tissues, culture of neural crest explants in vesicles derived from hypomorphic tissues and radioactive marking of neural crest cells.

Somite-situated grafts of UHT, MHT and LHT were found to possess melanophore densities similar to those exhibited by such hypomorphic tissues when in their normal situation.

The number and distribution of trunk melanophores in 'crestless' second host larvae bearing grafts of UHT, MHT and LHT transferred from the somites of primary host embryos indicated that (a) many melanoblasts entered all transplants during neural crest migration in the primary host: subsequently, a small number of melanoblasts were lost from transplants of UHT, a greater number from transplants of MHT and almost all from transplants of LHT; (b) almost all melanoblasts migrated out from transplants of MHT and LHT and entered the tissues of the 'crestless' host, whereas a considerable number of melanoblasts remained in the transplant when it was formed from UHT.

Grafts of UHT placed mid-ventrally in the hypomere failed to exhibit melanophores.

Vesicles of (a) UHT + MHT and (b) LHT containing neural crest tissue possessed similar numbers of melanophores. Vesicles of LHT differed from those of UHT + MHT in that melanophores were densely aggregated in the implanted neural tissues.

Following radioactive marking of neural crest cells labelled nuclei were found on the dorsal ridges of the somites, the surfaces of the neural tube and notochord and in the mesoderm of the upper hypomere and the fin, but were absent from the lateral surfaces of the somites.

These results showed that the melanophore pattern in larval *Xenopus* depended upon melanoblast-tissue interactions, which influenced the migration, rather than the differentiation, proliferation or destruction, of melanoblasts and suggested that tissue selection by migrating melanoblasts enabled these cells to distribute themselves in embryonic tissues in accordance with a hierarchy of melanoblast-tissue affinities.

Melanoblast-tissue affinities appeared to be related to the adhesiveness of mesodermal cells: melanoblast extensibility appeared to facilitate exploration of the surrounding tissues.

The formation of pigment pattern in larval *Xenopus* appeared to depend upon the interaction between the melanoblast population pressure and melanoblast-tissue affinities.

The present results and those of other workers on amphibian pigmentation were used to construct a model capable of accounting for species-specific differences in larval amphibian pigment patterns, in terms of interactions between species-specific differences in melanoblast-tissue affinities and melanoblast population pressure.

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INTRODUCTION

The melanophores of Amphibia are derived from the neural crest (DuShane, 1935). On completion of neurulation the neural crest lies dorsal to the neural tube and immediately below the epidermis. Colourless melanoblasts migrate from this position to their definitive locations in the embryo where they differentiate and give rise to a species-specific larval pigment pattern. Many attempts have been made to discover the mechanisms which control such patterns. However, present knowledge of these mechanisms is plagued with uncertainty, partly due to a tendency for earlier workers to place undue emphasis on differences in pigment pattern formation in different species. While one must exercise caution in generalizing from one species to another, it is probable that fundamental control mechanisms are common to all amphibians, and the variety of pigment patterns observed in this group depend upon the degree to which such mechanisms are expressed, rather than upon the existence of separate and distinct systems controlling pigment pattern formation. The present work attempts to analyse the several factors underlying pigment pattern formation in the hypomere of *Xenopus laevis* and examines the experimental findings in relation to the diversity of mechanisms for pigment pattern formation previously postulated with the aim of finding some common ground.

Regional differences in pigmentation are apparent in the hypomeric mesoderm of *Xenopus*. In the present study the hypomere was considered to be divisible into upper, median and lower regions in accordance with the density of pigmentation observed in each. Melanophores appear initially in the upper hypomeric tissues (UHT) immediately ventral to the somites. Subsequently melanophores spread ventrally but less densely into the median hypomeric tissues (MHT). In general the lower hypomeric tissues (LHT) do not become pigmented.

The first objective in analysing a pigment pattern is to determine the extent to which the intrinsic properties of melanoblasts (melanophores prior to melanogenesis) and the interactions between melanoblasts and the tissue environment contribute to such patterns. The results of some previous investigators suggest that pigment patterns are mainly attributable to factors located in the tissue environment. Transplants of neural crest between several anuran species including *X. laevis* (Andres, 1963) and between black and white strains of the urodele *Ambystoma mexicanum* (DuShane, 1935, 1943; Dalton, 1949, 1950) were observed to result in essentially host-type patterns. In species of the urodele *Taricha* there is also good evidence that the ability of tissues to support pigmentation varies according to the region of the embryo (DuShane, 1935, 1939; Twitty, 1936, 1945; Twitty & Bodenstein, 1939, 1944; Lehman, 1951, 1953). However, neural crest transplants between species of *Ambystoma* and species of *Taricha* and between the latter and their hybrids suggested that species-specific differences in pigment pattern are dependent upon properties intrinsic to melanoblasts (Twitty, 1936; Twitty & Bodenstein, 1939). The
environmental control of pigment pattern in *Xenopus* postulated by Andres (1963) suggests that the distribution of melanophores in the hypomere of this species might arise from regional variations in the properties of the hypomeric tissues. Such properties might be expected to influence one or more of the following aspects of melanoblast development:

(a) Melanoblast differentiation. Melanophore patterns may depend upon the environmental tissues providing certain prerequisites of melanogenesis. A variety of evidence indicates dependence upon and competition for such an environmental contribution (Twitty & Bodenstein, 1939, 1944; Twitty, 1944, 1945, 1953; Baltzer, 1947; Lehman, 1951; Andres, 1963). In this way melanoblasts might be evenly distributed throughout the hypomere but would differentiate only in tissues capable of supplying these factors.

