

# **Volume TWO (2)**

THE MORPHOGENESIS OF FEATHER PRIMORDIA

IN CHICKEN SKIN

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CHAPTER 5

THE DYNAMICS OF DERMAL MORPHOGENESIS

1.

INTRODUCTION

The evidence presented in this chapter relates to the morphogenesis of dermal condensations. In a series of parallel studies, attention is focussed on the rôles of cell division and movement. This is followed in the last experimental section by an investigation of strong adhesions formed by dermal cells which may play a part in the structural stability and morphogenesis of the condensation.

2.

DERMAL MORPHOGENESIS IN VIVO2.1 Introduction

This section deals with particular features of dermal morphology in vivo which have not been previously described, from the point of view of gaining some insight into the forces which underlie the structural changes.

The account is based on 23 specimens of freshly excised skin (Stages 29+ to 32; at least 3 specimens from each stage). Correspondence between the number of rows detected in the dissecting microscope and the number observed in serial sections was checked in each specimen. Some of these specimens were the same ones as used in the study described in Chapter 4.

2.2 ObservationsArrays

Comparison of the structure of arrays with that of lateral dermis provides some information on the forces responsible for cell orientation and alignment (Fig. 5.1a and b).

Cells of the lateral, dense dermis are typically broad and flat in the plane of the skin surface and lack orientation (see also Fig. 4.1c). Their cell processes extend about 15  $\mu\text{m}$ , in the same plane, making contact with neighbouring cells all around. In tangential sections, these cells are seen to lie in a tangled mesh of fibres or processes, but transverse sections show that some of these fibres are associated into long, wavy bundles lying parallel to the skin surface. Cell density is greatest immediately below the epidermal-dermal interface. At the medial boundary of the dense dermis, though cells are not mutually aligned, the frequency of bipolar, spindle-shaped cells is greater than in lateral regions.

At the lateral extremities of forming arrays, spindle-shaped cells point in all directions and only a few groups of cells follow a projection of the main axis of the array. Arrays which extend towards presumptive condensation sites are always more highly organised medially where their mutually aligned cells are typically spindle-shaped and oriented parallel to the skin surface, with long, almost straight processes extending 20 to 25  $\mu\text{m}$  from either or both ends of a cell body about 15  $\mu\text{m}$  long which contains a long, cylindrical nucleus. Medially these arrays appear to merge with the radially oriented cells which surround definitive condensations. Transverse sections indicate that some cells may pass below the condensation and that the array may be continuous with an array extended from the medial side of the condensation.

Well developed arrays - for example, those joining formed condensations (see, for example, Fig. 5.1) - are between 30 and 45 cells (100 to 150  $\mu\text{m}$ ) wide, 3 to 4 cells (about 15  $\mu\text{m}$ ) deep, and, in their length of around 150 to 200  $\mu\text{m}$ , span 5 or 6 cell lengths. They thus contain between 500 and 1000 cells. Orientation is most

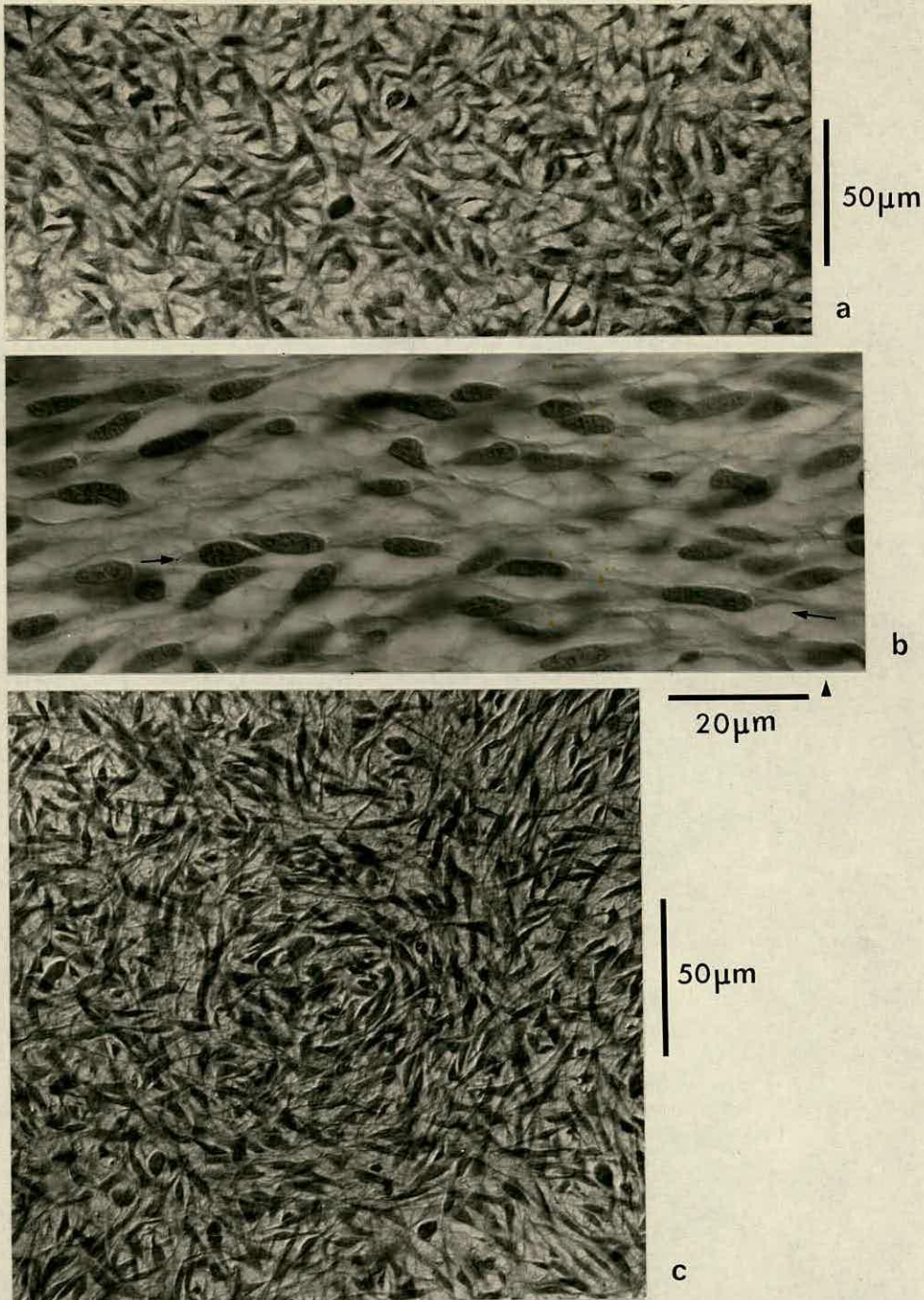


Fig. 5.1

Dermal morphogenesis in freshly excised skin.

- a. The starting point for morphogenesis; dense dermis.
- b. A well-developed intercondensation array: arrows indicate narrow files of cells arranged end-to-end.
- c. A definitive condensation in an early stage of development. The circumferential arrangement of some of the cells is especially clear.
- d-f. see over.

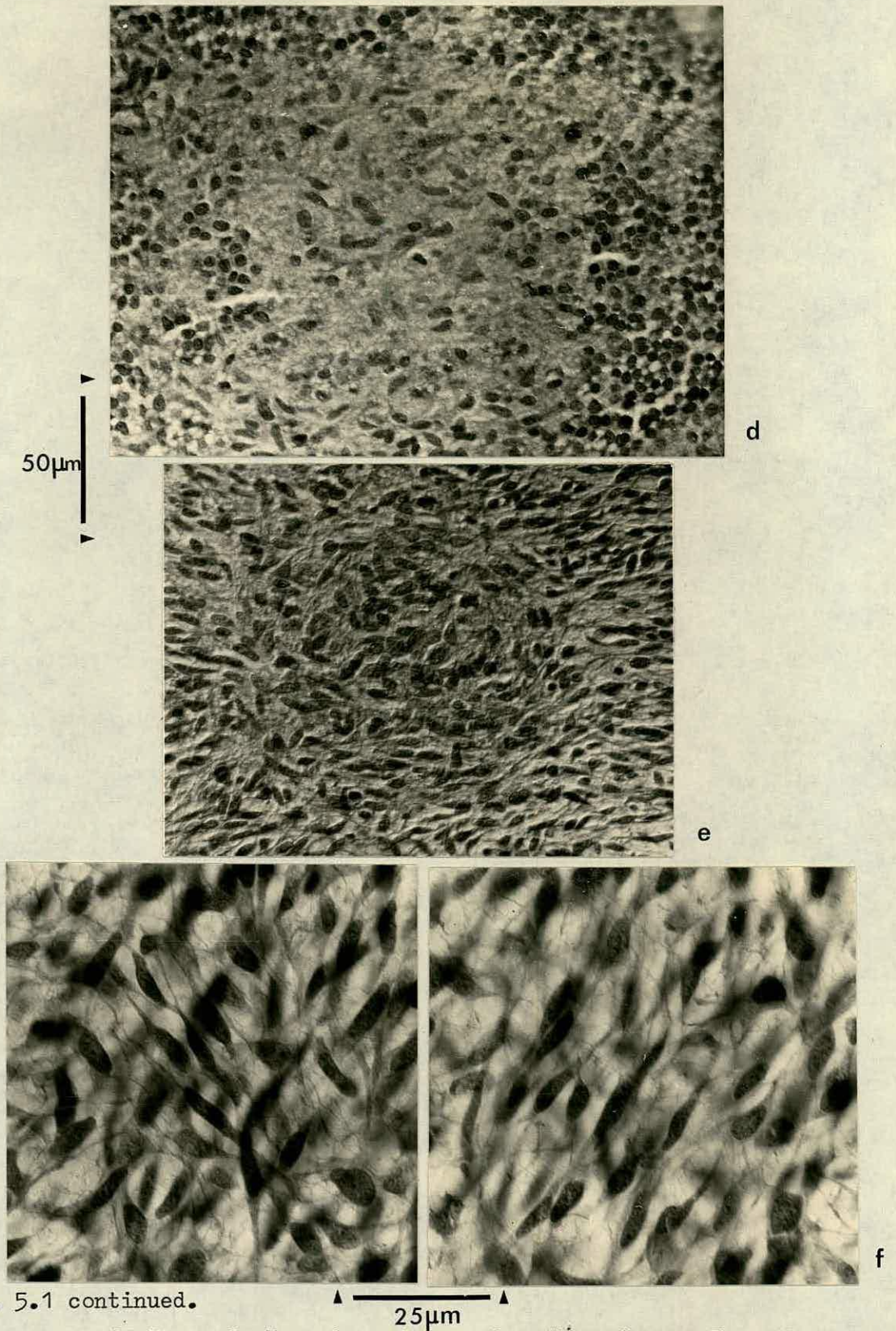


Fig. 5.1 continued.

- d. The apical region of a condensation in row 3. There are few nuclei, but fibres or cell processes are abundant.
- e. The central mass of cells in a row 3 condensation. Compare with a and c.
- f. The basal region of a condensation in row 5. These photographs illustrate arrays crossing: they were taken with the specimen in the same orientation, by focussing on different planes.

highly ordered about 20  $\mu\text{m}$  below the dermal-epidermal interface. Cells in the array are not closely packed. End-to-end arrangements and converging cell processes are commonly observed: in many places, cells lie precisely in the same straight line, though it is not clear if their cell processes which extend towards one another actually touch, or, for example, follow a common extracellular fibre. In addition, small groups of cells arranged end-to-end, two or three cells wide, lie along straight or slightly curved paths extending at least 300  $\mu\text{m}$  through the dermis. These lie parallel to the skin surface and at various angles, often close to, but distinct from, the predominant direction of the array. Each of these groups lies in a plane distinct from the rest of the cells in the array. These groups or files of cells are especially common where arrays meet. It is not possible to determine whether or not cells in arrays or in narrower files follow any particular fibres or general orientation of the matrix, for extracellular fibres are generally indistinguishable from long cellular processes. However, it is clear that fibrous material in general forms no grossly visible meshwork in arrays, but is aligned with the main bodies of the cells.

In addition to diagonal arrays, a major array lies along the midline from early in the development of the pteryla. Joining condensations on either side of the midline, well formed arrays develop which cross the midline array perpendicular to it. This is the only site, apart from presumptive condensation sites, where two arrays were observed to cross. In specimens at Stages 31 to 32, well developed arrays were sometimes observed to lie antero-posteriorly and medio-laterally between condensations: these did not cross one another in the specimens examined. These arrays were just as well formed as the more common diago-lateral arrays.

In several regions examined, cells of the dermis immediately adjacent to an array were oriented parallel to the skin surface and perpendicular to the array, but lay in a plane immediately below it. The remainder of the dermis between arrays is sparsely populated by scattered, non-polarised cells.

#### Intersections of Arrays

In most specimens examined in tangential section, where arrays were seen to intersect at the presumptive sites of condensations, there was no indication of the forthcoming condensation process. Arrays overlap in distinct, adjacent planes, typically at an angle of about  $120^{\circ}$ , but markedly different angles were observed in some cases. Arrays do not interweave, and their cells mingle only where a few curve round the medial angle of the intersection.

However, in about one third of the intersections examined, cell arrangements were observed which may reflect the forces involved in the beginning of the condensation process. Near the presumptive condensation site, it was possible to distinguish a ring, two or three cells wide and 70 to 80  $\mu\text{m}$  in diameter, composed of long, curved cells. A small cluster of short, unoriented cells with oval or nearly circular nuclear profiles was often observed within this ring. Examination of transverse sections suggested that these cells are not oriented perpendicular to the skin surface and that their nuclei are ovoid in shape, although this observation is based on only a few cases where such arrangements were discernible in transverse section. These structures resemble the more highly organised arrangements found in the first definite stages of condensation. However, one cannot be certain that these rings and clusters are exclusively involved in the condensation process, for they are sometimes found in other parts of the dermis.

## Condensations

When viewed at low magnification, several tangentially sectioned initiating condensation sites gave the impression of a higher tissue density than the surrounding dermis, without signs of any recognisable condensation at the cellular level (see, for example, Fig. 4.1). The earliest recognisable stage in the condensation process, as seen at high magnification, is the appearance of distinctive radial and tangential cell orientation round a central region where, though cell density is not appreciably greater than in the surrounding dermis, cells lack consistent orientation and many appear (from examination of both transverse and tangential sections) to be shorter than those of the surrounding regions (Fig. 5.1c).

The boundaries of condensations at this stage do not touch the boundaries of either neighbouring condensations in the preceding row.

A picture of the three-dimensional structure of early condensations (rows 1 to 3) can be reconstructed from observations on serial tangential sections coupled with information from transverse sections. Immediately below the placode, an area about 6  $\mu\text{m}$  thick is sparsely populated with nuclei, but filled with fibrous, eosin-staining material at least partly composed of cell processes which show no consistent orientation. Below this, cells form patterns which become more pronounced over the next 10 to 20  $\mu\text{m}$ . Thus, about 25  $\mu\text{m}$  below the dermal surface lies a prominent central mass of cells between which there is little eosin-staining extracellular space by comparison with intercondensation dermis. This mass is surrounded by radially oriented cells which are found down to about 40  $\mu\text{m}$  below the dermal surface. A smaller number of cells lie parallel to the circumference of the condensation. These are most

frequent at about 30  $\mu\text{m}$  below the dermal surface. Cells are often stacked in mutually orthogonal arrangements: for example, individual circumferentially oriented cells may be found above and below radially oriented cells. However, individual cell bodies are not interwoven. None of these arrangements of oriented cells is based on close-packing. (These features are shown in Fig. 5.1c-e.) A few elongated cells are present at the base of the condensation: in some cases, these compose small groups of common orientation, however arrays are not clearly observed below condensations at this stage. Transverse sections do, however, show that a few cells from adjacent arrays point downwards towards elongated cells at the base of the condensation.

Older condensations (rows 4 and 5) showed a similar structure with the following additional features. Cells appeared (without quantitative analysis) to be much more closely packed in the central mass. Orientation at the periphery of the condensation is more marked than in earlier stages. The base of the condensation is made up of cells in two striking and distinct arrays which lie on adjacent planes and are moulded round the sides of the condensation and continuous with intercondensation arrays so that the core is contained within a basket-like structure of oriented cells (Fig. 5.1).

### 2.3 Discussion

These observations will be discussed in relation to two questions: first, how do dermal arrays form; second, do arrays play a part in the formation of condensations? These questions concern dynamic mechanisms, and it must be emphasised that static morphological evidence alone cannot give complete answers. Nevertheless, the evidence does suggest that some possibilities are more likely

than others and thus point the way to further analysis by experiment. Experimental investigation of some of these possibilities - particularly those related to the second question - is described in the following sections of this chapter; others, concerning the formation of dermal arrays, have not been investigated further, but are discussed here because of their importance to the overall process of dermal morphogenesis.

The problem of how dermal cells become mutually aligned, and in particular whether or not their orientation is guided by extracellular fibres, has been widely discussed in relation to the morphology of the matrix (Wessells and Evans, 1968; Stuart et al, 1972; Overton and Collins, 1976; see Chapter 1 for review), but attention is focussed here on the detailed arrangement of the cells.

The observation that narrow files of cells in end-to-end arrangement commonly run for several hundred microns through the dermis and are oriented independently of neighbouring cells indicates that the orienting influence acts on several cells along a common line. The most obvious explanation is that cells in a file are oriented along a common matrix fibre. Circumstantial evidence supports this idea: cells form very similar arrangements by aligning along fibres in the substratum or matrix in culture (for example, on a substratum of fish scale (Weiss, 1959) or in a matrix of hydrated collagen (Elsdale and Bard, 1972)), while the conditions for such alignment are present in the dermal matrix, for fibres in the isolated matrix are seen in the SEM to follow curved paths similar to those followed by the files of cells (Overton and Collins, 1976). At least two alternative possibilities do not seem to hold: the cells are not closely packed side by side, nor do they seem to be simply stretched between adhesive contacts with preceeding and

succeeding cells in the file, for the file - and even individual cell bodies - follow smooth curves.

What forces align cells side by side in the major intercondensation arrays? The arrangement is not based on packing, rather the array resembles several narrow files of cells arranged in parallel. The observation that individual bipolar cells and files of cells are haphazardly oriented near the medial boundary of the dense dermis, but become progressively organised in parallel arrays more medially, suggests that the orientation of cells in files and the parallel arrangement in arrays occur by different processes. The possibility that cells within a file are aligned along a fibre suggests that arrays are produced by forces that align fibres. One explanation is that fibres are aligned by tension forces in the dermis directed between condensation sites. Tension has been suggested by Garber (1976) to account for alignment of cells along the dorsal midline.

On one hand, there is circumstantial support for this hypothesis. General arguments show that such a mechanism is possible. Weiss (1959) has shown that fibres in a plasma clot can be oriented by tension between contracting centres (for example, between fibrocyte cultures), and that cells align along these fibres to form structures which are very similar to intercondensation arrays. Weiss (1933) has also reviewed evidence that tension (and other physical factors) influences cell patterns in vivo; more recently, Belousov, Dorfman and Cherdantzev (1975), for example, have shown that stress patterns occur during the development of the Amphibian neural plate which are later followed by actively elongating and migrating cells. It is likely that such a mechanism would operate

in the feather system if the appropriate tension forces were present; Overton and Collins (1976) have emphasised the sensitivity of isolated dermal matrix to stress forces, imposed during its preparation, which produce alignment in groups of fibres. The fact that Overton and Collins could not detect natural alignment in these matrices could be due to relaxation of the matrix as cells (which might be supposed to generate the tension) are removed. The present observations also provide support for this hypothesis: the files in intercondensation arrays are very straight and certainly have the appearance of being stretched, and the formation of antero-posterior and medio-lateral arrays between forming condensations might suggest that the condensation is a contracting centre responsible for the development of the array. The radial orientation of cells around forming condensations might also be accounted for by this mechanism.