(b) Melanoblast migration. The environmental tissues may influence melanoblast migration in such a manner that melanoblasts come to rest only in those regions which subsequently exhibit melanophores. Inhibition of melanoblast migration in the skin of the white axolotl has been reported (Dalton, 1950) and has also been postulated to occur in the hypomere of *Taricha torosa* (DeLanney, 1941; Finnegan, 1958). Furthermore, there is evidence that in the chick the environmental tissues influence the migration of neural crest cells (Weston, 1963; Noden, 1975).

(c) Melanoblast proliferation. Different regions of the hypomere may vary in the extent to which they permit the proliferation of melanoblasts.

(d) Melanoblast destruction. It is possible that the absence of melanophores from *Xenopus* LHT results from the destruction of melanoblasts in these tissues. However, although melanoblast destruction has not been reported from any species, degeneration of melanophores has been observed in urodèles (DeLanney, 1952; Finnegan, 1955).

**MATERIALS AND METHODS**

*Xenopus* eggs were obtained by standard methods (New, 1966). Most experiments employed stage-22 embryos (staging according to Nieuwkoop & Faber (1956)), at which stage trunk neural crest is situated above the closed neural tube but has not yet begun to disperse. Hypomeric tissues used in experiments consisted of epidermis and all subadjacent mesoderm. Experimental data was obtained from larvae and tissues at or equivalent to stage 40, at which stage the essential features of pigment pattern in normal larvae are apparent.

Jelly coats and vitelline membranes were removed using the technique of Jones & Elsdale (1963). Surgery was carried out on embryos immersed in the full strength saline medium of Niu & Twitty (Flickinger, 1949) containing 0.25% sodium sulphadiazine. Following tissue excision or transplantation experimental embryos were kept in full strength saline in the operating dish until wound healing had taken place, then transferred to 10% saline with added 0.1% sodium sulphadiazine and allowed to develop at room temperature (18–21 °C).
Heterotopic transplantation of UHT, MHT and LHT to the lateral surface of the trunk somites

To determine the extent to which the tissue environment controls the distribution of melanophores in the hypomere a piece of hypomere not yet colonized with melanoblasts was transplanted to a site near the neural crest of a host larva and allowed to remain in this situation throughout the period of melanoblast migration. Any subsequent appearance of melanophores in the graft is indicative of a prior immigration of host melanoblasts. The influence of hypomeric tissues on melanophore patterns was measured by comparing the amount of pigmentation which developed in the graft with that observed in tissues at a similar site in the normal animal.

Similar-sized pieces of UHT, MHT and LHT were transplanted separately from stage-22 donor embryos to the lateral surface of the anterior somites of host embryos of the same age.

Heterotopic transplantation of somite-situated grafts of UHT, MHT and LHT to the median hypomere of second host ‘crestless’ embryos

To aid in discovering if melanophore patterns are determined by an environmental control of differentiation, migration, proliferation or destruction of melanoblasts, the distributions of melanoblasts in somite-situated grafts of hypomeric tissues were examined at several stages during melanoblast migration. The method entailed the transfer of such grafts between host embryos, the trunk of the recipient having been experimentally deprived of melanoblasts. The number of melanophores subsequently observed in the trunk of the second host gave a measure of, although not necessarily being identical to, the number of melanoblasts which were present in grafts at the time of transfer.

 Portions of UHT, MHT and LHT were transplanted separately from stage-22 donor embryos to the lateral surface of the somites of host embryos of the same stage. Experimental embryos were divided into three groups which were allowed to develop until stages 29/30, 32 and 33/34, stages selected to coincide with (a) the final stages of neural crest migration, (b) completion of neural crest migration and (c) the onset of pigmentation of host embryos respectively. At these stages grafts were transferred to the median region of the hypomere of stage-22 ‘crestless’ host embryos (i.e. embryos from which neural crest and neural tube had been removed from the trunk). ‘Crestless’ embryos without grafts were employed as controls. The experimental procedure is illustrated in Fig. 1.

Heterotopic transplantation of UHT to midventral LHT

In order to establish whether or not melanoblasts colonize the normally unpigmented LHT during normal development a piece of hypomere known to support melanoblast localization and differentiation was transplanted prior to
Fig. 1. Diagram showing the sequence of events in transplantation studies. (A) Pieces of UHT (■), MHT (□) and LHT (□) were transplanted from donor embryos to (B) the lateral surfaces of the somites of primary host embryos. (C) These embryos were allowed to develop to various larval stages. (D) At the required larval stage grafts were transferred to the median region of the hypomere of 'crestless' host embryos from which neural crest and neural tube had previously been removed (indicated by interrupted arrows).

melanoblast migration to a position on the hypomere where it was isolated in LHT. If melanoblasts colonize LHT during migration some such cells might be expected to settle and differentiate in the graft.

Portions of UHT from stage-22 embryos were transplanted to the midventral hypomere of host embryos of the same age.

Culture of neural crest in vesicles derived from hypomeric tissues

To determine whether or not melanoblast destruction accounts for the absence of pigmentation from LHT and to obtain further information on the differentiation and migration of melanoblasts in hypomeric tissues the development of melanoblasts isolated in LHT was compared with that of melanoblasts isolated in UHT and MHT by culturing neural crest explants in vesicles derived from different hypomeric regions, using a technique similar to that described by Brick & Dalton (1963).

Similar-sized portions of neural crest and underlying dorsal sector of neural tube were excised from the anterior trunk of stage-22 embryos. Such explants were cultured in vesicles derived from uniformly sized square-shaped portions of (a) UHT + MHT and (b) LHT explanted from stage-22 embryos. Newly formed vesicles were placed in 50 % saline and maintained at 20 °C for the
duration of the experiments. Other stage-22 embryos were allowed to develop normally for use as controls in determining the developmental age of the vesicles.