On the other hand, the tension hypothesis suffers particular difficulties. At the intersections of arrays, cells are not simply pulled towards the centre of the condensation site: files and arrays lying in adjacent planes point in different directions, so the pattern of forces orienting the cells must be quite complex. Moreover, arrays intersect before condensations form and some files of cells lie across the presumptive condensation site and run into lateral unpatterned dermis. There is no morphological evidence to suggest where the tension responsible for such alignments might be generated.

One alternative to the tension hypothesis is that matrix fibres are laid down in their final orientation. The assembly of aligned collagen fibres appears to be possible: the most striking example is the formation of layers of mutually orthogonal collagen fibres in the chicken corneal stroma (Trelstad and Coulombre, 1971)

which eventually dictates the alignment of invading secondary mesenchyme cells (Bard and Higginson, 1977). However, this hypothesis pushes the problem only a little further back, since we have no idea what cellular or molecular mechanism is responsible for such an ordered assembly of matrix.

A second alternative is that cell behavior alone is responsible for the formation of arrays. Ede et al (1971) have suggested that cells shuttle back and forth between attractive (chemotactic) centres in condensations. However, the alignment of cells in very straight files appears to be more precise than this mechanism would suggest.

None of these mechanisms is supported by strong evidence and each is quite difficult to test in a positive way. The tension hypothesis is, perhaps, most interesting in the light of the present results, since it emphasises the possibility that condensations may be contracting centres, and this accords well with the evidence presented in the remainder of this chapter. Experimental investigation of the rôle of tension is simple in principle - for example, a cut between condensations would be expected to result in the rapid disorganisation of the whole array: preliminary experiments indicate, however, that it is difficult in practice to obtain a clean result because arrays are quite short and the operation itself generates tensions.

With regard to the rôle of dermal arrays in the formation of condensations, the situation is clearly more complex than has previously been thought. Though it has been suggested that cells migrate along arrays till their movement is restricted at the inter-sections (Stuart et al, 1972; Garber, 1976) there is no

morphological evidence to suggest that movement is more restricted at intersections than elsewhere in the array. Cells in intersecting arrays do not collide but lie in different planes. Moreover, orientation is not confused at the intersection; on the contrary, the organization of the array increases with time at the condensation site, and as the condensed mass of cells forms immediately below the epidermis arrays become increasingly organised at its base. It seems likely that this latter development is associated with the increased rate of deposition of newly synthesized acid mucopolysaccharides at the base, observed by Sengel, Bèscol-liversac and Guillam (1962). These observations emphasise that arrays may play a structural rôle rather than acting as pathways of cell movement into the condensation.

### 3. QUANTITATIVE ANALYSIS OF DERMAL CONDENSATION IN CULTURE

#### 3.1 Introduction

In this and the following sections, evidence is presented from studies on the development of condensations in culture where analysis and experimentation are facilitated. Although the initial stages of morphological development in culture differ from those in vivo (Chapter 3), some insight into the essential mechanisms of the condensation process may be gained.

The object of the present section is to estimate the increase in cell numbers in the dermal condensation during the early stages of its development in culture, and the maximum proportion of this increase which could be the result of local cell proliferation below the placode.

Estimates of cell numbers and cell density in developing condensations have been made by Wessells (1965), but though these give comparative values they cannot be used to analyse the dynamics of the condensation process for they do not give a realistic estimate of the number of cells in a condensation, which is required to assess the contribution of cell division to the process. They are based on simple nuclear counts on transverse sections together with an estimate of the volume counted. The estimate of cell numbers obtained in this way is not accurate: for many nuclei counted, less than half the nucleus will lie in the section examined and the number of cells will therefore be overestimated. Abercrombie (1946) has shown that when the section thickness approaches the nuclear diameter the over-estimate of cell numbers approaches 100%: nuclear diameters in the condensation are in the range 5 to 7  $\mu\text{m}$  and section thickness used by Wessells was approximately 5  $\mu\text{m}$ .

Cell division within developing condensations has not been examined in a way which allows the rate of proliferation to be estimated (the evidence is reviewed in Chapter 1).

Attention is focussed here on the period of development beginning when the condensation first becomes clearly defined in transverse sections (represented by the most lateral row: row 1) and ending when the condensation is well developed (represented by the second most lateral row: row 2). Cell density and numbers are estimated from corrected nuclear counts and the rate of cell division is estimated from the accumulation of mitotic figures in the presence of colcemid.

### 3.2 Method and Calculations

#### Specimens

Four specimens were used in the present analysis. These were explanted at Stages 29 to 29+ in three independent experiments, and cultured till several rows of primordia were present (Table 5.1).

TABLE 5.1 Specimens used in the Present Analysis

Specimen	Time in culture (days)	Time of day fixed	Living Specimens		Transverse Sections	
			No of rows primordia	Individual primordia	No of rows primordia	Individual primordia
I	2	noon	5	-	5	-
II	2	noon	6	(20)	6	(20)
III	2	noon	9	-	9	-
IV	2	11:00pm	7	(17)	7	(17)

Specimens I and II were from the same experiment.

### Regions examined

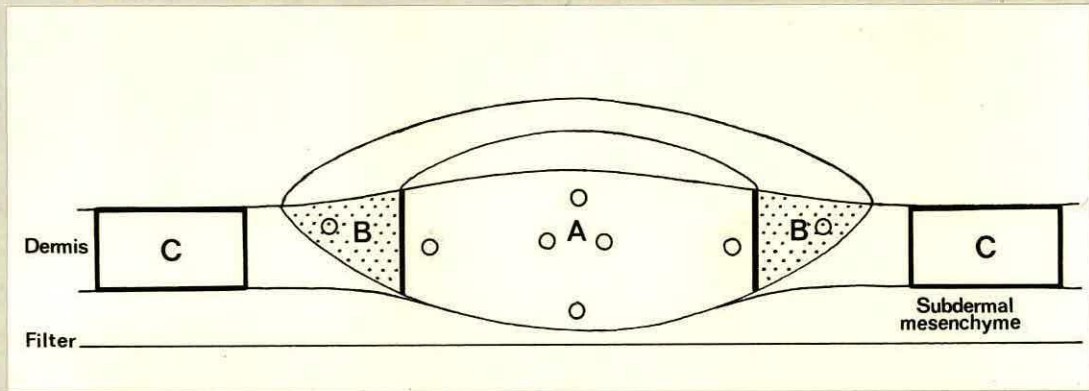
Two condensations in each of rows 1 and 2 were examined in transverse sections (except specimen 3; where 3 row 1 condensations were examined). Three of the four most central sections in each condensation were selected for counting on the basis of technical quality (except specimen 2, condensation P; two sections, see Table 5.5). Within each section 3 regions were analysed (Fig. 5.2a).

- A. Central region, 111  $\mu\text{m}$  (15 graticule squares) in diameter.
- B. Remainder of the condensation.
- C. Rectangular sample areas in the adjacent dermis on either side of the condensation, each beginning 15  $\mu\text{m}$  from the edge of the condensation. The area varied between approximately 500 and 1300  $\mu\text{m}^2$  according to the space available between adjacent condensations. In a few cases (Table 5.7) this area was too small to analyze. C. regions lateral to row 1 are termed 0/1; regions between rows 1 and 2 are termed 1/2; and regions medial to row 2 are termed 2/3.

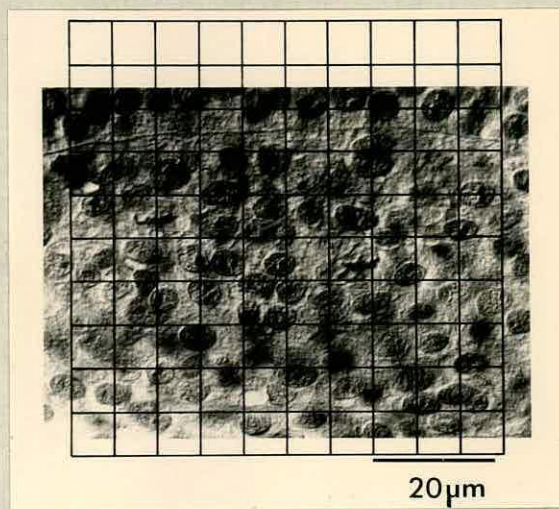
The size of region A was chosen arbitrarily for the purposes of comparison between different condensations, because all condensations examined were of greater diameter than 111  $\mu\text{m}$ ; the condensation in this region occupies approximately the entire depth of the dermis.

### Cell numbers in the section

Cell numbers in each section were estimated from nuclear counts made at a magnification of x 1250 using a squared graticule eye-piece as a guide (one side of square = 7.4  $\mu\text{m}$ , see Fig. 5.2b) (see Chapter 2 for details). The condensation profile, though clearly defined at low magnification, was not distinct at the



a



b

Fig. 5.2 Illustration of sampling methods.

- a. Regions sampled. The diagram represents a condensation cut transversely in half and viewed face-on from above. See text for meaning of symbols;  $\circ$  section thickness measurement points.
- b. View of cells in a condensation and the eye-piece graticule used as an aid to counting nuclei. Each square or column of squares represents a square or column referred to in the Tables.

cellular level; the boundary of the condensation was judged approximately according to cell morphology and is likely to represent the true boundary to within about 1 cell diameter. Nuclei were counted in successive columns of graticule squares (Fig. 5.2b) throughout the entire width of the condensation, the columns ranging in height to fit the profile of the condensation.

Mitoses were also counted in the same regions and percent mitosis calculated. (All percent mitosis quoted are calculated from the uncorrected number of mitoses counted and the uncorrected number of nuclei: since the diameter of a mitotic figure is approximately the same as the diameter of a nucleus, this value provides an adequate estimate of the proportion of mitotic figures in the nuclear population.)