Radioactive marking of trunk neural crest

To determine the migratory pathways of neural crest cells and hence the possible destinations of melanoblasts larvae bearing grafts of ³H-labelled neural crest were examined by autoradiography during the final stages of neural crest migration (cf. Weston (1963)).

Early gastrulae freed of jelly and lying within the vitelline membrane were placed in full strength saline containing 7.5 µCi/ml of ³H-thymidine (Loeffler & Johnston, 1964). Embryos were removed from this solution at stage 22 and washed in several changes of full strength saline. Some labelled embryos were allowed to develop and employed as controls. The anterior region of the trunk of other labelled embryos provided portions of ³H-labelled neural crest and underlying dorsal sector of neural tube which were transplanted homotopically to unlabelled host embryos of the same age. Larvae were maintained until they reached stage 31 then were fixed in Bouin's fluid and embedded in paraffin wax. Seven µm sections were cut, mounted and coated with Kodak AR 10 stripping film, then exposed for periods of up to 8 weeks (Rogers, 1967). Only larvae which showed normal development and bore well-healed grafts were used for autoradiographic examinations. Some experimental larvae were allowed to develop into fully formed larvae.

RESULTS

The normal development of pigment pattern in Xenopus

Melanophores were first observed at about stage 33/34 on the dorsal surface of the head and as a horizontal band in the upper hypomeric mesoderm. As development progressed pigmentation increased in the hypomeric mesoderm and spread ventrally into the median region. Melanophores were also observed on the dorsal surface of the neural tube, on the dorsal ridges of the somites and on the lateral surface of the tail somites. The lateral surface of the trunk somites and the lower hypomeric region remained unpigmented. The essential features of the pigment pattern of the normal larva are illustrated in Fig. 2A.
Heterotopic transplantation of UHT, MHT and LHT to the lateral surface of the trunk somites

The numbers of melanophores found in grafts are recorded in Table 1. Grafts of UHT exhibited dense pigmentation, while those of LHT remained essentially unpigmented (Fig. 2B, D). Although the amount of pigmentation in grafts of MHT varied it was for the most part intermediate between that of grafts of UHT and LHT (Fig. 2C).

Heterotopic transplantation of somite-situated grafts of UHT, MHT and LHT to the median hypomere of second host ‘crestless’ embryos

The trunks of ‘crestless’ control larvae were observed to remain unpigmented until stage 42. Subsequently, small numbers of melanophores were observed in anterior UHT. The numbers of melanophores present in the trunks of experimental larvae were found to reach a maximum when such larvae reached stage 37/38 (i.e. transplanted tissues were of an age equivalent to stage 40 of the donor animal), enabling the studies on experimental larvae to be completed prior to any contamination of the trunk region by host melanophores.

The numbers of melanophores observed in the trunks of experimental larvae are given in Table 2.

(a) Grafts removed from first hosts at stage 29/30. Large and approximately equal numbers of melanophores were present in the trunk regions of all larvae. The proportion of these cells located in the graft varied according to the hypomeric region which provided the graft. Grafts of UHT contained approximately one-third of the trunk melanophores whereas grafts of MHT and LHT contained virtually none.

(b) Grafts removed from first hosts at stage 32. The numbers of trunk melanophores observed differed according to the hypomeric origins of the grafts. Many
Table 2. Numbers and distribution of melanophores in the trunk regions of experimental larvae bearing transplants of UHT, MHT or LHT removed from primary hosts at stages 29/30, 32 or 33/34

<table>
<thead>
<tr>
<th>Transplants removed from primary hosts at</th>
<th>Number of melanophores in</th>
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<tbody>
<tr>
<td></td>
<td>Transplant</td>
<td>Host trunk</td>
</tr>
<tr>
<td><em>(a) Stage 29/30</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UHT (17)</td>
<td>8.6 ± 2.1</td>
<td>16.3 ± 2.5</td>
</tr>
<tr>
<td>MHT (17)</td>
<td>1.8 ± 1.3</td>
<td>20.2 ± 3.1</td>
</tr>
<tr>
<td>LHT (18)</td>
<td>1.7 ± 1.2</td>
<td>23.0 ± 4.1</td>
</tr>
<tr>
<td><em>(b) Stage 32</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UHT (20)</td>
<td>9.6 ± 2.4</td>
<td>11.9 ± 2.9</td>
</tr>
<tr>
<td>MHT (22)</td>
<td>1.5 ± 1.1</td>
<td>11.2 ± 2.7</td>
</tr>
<tr>
<td>LHT (22)</td>
<td>0.2 ± 0.05</td>
<td>4.2 ± 1.8</td>
</tr>
<tr>
<td><em>(c) Stage 33/34</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UHT (14)</td>
<td>10.6 ± 2.7</td>
<td>10.8 ± 3.1</td>
</tr>
<tr>
<td>MHT (13)</td>
<td>4.0 ± 1.6</td>
<td>9.3 ± 2.2</td>
</tr>
<tr>
<td>LHT (15)</td>
<td>0.66 ± 0.08</td>
<td>2.8 ± 1.5</td>
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</table>

The numbers in parentheses in the first column refer to the number of experimental larvae.

Trunk melanophores were found in larvae bearing grafts of UHT, a lesser number in those bearing grafts of MHT and few or none in those bearing grafts of LHT. The proportion of trunk melanophores located in grafts was greater in grafts of UHT than in those of MHT or LHT.

*(c) Grafts removed from first hosts at stage 33/34.* The numbers of trunk melanophores observed in experimental larvae and the proportion of these cells in the grafts were similar to those observed in larvae bearing transplants which had been transferred at stage 32.

_Heterotopic transplantation of UHT to midventral LHT_

Grafts of 28 experimental larvae failed to exhibit melanophores. Three larvae displayed grafts possessing one, two and three melanophores respectively. In the solitary larva possessing three melanophores, two of the latter were greatly elongated, one end of each melanophore being located in UHT of the host and the other in the graft.

_The culture of neural crest in vesicles derived from_  
*(a) UHT + MHT and (b) LHT*_

Similar numbers of melanophores were observed in both groups of vesicles (Table 3). Many individuals in both sets of vesicles survived for more than 2 weeks, during which time degradation of melanophores was not observed. Although melanophores were distributed throughout both vesicular mesoderm and neural implant in all vesicles, in vesicles of LHT many melanophores were
Table 3. Number of melanophores in vesicles formed from (a) UHT + MHT and (b) LHT equivalent to stages 40 or 41

<table>
<thead>
<tr>
<th></th>
<th>UHT + MHT (50)</th>
<th>LHT (50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of melanophores</td>
<td>48.1 ± 9.7</td>
<td>42.8 ± 9.3</td>
</tr>
</tbody>
</table>

Numbers in parentheses refer to the number of experimental vesicles.

![Figure 3](image)

Fig. 3. Vesicles equivalent in age to stage 40 of normal larvae derived from (A) UHT + MHT and (B) LHT, each containing neural crest implants. Note that although melanophores are distributed throughout both types of vesicle, in (A) melanophores are dendritic whereas in (B) melanophores are punctate and many melanophores are concentrated within the implanted neural tissues.

Concentrated in the form of an aggregate within the implanted neural tissues. This phenomenon was not observed in vesicles of UHT + MHT in which vesicles melanophores were more evenly distributed throughout implant and mesoderm (Fig. 3).

The degree of dispersion of pigment in the melanophores differed between the two sets of vesicles. Pigment was fully dispersed in vesicles of UHT + MHT at all stages, whereas in vesicles of LHT pigment became concentrated at stages 39 or 40.

Radioactive marking of trunk neural crest

Labelled nuclei were observed on the dorsal ridges of the somites, the surface of the ventral sector of the neural tube, the surface of the notochord, in the mesenchyme between the somites and endoderm, in the upper hypomeric mesoderm and in the mesoderm of the fin. Only a few labelled nuclei were observed in host tissues in any one section. To illustrate clearly the positions of labelled nuclei and thus the pathways followed by neural crest cells all labelled nuclei observed in all sections through a single larva are represented as occupying a single section (Fig. 4). All labelled larvae employed as controls were found to develop normally.
DISCUSSION

The nature of melanoblast-tissue interactions

An attempt was made in the present study to determine whether or not the local tissue environment was capable of influencing the distribution of melanophores in the hypomere, and, if so, the nature of the melanoblast-tissue interactions involved.

The results of experiments employing heterotopic transplantation of hypomeric tissues revealed that the melanophore densities in somite-situated grafts of UHT, MHT and LHT were similar to those exhibited by these tissues when in their normal situations, suggesting that melanophore patterns depend indeed upon factors present in the local tissue environment.

In order to identify the component of melanoblast development susceptible to the influence of environmental factors, e.g. melanoblast differentiation, proliferation, migration, or destruction, two approaches were adopted. The first approach necessitated the determination of the distribution of melanoblasts in somite-situated grafts of hypomeric tissues at several stages after the onset of neural crest migration but prior to the appearance of the definitive pigment pattern and comparing these experimentally obtained distributions with the hypothetical distributions predicted by postulating hypomeric tissue control of the different components of melanoblast development previously described. The second approach entailed a comparison of the development of
Melanoblast-tissue interactions in *Xenopus* larvae

Melanoblasts isolated in vesicles of LHT with that of melanoblasts isolated in vesicles of UHT + MHT.

The results of experiments designed to determine the distribution of melanoblasts in somite-situated grafts of UHT, MHT or LHT showed that in grafts from host larvae at stage 29/30 (i.e. during neural crest migration) large and approximately equal numbers of melanoblasts were present in all grafts, whereas in grafts from stage 32 and 33/34 host larvae (i.e. following cessation of neural crest migration and at the onset of pigmentation respectively) the numbers of melanoblasts contained in the grafts varied according to the region of the hypomere which provided the graft, i.e. many melanoblasts were found in grafts of UHT, a lesser number in grafts of MHT and few or none in those of LHT. These findings showed that the numbers of melanoblasts contained in the grafts decreased between stages 29/30 and 32 and that the distribution of these cells after cessation of neural crest migration became similar to that of melanophores in somite-situated grafts and normally situated hypomeric tissues.

In view of these findings the proposal that melanophore patterns in *Xenopus* are dependent upon a regional variation in the ability of hypomeric tissues to initiate differentiation in an evenly distributed melanoblast population (i.e. arise from underlying melanogenic patterns in the tissues) cannot be maintained. This conclusion is further supported by the finding that tissues which remain unpigmented in normal animals are quite capable of supporting melanoblast differentiation, as shown in the present study by the presence of considerable numbers of melanophores in the mesoderm of vesicles of LHT, and also by the work of Stevens (1954) who reported a capacity for pigmentation in the tissues at the surface of the trunk somites of *Xenopus*. These findings are somewhat at variance with those of Andres (1963) who carried out reciprocal transplantations of neural crest between members of several species of anurans (*X. laevis, Hyla arborea, Bombina variegata, Discoglossus pictus, Rana esculenta*) and concluded from these studies that pigment pattern formation in these anurans was influenced by the availability of chromogenic substances in the environmental tissues. However, the widely different developmental rates of the species employed, the lack of physiological harmony between donor and host melanophores and between donor melanophores and host tissues, culminating in atypical differentiation or even degeneration of melanophores in host tissues, make it unrewarding to attempt to compare the results of Andres with those of the present studies.