Nuclear counts were corrected to obtain an estimate of the true number of nuclei in the section using Abercrombie's correction factor (Abercrombie, 1946):

$$n = c \left( \frac{T}{T + l} \right)$$

where  $n$  is the corrected number of nuclei,  $c$  is the counted number of nuclei,  $T$  is the section thickness ( $\mu\text{m}$ ), and  $l$  is the mean length of nuclei ( $\mu\text{m}$ ) perpendicular to the plane of the section.

The length of nuclei was measured parallel to the midline in tangential sections through the dermis of cultured skin in specimens from an independent experiment (the approximation involved in using measurements of the length of nuclear profiles in section as an estimate of true nuclear length does not lead to serious errors (Abercrombie, 1946)).

Section thickness was measured using fine focus of the Universal microscope (see Chapter 2) at 8 points over the condensation in each section (Fig. 5.2b). To calibrate the fine focus

adjustment, pieces of one glass coverslip were measured in the Universal microscope, and using a micrometer screw gauge and by examination in the SEM. (Table 5.2). These measurements confirm the correspondence between the graduations on the fine focus adjustment and the  $\mu\text{m}$  units as measured with a screw gauge and in the SEM. In addition, there was little variation between repeated measurements of sections in the order of 5 to 8  $\mu\text{m}$  thick (see for example, Table 5.3), indicating that the mean values of section thickness presented in the results can be taken as sufficiently precise for the present purposes.

#### Cell density

Cell density was calculated directly, by dividing the estimate of cell numbers in the section by the estimated volume of the section (area counted x section thickness).

#### Volume of Region A

Trial calculations showed that the volume of region A cannot be reliably estimated from the volume of geometric shapes approximating to the shape of region A: the condensation is too irregular in shape and the geometrical estimates are too sensitive to small differences in axis measurements. The volume was therefore estimated directly from the area of region A in the sections counted, as follows. For each column of graticule squares in the left half of region A, the volume of rotation about the centre of the condensation was calculated. This was done by calculating the difference between the volume of two solid cylinders, both the same height as the column, one with radius equal to the distance from the centre of the condensation to the peripheral side of the column, the other with radius equal to the distance from the centre of the condensation to the central side of the column (i.e. for column height  $h$   $\mu\text{m}$  with a

TABLE 5.2 Measurements of the Thickness of a Coverslip Using the Universal Microscope, the SEM, and a Micrometer Gauge.

Fragments of one Coverslip	Measurement of Thickness ( $\mu\text{m}$ )		
	Universal Microscope Mean of 5 measurements; SE	SEM Mean of 5 measurements; SE	Micrometer Gauge Mean of 10 measurements; SE
1	128 ; 0.51		
2	123 ; 1.0	123 ; 0.49	
3	129 ; 1.3	131 ; 0.37	
4			134 ; 0.60

peripheral edge  $r \mu\text{m}$  from the centre of the condensation, the volume of rotation is  $\pi h (r^2 - (r - 7.4)^2) \mu\text{m}^3$ . Volumes of rotation of successive columns were summed to obtain an estimate of the volume of the region. This procedure was repeated for the right half of region A to obtain a second estimate. The mean of these two estimates is quoted as the volume of region A.

#### Number of cells in region A

The number of cells in region A was estimated by multiplying the cell density for each column by the volume of rotation of the column and the results summed for columns in each half of region A. The mean for the two halves of region A is quoted as the estimated cell numbers.

### Cell Proliferation

To estimate the rate of cell proliferation in the condensation, and to compare this with the rate of cell proliferation in the surrounding dermis, the accumulation of mitoses in the presence of colcemid was measured. This approach has been used similarly in other systems (for example in plant meristems (Evans, Neary, and Tonkinson, 1967)). The use of this method raises the need to estimate the effectiveness of the drug in arresting mitosis. The drug arrests mitosis in metaphase by interfering with spindle assembly (Brinkley, Stubblefield, and Hsu, 1967): its efficiency at the time of fixation has therefore been estimated by comparing the number of anaphase plus telophase (a + t) figures in treated and control explants. In addition, an estimate of the effectiveness of the drug over the period of treatment has been made by measuring percent mitosis as a function of time in colcemid.

The rate of cell division in condensations and inter-condensation dermis, and the effectiveness of colcemid at the time of fixation, were estimated in specimens cultured from Stage 29 till 7 rows had developed then treated for  $5\frac{1}{2}$  hours in colcemid ( $5.4 \times 10^{-6}M$ ), and fixed (fixation time, noon). Mitoses were counted in transverse sections. To estimate the accumulation of mitosis as a function of time in the drug, specimens cultured from Stage 29 till several rows had formed were treated with colcemid ( $5.4 \times 10^{-6}M$ ), for periods of between 2.5 and 10.75 hours (two independent experiments; specimens are shown in Table 5.18; fixation time, 5 p.m. till midnight).

### 3.3 Results

#### General Morphology of Condensations

The morphology of condensations used in this analysis (Fig. 5.3) was the same as that described for other cultured specimens examined in transverse section (Chapter 3).

The chief difference between condensations of the two rows was in their size and shape (mean diameter and depth; row 1, 173 and 40  $\mu\text{m}$  respectively; row 2, 238 and 51  $\mu\text{m}$ ; see Table 5.4).

The relevant morphological details are as follows:

Row 1 condensations: The apical surface was only slightly domed while the base of the condensation lay on, or within one cell diameter of, the upper surface of the subdermal mesenchyme.

Row 2 condensations: The apices were, without exception, markedly domed. The bases lay on the subdermal mesenchyme.

Cells in the central mass of condensations in either row had large nuclei with prominent nucleoli, and were apparently closely packed with no unstained extracellular space. These cells occupied almost the entire volume of condensations in row 2. In the remainder of the dermis, nuclei were either small and intensely stained, or intermediate in size and staining: the former were most common in the peripheral regions of the condensation.

#### Cell Counts: Raw Data

An example of the measurements made on one row 2 condensation is given in Table 5.3\*. Region A encompasses most of the

\* Tables are shown on pages 182 to 198.

Abbreviations used in Tables in this chapter:

Cond. - condensation; No. - number of; Region A,B,C - refers to regions shown in Fig. 5.2a. SE - standard error (standard deviation divided by  $\sqrt{n}$ , where n is the sample size); Sp. - specimen; Sq. - graticule squares (7.4  $\mu\text{m}$  x 7.4  $\mu\text{m}$ ); T - section thickness; 0/1, 1/2, 2/3 - see text.

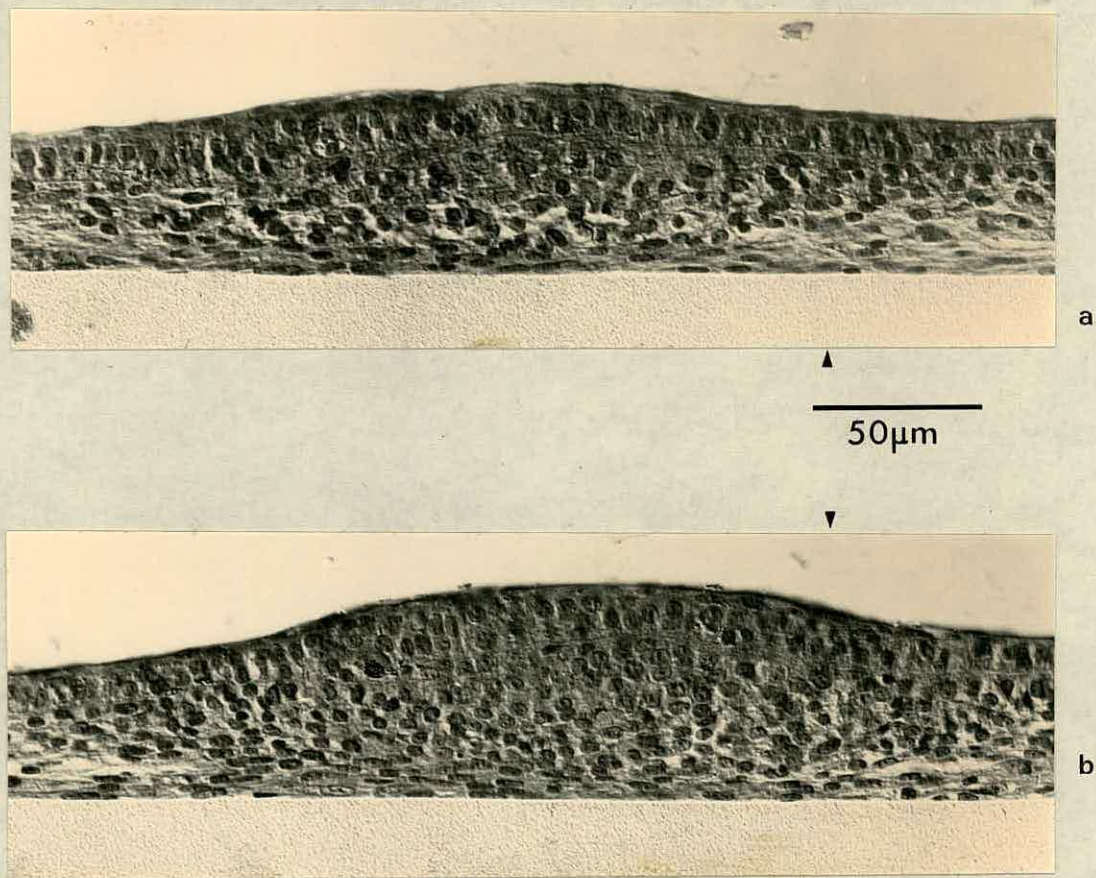


Fig. 5.3 Primordia in cultured skin.