In considering whether or not a differential proliferation of melanoblasts in hypomeric tissues contributes towards melanophore patterns one might predict, assuming such proliferation took place, that the numbers of melanoblasts present in grafts at the onset of pigmentation would exceed those found at earlier stages in such grafts. However, temporal increases in melanoblast numbers were never observed in the present studies. Indeed, the graft experiments showed that the definitive distribution of melanoblasts resulted from
losses of melanoblasts from grafts. In addition, the observation of similar numbers of melanophores in vesicles of LHT and in vesicles of UHT + MHT is consistent with the existence in both groups of vesicles of similar levels of melanoblast proliferation. It is therefore unlikely that differential proliferation of melanoblasts contributes to hypomeric melanophore patterns.

In accounting for the development of a disparate distribution of melanoblasts in grafts of the different hypomeric tissues the relative importance of melanoblast migration or destruction, under the influence of a regional variation in environmental factors, alone remains to be assessed. In other words, reduction in the numbers of melanoblasts in the various grafts between stages 29/30 and 32 must be accomplished by emigration or destruction of melanoblasts. However, the observed similarity between the numbers of melanophores in vesicles of LHT and of UHT + MHT indicates that the capacity for melanoblast destruction is no greater in LHT than in other hypomeric tissues. Thus, even if melanoblasts are destroyed during normal development the phenomenon neither imposes nor controls melanophore patterns. The observation that pigment was concentrated in melanophores (i.e. punctate melanophores) contained in vesicles of LHT was considered unlikely to be indicative of melanophore degeneration, as the melanophore pigment became fully dispersed on illuminating these vesicles with a bench lamp, a phenomenon which suggested that such punctate melanophores retained both physiological and structural integrity. These observations will be discussed more fully elsewhere.

Such reasoning suggests that the apparent losses of melanoblasts from grafts between stages 29/30 and 32 can only be attributed to melanoblast emigration. It may be significant that losses of melanoblasts between these stages coincides not only with the exhaustion of the neural crest source of melanoblasts in the host animal in these experiments but also with the stage of migration of melanoblasts into the hypomere in the normal embryo. The latter emigration was shown by Stevens (1954) to commence at stage 31. The present evidence indicates that a transitory stream of host melanoblasts entered all grafts during neural crest migration, giving rise to the large numbers of these cells found at stage 29/30. Subsequently, a proportion of these cells emigrated from the grafts, and, like their counterparts in normal embryos, may have continued their migration into the host hypomere. The data listed in Table 2 suggest that of the graft melanoblast complements few melanoblasts emigrated from UHT, about one-half their number emigrated from MHT, and almost all emigrated from LHT. Hence the tendencies of melanoblasts to emigrate from hypomeric tissues appears greatest in LHT, less so in MHT and least of all in UHT.

These conclusions are borne out by examination of the distributions of donor melanophores in the trunks of 'crestless' larvae bearing grafts of the various hypomeric tissues which had been transferred from the somites of the first host at stage 29/30. In all larvae the numbers of trunk melanophores observed in
host tissues indicated that many melanoblasts had emigrated from grafts. However, the proportion of trunk melanophores contained in the grafts (Table 2) suggested that whereas almost all melanoblasts had emigrated from grafts of MHT and LHT, a considerable number of these cells had failed to emigrate from grafts of UHT. Furthermore, a comparison of the numbers of melanophores found in grafts situated on the somites (Table 1) with those observed in grafts situated on the hypomere of 'crestless' host larvae (Table 2) suggests that emigration of melanoblasts from grafts occurred more readily when grafts were situated in the latter environment.

The existence of regional differences in the tendencies of melanoblasts to remain in hypomeric tissues can be interpreted in terms of regional differences in melanoblast-tissue affinities in the hypomere, such affinities being greatest in UHT, less in MHT and least in LHT. The finding that the emigration of melanoblasts from grafts was influenced by the tissues surrounding the grafts supports the concept of melanoblast-tissue affinities, and suggests that the tendency for melanoblasts to migrate out of one tissue and enter others depends upon differences in the affinity of melanoblasts for the tissues in which they are situated and the affinities of these cells for other available tissues.

These interpretations are supported by the observation that the implanted neural tissues in vesicles of LHT were densely populated by melanophores: this phenomenon was not observed in vesicles of UHT + MHT. Hence melanoblasts will accumulate in neural tissues if LHT is the sole alternative, but will not do so if UHT + MHT are available. Such behaviour is consistent with the indicated regional differences in melanoblast-tissue affinities in the hypomere, and suggests that the affinity of melanoblasts for neural tissues is greater than the affinity of these cells for LHT but is similar to or less than their affinity for UHT + MHT. The presence of melanophores on the neural tube in normal Xenopus is consistent with the existence of such an affinity between melanoblasts and neural tissues.

The absence of melanophores from the lateral surfaces of trunk somites may similarly be ascribed to relative differences in melanoblast-tissue affinities. Following the marking of neural crest with [3H]thymidine labelled-nuclei were not observed in tissues lateral and external to the somites, suggesting that melanoblasts had not colonized these tissues. However, the report by Stevens (1954) that these tissues can support melanoblast migration, and the finding in the present study that somite-situated grafts of hypomeric tissues are colonizable by melanoblasts suggests that the absence of melanoblasts from tissues at the surfaces of the somites during normal development results from a relatively low affinity between the latter tissues and melanoblasts.

It seems then that the melanophore pattern in Xenopus depends upon tissue selection by migrating melanoblasts, by which means melanoblasts distribute themselves in embryonic tissues in accordance with a hierarchy of melanoblast-tissue affinities.
The cellular basis of melanoblast-tissue affinity

A considerable body of evidence indicates that the movements of migrating cells are related to the adhesive properties of such cells and to the adhesive properties of the substratum (Rosenberg, 1963; Carter, 1965; Steinberg, 1970). The in vitro studies of Harris (1973) have a particular bearing on the present work. By culturing a variety of cell types on an artificial substratum consisting of a meshwork of varying adhesiveness Harris was able to demonstrate that migrating cells accumulate and become confined within the more adhesive components of the meshwork. It is possible that in Xenopus a similar mechanism underlies the preferential accumulation of migrating melanoblasts in embryonic tissues. In other words, the regional differences in melanoblast-tissue affinities in the hypomere described in this study may be governed by differences in the adhesive properties of the mesodermal cells. An examination of the behaviour of mesodermal tissues in vesicles of UHT + MHT and in vesicles of LHT was found to strengthen this hypothesis. Mesodermal cells contained in vesicles of UHT + MHT became closely associated, forming cellular strands and sheets which eventually differentiated into epithelial membranes and nephron-like tubules. In contrast, mesodermal tissues contained in vesicles of LHT mainly formed loose associations of cells, which subsequently differentiated into erythropoietic tissues. These observations suggest that the strength of the adhesion between mesodermal cells is greater for UHT + MHT than for LHT, or, more generally, that upper and median hypomeric mesoderm is more adhesive than lower hypomeric mesoderm.

It is possible that melanoblasts select the tissues in which they migrate by forming cell extensions which enable them to make contact with tissues not in their immediate environment, and that melanoblasts migrate into those tissues with which their extensions make the strongest adhesions. Gustafson & Wolpert (1967) proposed such a mechanism in order to account for the behaviour of mesenchymal cells during gastrulation in the sea urchin. In the present studies it was noted that a single larva bearing a mid-ventral graft of UHT possessed greatly elongated melanophores. Each melanophore was so oriented that one extremity was located in normally positioned host UHT and the other in graft UHT, giving the impression that prior to differentiation these cells as melanoblasts were in a state of flux between two tissues for which they had a similar affinity, and that the strength of the adhesion between each cell extremity and the local tissues was such that neither adhesion was broken during melanoblast differentiation. This situation appears comparable with that described by Harris (1973) from which he reported the ability of cells in culture to bridge two adhesive areas of substratum across a less adhesive gap.

It appears that on entering a tissue for which they have a relatively high affinity melanoblasts will be reluctant to relinquish their adhesion to such a tissue for other less strong adhesions which their cell extensions might make
with other tissues and hence remain in the first tissue. The finding that melanoblasts of stage 33/34 larvae which ordinarily would remain and differentiate in somite-situated transplants of UHT or MHT (see Table 1) retain the ability to migrate out from the graft, upon the latter's transfer to the hypomere of 'crestless' embryos (see Table 2), suggests that the constancy of a melanoblast distribution depends upon the relative affinities which originally determined the distribution remaining unaltered, until such time as the melanoblasts begin to differentiate and lose their capacity for migration. However, changes in the relative affinities occasioned by, for instance, graft transfer, might well lead to a corresponding change in the distribution of melanoblasts.

The role of melanoblast population pressure

The postulation that differences in melanoblast-tissue affinities are responsible for melanoblast distributions does not explain the ability of melanoblasts to migrate from UHT to MHT during normal development, or the finding that in experiments employing vesicles of LHT not all melanoblasts showed a preference for the enclosed neural tissues. Such apparent anomalies are, however, explicable if the effect of melanoblast population density upon migration is considered. The migration of melanoblasts in species of the urodele *Taricha* has been shown to be motivated by a mutual repulsion among these cells. The extent of the migration was found to be proportional to the size and density of the melanoblast population (Twitty, 1944; Twitty & Niu, 1948, 1954). The relevance of such mechanisms in controlling the distribution of melanoblasts in *Xenopus*, by enabling these cells to overcome differences in tissue affinity, was indicated by the work of Stevens (1954), in which the subsequent culture of portions of dorsal trunk (i.e. trunk excluding hypomeric and endodermal tissues) taken from *Xenopus* embryos prior to neural crest migration revealed large numbers of melanophores on the lateral surfaces of the somites, a region not normally colonized by melanoblasts. The same author concluded that in the absence of a ventral region into which melanoblasts might spread the melanoblast population pressure compelled these cells to colonize tissues not ordinarily colonized by melanoblasts, or, in the concepts of the present paper, tissues for which they have a low affinity. The presence in *Xenopus* of melanophores on the lateral surfaces of the tail somites appears to represent a similar situation. Work on PET strains of mice, in which melanocytes are present not only in the skin but also, unusually, in connective tissue and muscle, seems relevant. Mayer & Reams (1962) found that melanoblasts left the skin and entered other tissues only if the melanoblasts had fully colonized the skin. Furthermore, Harris (1973) reported that cells cultured on a substratum composed of regions of different adhesive strengths and which had been confined to the more adhesive regions had, on reaching a critical population density, subsequently migrated out from such regions and entered less adhesive regions. Thus there is evidence for the migration of melanoblasts from one tissue into another being dependent
not only upon melanoblast-tissue affinities but also upon melanoblast population pressure.