- a. Row 1 (from Specimen IV)
- b. Row 2 (from Specimen I)

width of this condensation: in several other row 2 condensations region A encompassed a smaller part of the width (see Table 5.4). Data for each section counted in each condensation is shown in Table 5.5. Table 5.7 gives equivalent measurements in region C. The lengths of nuclei in regions A, B and C are given in Table 5.9. From Table 5.9 it is clear that cells in the condensation are nearly spherical or are randomly oriented, those at the centre of the condensation being significantly larger than those at the periphery. Cells in the intercondensation region are, on average, longer parallel to the midline than perpendicular to it.

#### Cell Density and Numbers, and Region Volume: Derived Estimates

An example of the estimates of cell density, region volume, and cell numbers in region A for 3 sections through a single condensation is given in Table 5.10. There is considerable variation within the estimated volumes, even within the same section, reflecting the irregularity and asymmetrical shape of the condensation. Generally, calculated cell densities and cell numbers showed less variation between sections.

The estimated cell density, volume and numbers for region A, in each condensation examined, are shown in Table 5.11. The most striking feature of these values is the consistently greater number of cells in region A in row 2 condensations than in row 1. The increase in the number of cells in this region, between the two rows, amounts to about 60% of the number of cells in row 1 (Table 5.14a). The estimated value for this increase does, however, show considerable variation. A randomised block test, in which the effect of variation between specimens is eliminated in a comparison between rows, shows that this difference in cell numbers is significant at

the 5% level (Table 5.15). The volume, too, is greater in row 2 than in row 1. In contrast, the cell density shows no consistent difference between the two rows.

Estimates of cell density in region B also show no consistent difference between rows (Table 5.12), and the estimated values for cell density in region C (Table 5.13) show no consistent differences between regions in 0/1, 1/2 and 2/3.

Comparing cell density values in different specimens, (Tables 5.11, 5.12, and 5.13), a certain amount of consistent variation may be noted: for example, specimen IV is generally higher in cell density than specimen II.

Comparing cell density within regions and between rows (Table 5.14b) shows little difference, but for a possible slight increase in cell density in region A between rows 1 and 2. To test the significance of this further would require that a larger number of specimens be counted. Comparing regions within rows (Table 5.14b) the most striking feature is the lower cell density in region A than elsewhere in the dermis. Comparison of Tables 5.11 and 5.12 shows that this difference is highly consistent within the condensation.

#### Data on Mitosis

Counts of mitoses in the same sections as were used for cell counts are shown in Table 5.6 and 5.8 for condensation and intercondensation dermis respectively. There is little difference between the frequency of mitosis in condensations and intercondensation areas in these untreated specimens, although the former show a slightly higher value than the latter. Estimates of the frequency of mitosis in intercondensation regions of other untreated specimens (Table 5.18) give approximately the same values. In the

course of counting mitoses in these specimens it was clear that their frequency was approximately uniform throughout the condensation (as judged without quantitative analysis).

The same mean percent mitoses was observed in condensations as in intercondensation dermis, after  $5\frac{1}{2}$  hours in colcemid (Table 5.17). The distribution of mitoses through the condensation and the immediately surrounding intercondensation dermis is approximately uniform (Fig. 5.4): there is no depression in the number of mitoses near the centre of the condensation such as is found in vivo (Wessells, 1965) and, though there is a suggestion of a peak in the number of mitoses in the intercondensation dermis immediately surrounding the condensation, this is not as high as might be expected from in vivo studies (Wessells, 1965) and more data will be required to establish whether or not this is a real phenomenon. The proportion of a + t figures in the same specimens is shown in Table 5.16, and may be compared with the proportion of a + t figures in controls for the same experiment (this is discussed further in Section 3.4).

Percent mitoses in intercondensation dermis as a function of time in colcemid (Table 5.18; Fig.5.5) shows considerable variation over the first 6.5 hours: accumulation of mitotic figures shows a very approximately linear increase with time in the drug. (Since proportions (e.g. percent mitosis) do not satisfy the assumptions on which regression analysis is based, it is necessary to transform the data ( $x$ ) to the form  $\arcsin \sqrt{x}$  to perform regression analysis (Aherne, Camplejohn and Wright, 1977). A least squares regression line for the data  $\arcsin \sqrt{\% \text{ mitosis}}$  versus time from 2.25 to 6.5 hours has a slope of 1.1 mitosis per hour and an intercept of 1.5% mitosis (correlation coefficient=0.8412).) The data correlate well with the

TABLE 5.3 Example of Measurements on One Condensation  
(Condensation g)

Nuclei in each of three sections were counted in successive columns of graticule squares, each one square (7.4  $\mu\text{m}$ ) wide and of variable height. The Table shows data on each section for each region of the condensation. The height of each column and the number of nuclei in each column are presented. Section thickness, measured at 8 points in the condensation (Fig. 5.2a) is shown together with the number of mitoses in each section of the condensation.

T ( $\mu\text{m}$ )	SECTION 1		SECTION 2		SECTION 3	
		9.0		8.2		9.1
	8.3		8.4		9.6	
	9.0		7.9		9.3	
	8.8		7.3		9.2	
	8.8		8.3		7.6	
	8.2		9.0		8.8	
	8.2		8.5		9.4	
	8.5		8.7		7.0	
Mean T ( $\mu\text{m}$ )	8.6		8.3		8.8	
No. Mitoses	2		3		3	
REGION	Column height (Sq)	Nuclei	Column height (Sq)	Nuclei	Column height (Sq)	Nuclei
B	2.0	2	1.5	4	2.0	5
	2.0	6	2.6	3	3.0	7
	4.0	8	3.4	6	3.6	7
	4.3	8	3.6	8	4.0	9
	4.9	13	3.9	9	4.2	12
A	5.4	12	5.3	13	6.3	14
	5.6	10	5.3	13	6.3	14
	5.7	8	5.3	14	5.3	15
	5.7	14	5.3	11	5.4	12
	5.8	14	5.2	11	5.2	12
	5.0	10	5.3	10	5.5	7
	5.0	10	5.3	12	5.5	13
	5.0	12	5.6	12	4.5	14
	5.1	12	5.6	13	4.7	11
	5.3	13	5.6	15	4.9	11
	5.3	12	5.6	14	4.6	8
	5.3	8	5.6	12	4.7	9
	5.4	13	5.0	13	4.7	10
	5.5	11	4.7	10	4.5	14
	5.5	12	4.3	9	4.2	10
B	4.3	8	4.3	10	3.6	9
	3.9	9	4.0	6	3.3	8
	3.6	8	3.5	7	2.8	6
	2.2	7	3.3	7	2.4	5
	2.0	4	2.2	3	2.0	2
	2.0	4	1.0	2		

TABLE 5.4 Measurements of Condensation Diameter and Depth

Sp. No.	Cond. row	Cond.	Diameter ( $\mu\text{m}$ )		Maximum Depth ( $\mu\text{m}$ )	
			Values	Mean	Values	Mean
I	1	a	178, 163, 170	170	27, 38, 38	38
	1	b	185, 207, 200	197	23, 33, 30	29
	2	c	266, 296, 303	289	44, 43, 49	45
	2	d	222, 229, 222	225	38, 37, 39	38
II	1	e	148, 170, 155	158	32, 38, 37	36
	1	f	126, 133, 133	131	40, 39, 38	39
	2	g	192, 192, 185	190	41, 41, 41	41
	2	h	185, 178, 185	183	47, 47, 43	46
III	1	i	237, 222, 222	227	48, 52, 59	53
	1	j	148, 148, 155	150	49, 44, 42	45
	1	k	170, 170, 163	168	44, 44, 47	45
	2	l	266, 222, 266	252	56, 59, 53	56
	2	m	274, 281, 274	276	64, 67, 60	63
IV	1	n	178, 200, 207	195	30, 34, 32	32
	1	o	170, 170, 155	165	37, 37, 42	39
	2	p	244, 237	241	62, 60	61
	2	q	229, 244, 274	249	59, 56, 56	57

TABLE 5.5 Measurements on Condensations

This Table shows data from each of three sections through each condensation examined. Data for the whole condensation and for Region A are presented: (data for Region B may be obtained by subtraction). The area counted (calculated by summation of the column heights in each region) and the number of nuclei in this region are shown, along with the mean thickness of each section.

Sp. No.	Cond. row	Cond.	Whole Cond. (Regions A & B)		Centre (Region A)		T ( $\mu\text{m}$ )
			Area (Sq)	Nuclei	Area (Sq)	Nuclei	
I	1	a	91.3	154	66.1	102	4.4
			89.2	162	69.3	121	4.5
			91.5	159	68.1	91	4.6
	1	b	70.7	112	43.6	65	4.5
			94.7	140	57.9	70	5.2
			87.6	132	58.4	83	5.0
	2	c	153.3	336	82.3	174	4.4
			159.5	302	80.5	145	3.9
			163.8	332	89.9	177	5.1
	2	d	115.4	226	69.9	131	5.0
			116.8	229	67.4	102	4.9
			119.8	238	69.0	136	5.1
II	1	e	71.7	115	59.3	92	7.0
			74.3	142	60.2	115	7.6
			77.9	140	62.9	107	7.0
	1	f	64.0	149	61.0	141	7.9
			68.3	124	60.8	110	8.1
			65.6	138	59.0	127	8.0
	2	g	115.8	248	80.6	171	8.6
			112.3	247	79.0	182	8.3
			107.2	244	76.3	174	8.8
	2	h	112.7	232	79.9	165	7.4
			105.1	224	78.6	157	7.2
			103.7	210	74.6	141	8.1

(continued)

TABLE 5.5 (continued)

Sp. No.	Cond. row	Cond.	Whole Cond. (Regions A & B)		Centre (Region A)		T ( $\mu\text{m}$ )
			Area (Sq)	Nuclei	Area (Sq)	Nuclei	
III	1	i	180.7	259	95.2	134	5.3
			170.6	243	97.5	136	5.3
			176.8	241	104.6	126	5.3
	1	j	106.1	129	88.2	101	5.1
			98.5	131	80.2	99	5.5
			88.1	107	72.0	82	5.6
	1	k	106.6	164	80.8	121	6.0
			112.5	175	83.3	120	5.4
			99.3	173	76.8	129	5.5
	2	l	203.5	321	103.7	162	5.0
			183.9	264	108.6	146	4.9
			207.8	313	106.3	146	4.9
	2	m	234.2	369	124.2	187	5.9
			238.5	394	121.7	202	6.6
			235.0	358	117.1	181	5.7
IV	1	n	72.0	140	53.8	108	4.7
			99.1	188	65.1	124	4.6
			102.7	205	60.5	122	5.7
	1	o	87.7	175	66.4	128	5.4
			88.3	185	67.5	137	4.6
			78.7	155	66.2	128	5.7
	2	p	209.5	412	116.9	236	4.2
			192.9	389	113.6	223	5.3
	2	q	186.0	402	115.2	236	5.7
			187.0	407	106.1	230	6.0
			198.8	401	109.4	200	5.2

TABLE 5.6 Data on Mitosis in Condensations

This Table shows the number of nuclei and mitotic figures counted in each condensation. Figures are totals of counts on the sections sampled in each condensation. The number of nuclei has not been corrected by the Abercrombie factor (see text). Percent mitosis for each condensation, and overall per row, have been calculated from this data. Anaphase/telophase data are included to indicate control values as an aid to assessing the effectiveness of mitotic arrest in colcemid (see text).