It is seen then that the interplay of melanoblast-tissue affinities and melanoblast population pressure can account for the migration of melanoblasts in the hypomere during normal development. From a study of Fig. 4, which depicts the migratory pathways of labelled neural crest cells, it can be argued that melanoblasts arising from a ventral stream of neural crest cells can freely enter UHT, a tissue for which they have a high affinity. The population of melanoblasts will then continue to increase until it reaches a critical population density, whereupon the melanoblast population pressure at this density compels melanoblasts to spread into the relatively less attractive MHT. The absence of melanophores from most mid-ventrally situated grafts of UHT suggests that melanoblasts seldom attempt to colonize LHT during normal development. In this instance it is possible that the population of melanoblasts which enters the hypomere, though large enough to reach in UHT the critical density for the melanoblast population pressure there to compel melanoblasts to leave UHT and enter MHT, is not sufficiently large for a similar sequence of events to take place in MHT. The proposal that the small size of the migrating melanoblast population accounts for the absence of these cells from LHT is strengthened by the finding of MacMillan (1971) of a fixed melanophore complement in *Xenopus*, shown by experimental larvae possessing reduced numbers of melanophores following partial ablation of neural crest tissue.

The finding of large and approximately equal numbers of melanoblasts in somite-situated grafts of the various hypomeric tissues at stage 29/30 suggests that at this stage all hypomeric tissues possess high melanoblast-tissue affinities which, however, in normal larvae, are incapable of influencing the distribution of melanoblasts, as the latter do not ordinarily colonize hypomeric tissues until stage 31 (Stevens, 1954). Alternatively, the regional differences in the melanoblast-tissue affinities of the various grafts at stage 32 may, although present, be masked at stage 29/30 by the high population pressure of melanoblasts which at this earlier stage are migrating out from the nearby neural crest.

It is therefore proposed that the migration and localization of melanoblasts are controlled by:

1. Melanoblast population pressure, which, on reaching a critical level, causes melanoblasts to spread from regions of high population density into regions of low population density.

2. Melanoblast extensibility, enabling contact to be made between melanoblasts and tissues not in their immediate environment, and so enabling these cells to select from these tissues the site for which they have the greatest affinity.

3. Variation in the adhesive properties of the environmental tissues, determining whether or not melanoblasts (a) migrate into available tissues and (b) remain in tissues into which they migrate.

4. Loss of migratory capacity from differentiating melanoblasts.
The control of melanophore patterns in other Amphibia

The basic mechanisms found to control the distribution of melanophores in *Xenopus* which are described in this paper may also be relevant in interpreting investigations of pigment pattern formation in other amphibian species. For example, an analysis of experiments employing homografts and heterografts of embryonic tissues in order to demonstrate regional and species-specific differences in the abilities of particular tissues to support pigmentation in terms of tissue provision of prerequisites of melanogenesis (see, e.g., DeLanney, 1941; DuShane, 1943; Twitty, 1945) revealed that the experimental results might be better interpreted in terms of tissue selection by melanoblasts, such selection being effected by relative differences in melanoblast-tissue affinities. In *T. torosa* the melanophore distribution is characterized by a sparse population of melanophores in the uppermost region of the hypomere and a dense band of melanophores along the dorsal ridges of the somites. The latter band is formed by a secondary reaggregation of melanophores, following a phase in which some melanophores are found on the lateral surfaces of the somites. Twitty (1945) attributed this phenomenon to a mutual repulsion among melanoblasts during the migratory phase being replaced after melanoblast differentiation by a mutual attraction of melanophores, and proposed that the specificity of the somite ridges for reaggregation was due to a local increased availability of melanogenic substances, thus enabling a more rapid differentiation of melanophores in this region. He further proposed that these melanophores would serve as a reaggregation centre for subsequently differentiating cells.

However, the existence of the postulated increased availability of melanogenic substances has not been demonstrated. In the present work the author was able to show that in *Xenopus* pigment patterns are not dependent upon regional differences in the amounts of melanogenic substances, and suggests that Twitty's observations might be more readily explained in terms of the possession by melanophores of a higher affinity for tissues at the dorsal ridges of the somites than for tissues at the lateral surfaces of the somites. As a result of such relative differences in affinity melanophores situated in tissues at the dorsal ridges of the somites and under the influence of the stronger adhesive interactions taking place in this region would have a greater tendency to remain *in situ* than would melanophores situated at the lateral surfaces of the somites. Hence, during melanophore reaggregation, melanophores at the lateral surfaces of the somites would tend to be drawn towards those melanophores in the dorsal ridges of the somites, rather than the reverse.

In *T. torosa* the restriction of hypomeric melanophores to the uppermost region of the hypomere has been attributed to factors in the local tissues which prevent melanoblasts from migrating into the more ventral tissues (DeLanney, 1941; Finnegan, 1958). Finnegan found that following heterotopic transplanta-
tion of neural crest tissue to the hypomere the distribution of melanophores indicated a preferential dorsal migration of melanoblasts in hypomeric tissues, and suggested that these tissues possessed a gradient of physiological capability for supporting melanoblast migration which decreases dorso-ventrally. However, the existence of such a gradient has never been demonstrated, and Finnegan's observations might be better interpreted in terms of a dorso-ventral gradient of decreasing melanoblast-tissue affinity. The work of Rosin (1943) seems to support the proposal that relative differences in affinity can account for the banded pigmentation possessed by *T. torosa*. Following homotopic transplantation of integumental tissues from *A. mexicanum* to the upper hypomere of *Triturus alpestris*, the latter species having a banded distribution of melanophores similar to that of *T. torosa*, increased numbers of melanophores were observed in the hypomere, together with a decreased number of these cells in the dorsal band, suggesting that melanophores which normally would have been situated in the dorsal band had been attracted into the donor tissues on the hypomere. Such findings may best be interpreted in terms of melanoblast-tissue affinities, in that (a) banded distributions of melanophores are determined by relative differences in melanoblast-tissue affinities, and (b) the affinity of melanoblasts for the grafted tissues of *A. mexicanum* is greater than the affinity of these cells for upper hypomeric tissues of *T. alpestris*.