Sp.No.	Cond. row	Cond.	Sum of Nuclei	Mitosis			
				Total*	Metaphase*	Anaphase*/ Telophase	% mitosis
I	1	a	475	5	5	0	1.1
	1	b	384	13	10	3	3.4
	2	c	970	24	21	3	2.5
	2	d	693	4	4	0	0.6
II	1	e	397	3	1	2	0.8
	1	f	411	2	1	1	0.5
	2	g	739	8	6	2	1.1
	2	h	666	6	4	2	0.9
III	1	i	743	4	4	0	0.6
	1	j	367	9	8	1	2.5
	1	k	512	9	7	2	1.8
	2	l	898	8	8	0	0.9
IV	2	m	1121	31	28	3	2.8
	1	n	533	4	3	1	0.8
	1	o	515	3	2	0	0.6
	2	p	1172	24	19	5	2.0
	2	q	1210	15	12	0	1.2
Totals	1		4337	52	41	10	1.2
	2		7469	120	102	15	1.6

\* Note that total mitoses includes some mitotic figures which could not be unambiguously assigned to metaphase or anaphase/telophase; early prophase figures are not included. Metaphase and anaphase/telophase categories include only figures which could be unambiguously identified.

TABLE 5.7 Measurements on Non-Condensation Dermis

The data for non-condensation dermis (Region C) on either side of each condensation are presented in terms of the area (number of graticule squares) counted and the number of nuclei. A few regions could not be counted, for the adjacent condensations were too close to the condensation examined to allow an adequate sample, or for other, technical reasons.

Sp.No.	Cond.	0/1		1/2		2/3	
		Area (Sq)	Nuclei	Area (Sq)	Nuclei	Area (Sq)	Nuclei
I	a	9	21	18	36		
		9	27	21	40		
		9	19	21	51		
	b	12	23	9	21		
		12	19	15	27		
		9	19	18	32		
	c			-	-	21	39
				18	37	21	31
				18	38	18	33
	d			9	15	15	39
				9	16	12	27
				9	16	12	29
II	e	10	17	-	-		
		10	18	9	16		
		18	29	14	26		
	f	18	44	15	41		
		18	46	12	33		
		18	44	15	38		
	g			15	25	16	27
				18	31	14	26
				14	27	14	22
	h			24	39	16	25
				12	29	8	16
				18	38	16	26

(continued)

TABLE 5.7 (continued)

Sp.No.	Cond.	0/1		1/2		2/3	
		Area (Sq)	Nuclei	Area (Sq)	Nuclei	Area (Sq)	Nuclei
III	i	15	28	18	31		
		12	24	21	32		
		12	21	15	25		
	j	12	12	18	20		
		15	17	21	29		
		18	22	21	27		
	k	18	30	21	26		
		12	20	18	24		
		18	28	18	27		
	l			24	40	24	32
				24	34	24	30
				24	43	21	29
	m			21	30	24	29
				24	40	24	31
				24	32	24	33
IV	n	12	19	24	38		
		12	18	24	43		
		15	25	24	41		
	o	15	35	10	27		
		15	35	15	41		
		15	33	12	30		
	p			18	47	18	35
				-	-	16	29
				12	26	20	33
	q			18	41	18	38
				21	51	21	37
				18	43	15	25

TABLE 5.8 Data on Mitosis in Non-Condensation Dermis

The total uncorrected number of nuclei counted, and the number of identifiable mitoses in the sections sampled through each condensation are presented in this Table, together with the overall percent mitosis in non-condensation regions which is derived from this data. The data are comparable with those in Table 5.6.

Sp.No.	Cond. row	Cond.	Sum of Nuclei	Mitosis		
				Total*	Metaphase*	Anaphase/ Telophase*
I	1	a	194	2	1	1
	1	b	141	1	1	0
	2	c	178	1	0	0
	2	d	142	0	0	0
II	1	e	106	0	0	0
	1	f	246	2	2	0
	2	g	158	0	0	0
III	2	h	173	0	0	0
	1	i	161	1	1	0
	1	j	127	1	1	0
	1	k	155	1	1	0
IV	2	l	208	1	1	0
	2	m	195	4	3	0
	1	n	184	4	1	1
	1	o	201	2	0	1
	2	p	170	5	4	1
	2	q	235	2	1	0
Totals			2974	27	17	4
% mitosis				0.91		

\* See footnote to TABLE 5.6.

TABLE 5.9 Dimensions of Nucleia. Lengths of nuclei measured parallel to the dorsal midline in tangential sections.

These data are used in the Abercrombie correction factors for the respective regions (see text).

	Sp.	Condensation		Intercondensation (C)
		Central (A)	Peripheral (B)	
Sample size (n)	174/13	98	219	50
	174/15	77	93	
	174/16	74	51	50
	174/17	50	34	
Total Sample size		299	397	100
Nuclear lengths Mean ( $\mu\text{m}$ )		6.4	5.4	5.7
Standard Error		0.016	0.011	0.020

b. Lengths of nuclei measured parallel to the skin surface and perpendicular and parallel to the dorsal midline in transverse and tangential sections.

These data give a picture of the three-dimensional size of nuclei in different regions.

	Condensation						Intercondensation		
	Whole (A + B)			Centre (A)					
	n	$\bar{x}(\mu\text{m})$	SE	n	$\bar{x}(\mu\text{m})$	SE	n	$\bar{x}(\mu\text{m})$	SE
T S $\perp$	590 <sup>*</sup> (3)	<u>5.4</u>	0.0082	225(1)	<u>6.0</u>	0.014	307(1)	<u>4.9</u>	0.0095
Tang. S $\perp$	200(4)	<u>5.0</u>	0.016	-	-	-	100(2)	<u>4.8</u>	0.017
Tang. S $\parallel$	200(4)	<u>5.4</u>	0.014	299(4)	<u>6.4</u>	0.016	100(2)	<u>5.7</u>	0.020

\* ( ) = no. of specimens in sample

$\bar{n}$  = sample size

$\bar{x}$  = mean length

$\perp$  = perpendicular to the midline

$\parallel$  = parallel to the midline.

TABLE 5.10 REGION A: Example of Calculated Values for One Condensation. (Condensation g)

These figures provide an example of the values which were calculated for each condensation. Values for 3 sections are given. Corrected cell counts are derived from actual counts using the Abercrombie factor. Values for cell density, volume and cell numbers were calculated as described in the text.

Sp.No.	Cond. row	Cond.	Corrected Cell Counts	Estimated Values			
				Cell Density (cells per 1000 $\mu\text{m}^3$ )	Volume ( $\mu^3$ )		Cell Nos. (cells $\times 10^2$ )
					L $\times 10^5$	R $\times 10^5$	
II	2	g	98	2.6	4.0	3.9	9.8
			102	2.8	3.8	3.6	11
			100	2.7	4.1	3.2	10

TABLE 5.11 REGION A: Estimated Values for Cell Density, and for Volume and Cell Numbers in Region A

Calculated data presented for each condensation as a mean of 3 sections (except condensation p - mean of 2 sections).

Sp.No.	Cond. row	Cond.	Estimated Values		
			Cell density (cells per 1000 $\mu\text{m}^3$ )	Volume ( $\mu\text{m}^3 \times 10^5$ )	Cell Nos. (cells $\times 10^2$ )
I	1	a	2.6	3.1	8.2
	1	b	2.2	2.5	6.0
	2	c	3.3	3.9	13
	2	d	2.9	3.2	9.2
II	1	e	2.3	2.7	6.4
	1	f	2.7	2.6	6.9
	2	g	2.7	3.8	10
	2	h	2.6	3.5	9.2
III	1	i	2.1	4.6	9.8
	1	j	1.8	3.6	6.9
	1	k	2.3	3.6	8.7
	2	l	2.3	4.9	12
	2	m	2.3	5.7	13
IV	1	n	3.2	2.8	8.8
	1	o	3.1	3.0	9.3
	2	p	3.2	5.3	17
	2	q	3.0	5.1	16

TABLE 5.12 REGION B: Estimated Values for Cell Density

Calculated data is presented for each condensation as a mean of 3 sections (except for condensation p where the mean of 2 sections is given).

Sp.No.	Cond. row	Cond.	Estimated Values Cell density (cells per 1000 $\mu\text{m}^3$ )
I	1	a	4.3
	1	b	3.2
	2	c	4.0
	2	d	3.9
II	1	e	2.9
	1	f	2.8
	2	g	2.8
	2	h	3.3
III	1	i	2.6
	1	j	2.8
	1	k	3.1
	2	l	2.8
	2	m	2.6
IV	1	n	3.3
	1	o	3.9
	2	p	3.6
	2	q	3.8

TABLE 5.13 REGION C: Estimated Values for Cell Density

Calculated data is presented for dermis on either side of each condensation, as means of values calculated from the data on each section shown in Table 5.7.

Sp.No.	Cond.	Estimated Values for Cell Density (cells per 1000 $\mu\text{m}^3$ )		
		0/1	1/2	2/3
I	a	4.5	3.8	
	b	3.2	3.3	
	c		3.7	3.1
	d		3.0	4.2
II	e	2.4	2.6	
	f	3.3	3.6	
	g		2.3	2.2
	h		2.7	2.3
III	i	3.1	2.7	
	j	1.9	2.1	
	k	2.6	2.2	
	l		2.8	2.3
	m		2.3	2.0
IV	n	2.7	2.9	
	o	3.8	4.4	
	p		4.1	3.1
	q		3.8	3.0

TABLE 5.14 Summary of Estimated Values

This Table summarises the main results based on data from the whole of each region examined.

a) Mean values for cell numbers in Region A of each row

Cond. row	Mean Values* for Cell Numbers (Cells x 10 <sup>2</sup> ) Region A	
	Mean	SE*
1	7.8	0.57
2	12	1.4

b) Mean values for cell density in each region of each row

Cond. row	Mean Values* for Cell Density (Cells per 1000 $\mu\text{m}^3$ )									
	Region A		Region B		Region C					
					0/1		1/2		2/3	
	Mean	SE*	Mean	SE*	Mean	SE*	Mean	SE*	Mean	SE*
1	2.5	0.22	3.2	0.24						
2	2.8	0.20	3.3	0.29	3.1	0.28	3.1	0.31	2.8	0.35

\* Means of means from each specimen; standard errors are based on standard deviation between specimens and sample size of 4 specimens.

TABLE 5.15 REGION A: Cell Numbers  
 Randomised Block Test, Comparing Rows and Specimens

Cond. row	Mean Cell Numbers (Cells x 10 <sup>2</sup> )					Variance ratio $\frac{M}{S^2}$	DF	Significance level*	
	Sp.No.				Row totals			5%	1%
	I	II	III	IV					
1	7.1	6.6	8.3 <sup>+</sup>	9.1	31.1				
2	11	9.7	12	16	48.7				
Sp.totals	18.1	16.3	20.3	25.1					
Rows						26.8	$\frac{1}{3}$	✓	NS
Specimens						5.0	$\frac{3}{3}$	NS	

+ mean based on 2 values arbitrarily chosen from the 3 values available.

\*NS not significant.

✓ significant.

Standard error of difference between means row 1 and row 2 (SE) = 0.601..  
 Difference between means of cell numbers in rows 1 and 2 =  
 $4.4 \times 10^2 \pm 2.0 \times 10^2$  (90% confidence limits)  
 $\pm 2.7 \times 10^2$  (95% confidence limits).

TABLE 5.16 The Frequency of Mitotic Figures and of Anaphase and Telophase Figures in the Absence and Presence of Colcemid, Counted over the Whole Dermis

Sp.	Treatment	Nuclei (Total)	Total Mitoses ***	Metaphase Figures	A + T* Figures	Mitoses percent of Nuclei	A + T* percent of Mitoses
147/1/A	no c/m**	2000	15			0.75	
147/1/A	no c/m		250	210	40		16
147/1/B	c/m 5½ hrs	3214	217			6.75	
147/1/B	c/m 5½ hrs		3000	2996	4		0.13

\* A + T = Anaphase + Telophase

\*\* c/m = colcemid

\*\*\*Total mitoses = metaphase + A + T figures.

TABLE 5.17 Percent Mitosis in Condensations and Non-Condensation Dermis after 5½ hours in Colcemid

Region	Sp.No.	Cond. row or dermal region	Cells	Mitoses	Anaphase + Telophase	% Mitosis
Cond.	147/1	late 1	1000	59	0	5.9
	147/1	late 1	1055	77	0	7.3
	147/2	late 1	606	51	0	8.4
	Total		2661	187		
	Mean					7.0
Non-Cond. Dermis	147/1	0/1 and 1/2	655	46	0	7.0
	147/1	0/1 and 1/2	504	35	0	6.9
	147/2	1/2 and 2/3	282	21	0	7.4
	Total		1441	102		
	Mean					7.1

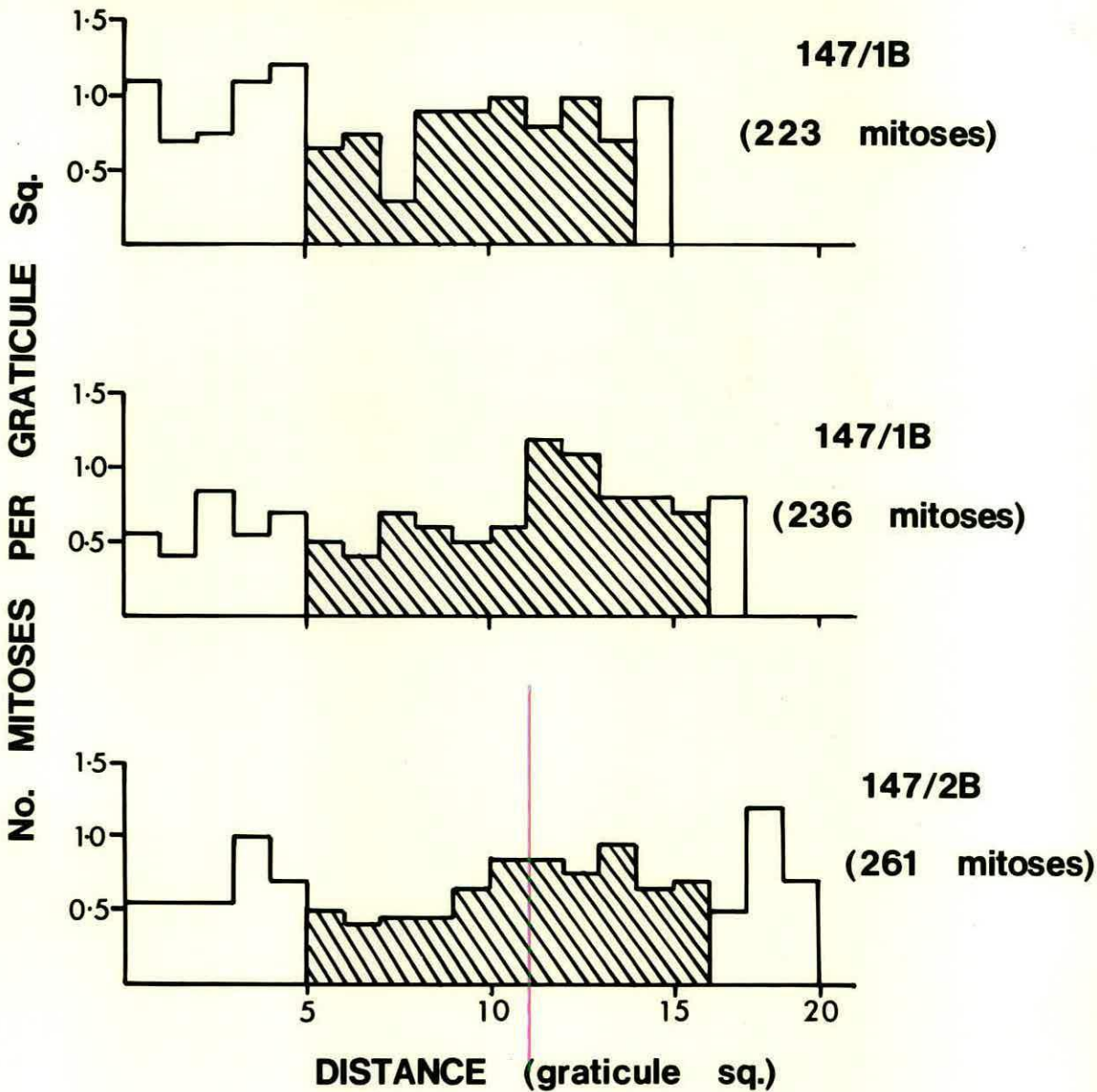


Fig. 5.4. Distribution of mitotic figures through the condensation and surrounding dermis after colcemid treatment. Three late, row 1 condensations from 2 specimens: 147/1B and 2B. Colcemid  $5.4 \times 10^{-6}$  M; 5.5 hrs. Mitoses were counted in successive columns of graticule squares at  $\times 640$  magnification (each column  $14.7 \mu\text{m}$  wide; each square  $216 \mu\text{m}^2$  in area) in 6 consecutive transverse sections through the centre of each condensation. Mean number of mitoses per square is plotted against distance from a point  $74 \mu\text{m}$  lateral to the edge of the condensation: condensation shaded; surrounding dermis plain.