This suggestion of the existence of differences in melanoblast-tissue affinities in similar tissues in different amphibian species receives support from other studies on *A. mexicanum*. The contrasting pigment patterns exhibited by black and white strains of this species of axolotl have been shown to arise from differences in the capacities of the integuments to support the migration of melanoblasts (Dalton, 1950). Furthermore, melanoblasts of either genotype contained in chimaeric vesicles were found to migrate preferentially into the black axolotl component, indicating a greater affinity of melanoblasts for black than for white integument (Brick & Dalton, 1963). Hence the affinity between melanoblasts and a specific tissue, in this instance integument, has been shown to exhibit genetic variation.

*A physical model for pigment pattern determination in Amphibia*

In seeking a fundamental mechanism which might underlie larval melanophore patterns in amphibians the findings of the present experiments were re-examined in relation to a careful analysis of the experimental results and conclusions of other workers on amphibian pigmentation.

The various amphibian pigment patterns may be placed in two groups, one in which melanophores are widely and uniformly distributed (uniform patterns), and another whose members exhibit regional differences in pigmentation (banded patterns). In many species in the latter group the trunk melanophores are restricted to the dorsal surface of the neural tube, the dorsal ridges of the somites and the upper region of the hypomere. As *Xenopus* possesses the latter
pigmentary characteristics, it may be appropriate to suggest at this point the possibility of a hierarchy of melanoblast-tissue affinities, similar to that shown in the present paper to be responsible for the melanophore pattern in larval *Xenopus*, being a general property of amphibian tissues.

In urodeles xenoplastic transplantation of neural crest from species which possess banded patterns to species which possess uniform patterns generally gives rise to essentially donor-type banded patterns (see, for example, Twitty, 1949). This generality is interpretable in terms of the present model as indicating that melanoblast-tissue affinities similar to those which operate in the donor species are present and capable of operating in the host, but are, however, suppressed in the normal animal. That is, a hierarchy of melanoblast-tissue affinities is present in the tissues of members of both pigmentary groups.

The size of the melanoblast complement appears to be a pertinent difference between members of the two groups. Amphibian species which exhibit banded pigment patterns appear to possess relatively small and fixed complements of melanoblasts (*T. torosa* — Lehman, 1951; *X. laevis* — MacMillan, 1971), which in *T. torosa* has been related to an inherently low division rate of melanoblasts (Youngs, 1957). In contrast, species such as *Triturus rivularis* and *Ambystoma punctatum* which possess uniform patterns possess relatively large melanoblast complements and a high capacity for melanoblast production, related to an inherently high division rate of melanoblasts (Lehman, 1951; Lehman & Youngs, 1952; Youngs, 1957). Accordingly, a further postulate is introduced to formulate a model for amphibian pigmentation, namely, the size of the melanoblast complement determines the extent to which melanoblast-tissue affinities are expressed in the tissues of the amphibian.

The proposal is then that species-specific differences in amphibian pigment patterns depend primarily upon the interaction between species-specific differences in melanoblast-tissue affinity and melanoblast population pressure. This proposal appears consistent with the experimental findings of many workers (see, for example, Twitty, 1936; Finnegan, 1958; Brick & Dalton, 1963): for brevity, only Finnegan’s work will be discussed here. Xenoplastic transplantation of neural crest from *A. punctatum* (uniform pattern) to the hypomere of ‘crestless’ embryos of early and late stages of *T. torosa* (banded pattern) indicated that the extent to which donor melanophores formed banded or uniform patterns was related to the number of donor melanoblasts which emigrated from the grafts, in that a small number of melanoblasts migrated into late stage host tissues and formed a banded pattern, whereas a greater number of melanoblasts migrated into early stage host tissues and gave rise to a uniform pattern. Furthermore, in *A. punctatum*, following homoplastic transplantation of neural crest to the hypomere, a uniform distribution of melanophores characteristic of this species was formed dorsal to the graft on the larval side bearing the transplant. However, a smaller number of melanophores formed a banded pattern on the side of the larva contralateral to the transplant. Although
Finnegan was unable to explain these findings, they are readily explicable in terms of the model proposed in this paper, i.e. in terms of the size of the migrating melanoblast complement and the degree of expression of melanoblast-tissue affinities. Thus, as a result of the relatively large number of melanoblasts migrating into the tissues in the vicinity of the graft, the melanoblast population pressure became sufficiently high to suppress the expression of tissue preference by melanoblasts, resulting in the observed uniform distribution of melanophores, whereas owing to the comparatively small numbers of melanoblasts which had migrated into the tissues on the side of the larva contralateral to the graft the melanoblast population pressure remained low, so permitting melanoblasts to distribute themselves in accordance with the relative differences in melanoblast-tissue affinities, and hence producing on this side of the larva the observed banded distribution of melanophores.

Hence the model described in this paper appears capable of accounting for the formation of melanophore patterns in larval Amphibia and to represent a fundamental mechanism underlying larval pigmentation, by which the distribution of melanophores is governed by a hierarchy of melanoblast-tissue affinities; the latter, however, depending upon the species, may be modulated or even suppressed by the melanoblast population pressure in the tissues.

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REFERENCES

Melanoblast-tissue interactions in Xenopus larvae


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