TABLE 5.18 Effect of Colcemid on Percent Mitosis in Intercondensation Dermis

Specimen	Time in Colcemid (hrs)	Nuclei (total)	No. of Mitoses	% Mitoses	Symbol*
85/2	2.5	1000	42	4.2	■
83/8	2.25	1000	32	3.2	▲
83/6	4.35	1000	72	7.7	▲
85/4	4.5	1000	41	4.1	■
149/1	5.5	1000	98	9.8	•
147/2	5.5	1000	70	7.0	⊙
85/15	6.5	1000	90	9.0	■
83/1	8.25	1000	167	16.7	▲
85/9	10.75	1000	215	21.5	■
85/11	10.75	1000	364	36.4	■
85/1	-	2000	27	1.35	 ◻
147/1/A	-	2000	15	0.75	
143/1/A	-	761	10	1.31	

\* See Fig. 5.5

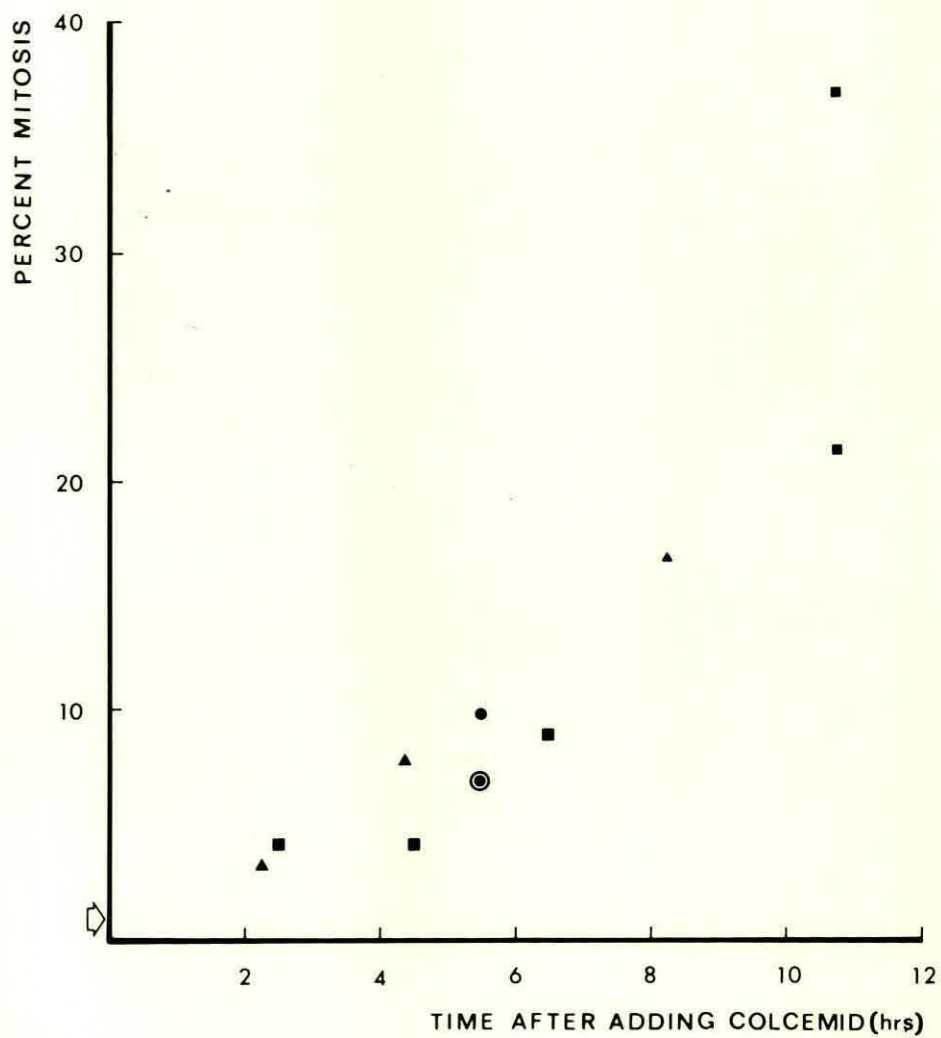


Fig. 5.5 Graph of the effect of colcemid on percent mitosis in intercondensation dermis. Percent mitosis versus time in colcemid. Data from Table 5.18, see Table for symbols.

values obtained in the previous experiment in condensation and intercondensation dermis (compare Tables 5.18 and 5.17). In specimens treated for longer than 8 hours, the data may suggest an increase in the rate of accumulation of mitoses with time. However, after treatment for more than 8 hours, nuclei were difficult to count, for their morphology was severely disrupted, while mitotic figures were more clearly discernible. Thus, percent mitosis estimated after 8 hours are not reliable.

### 3.4 Discussion

#### Cell Counts

In the present study all recognisable nuclei or parts of nuclei in the section were counted. The direct application of Abercrombie's correction factor to these counts is expected to yield a slight underestimate of the true number of cells in the section. This is because Abercrombie's correction is based on the principle that even the smallest part of a nucleus which lies within the section will be counted. In practice, however, the count will fall short of this ideal because some of the parts of nuclei will be too small to be recognised. The underestimate incurred may be in the order of 10 to 15%. This will lead to a corresponding underestimate of the absolute values of cell density, cell numbers and the increment in cell numbers in the dermis.

#### Cell Density

It is, at first sight, surprising to find that cell density is not higher at the centre of the condensation than in intercondensation dermis. Moreover, the density estimate appears to be unduly low at the centre of the condensation by comparison with

the figures published by Wessells (1965). Wessells estimated that cell density increases from  $2.60 \times 10^{-3}$  cells per  $\mu\text{m}^3$  in dense dermis to  $3.8 \times 10^{-3}$  cells per  $\mu\text{m}^3$  in young (Stage 30) condensations and finally, to  $5.52 \times 10^{-3}$  cells per  $\mu\text{m}^3$  in mature condensations just before elevation. However, these estimates are based on uncorrected counts. Abercrombie's correction may be applied directly using Wessells' estimate of section thickness ( $5 \mu\text{m}$ ) and the present estimates of nuclear length (Table 5.9a): the values become  $1.2 \times 10^{-3}$  cells per  $\mu\text{m}^3$  in dense dermis,  $1.8 \times 10^{-3}$  cells per  $\mu\text{m}^3$  in young condensations, and  $2.4 \times 10^{-3}$  cells per  $\mu\text{m}^3$  in mature condensations. The estimate for density for mature condensations in vivo is thus close to the estimated density at the centre of a condensation in culture. Since cells in both situations are of similar morphology and are tightly packed (as judged histologically, and by TEM of in vivo material (Kisher, 1968)) it seems that  $2.4$  to  $2.8 \times 10^{-3}$  cells per  $\mu\text{m}^3$  is close to the maximum mean density for cells of this size.

The main difference between cell densities in skin in vivo and in cultured skin is thus that the latter is much higher than the former in regions other than the centre of the condensation. This is to be expected for two reasons: first, the skin shrinks on explantation; second, while division continues in cultured skin, lateral growth does not.

Wessells (1965) noted that nuclei and cells in the condensation appear to be larger than those in the surrounding dermis and in dense, unpatterned dermis. The present measurements confirm, for cultured material, that nuclei at the centre of the condensation are larger than those in the surrounding dermis. Moreover, since central cells are packed at low density while those



in the surrounding dermis are not tightly packed at a higher density, it follows that the former are larger than the latter. Therefore, as the centre of a condensation forms in culture, it seems that the existing cells enlarge to fill the available space and may even contribute to some expansion of the condensation.

### Cell Numbers

The results indicate that there is an increase in cell numbers in the condensation below the central region of the placode and that this is due mainly to an increase in the thickness of the condensation.

The estimated value of this increase in cell numbers shows considerable variation between specimens: this is the least precise of the estimates used in this quantitative analysis. However, to obtain sufficient data to allow a much more precise estimate would be very time consuming, and the present results are sufficient to establish the probable relative roles of proliferation and cell movement in the condensation process. Since the variation is high, I have used not only the mean, but alternatively the upper and lower 90% and the lower 95% confidence limits of this estimate (Table 5.15).

### Rate of Cell Proliferation

#### A. Comparison between condensations and intercondensation dermis

The observation that the frequency of mitoses is nearly the same in condensations and intercondensation dermis suggests that there may be little difference in the rate of proliferation in these regions. This conclusion is dependent, however, on the condition that the duration of mitosis is the same in both cases.

Accumulation of metaphase figures in colcemid is a better guide since it is a function of the rate of entry of cells into

mitosis. The absolute value of the proliferation rate is determined from the colcemid data only after making several assumptions (discussed below), but comparison between regions is simpler. Making this comparison, it is necessary to assume first, that the rate at which colcemid penetrates and becomes effective in arresting mitoses is the same in both regions; and second, that the rate of change of the proliferation rate is the same in both regions. The first of these assumptions is likely to be nearly true: I argue below that colcemid is effective throughout the dermis by the end of the sampling period; although there may be a lag in its initial effectiveness throughout the dermis, colcemid rapidly penetrates cells and there is no reason to expect a marked difference in penetration of the two regions: the lag is more likely due to its ineffectiveness in arresting cells already in metaphase (Aherne, Campjohn, and Wright, 1977). The second assumption is also probably true; as discussed below, the rate of proliferation in both regions is probably nearly constant.

The fact that the same mean frequency of arrested mitoses was found in condensation and intercondensation regions treated for 5.5 hours with colcemid is thus interpreted to indicate that the mean proliferation rate is the same in these two regions. Thus, during the period between rows 1 and 2 the increase in cell numbers in the condensation is not caused by a locally high rate of cell proliferation.

Cell proliferation will, of course, contribute towards the observed increase in cell numbers in the condensation. To assess this contribution, it is necessary to estimate the absolute rate of proliferation in condensations.

## B. Rate of proliferation in condensations

I have used the measured value of the accumulation of mitoses over 5.5 hours in colcemid to estimate the proliferation rate of cells in condensation. Six assumptions underlie this approach.

1. I have assumed that colcemid is completely effective in arresting mitosis over the collection period.

A quantitative estimate of efficiency at the time of fixation may be gained from the data in Table 5.16 as follows. If colcemid was ineffective in mitotic arrest at the time of fixation, cells arriving in metaphase shortly before this would pass unhindered into anaphase and telophase and the same frequency of a + t figures would be observed as in controls. In addition, we might expect a contribution to the number of a + t figures from cells escaping from already partially arrested metaphase. This latter contribution cannot be directly assessed: I have therefore calculated a minimum estimate by comparing the numbers of a + t figures in control and treated explants.

In the control, 16% of mitoses were a + t figures (similar values were obtained in other specimens; for example, pooled data for 189 mitoses in specimens I to IV used in the quantitative analysis (Tables 5.6 and 5.8), gives 15% a + t figures). Thus, 0.12% (16% of 0.75%; see Table 5.16) of the cells were recognisable anaphase and telophase figures. In treated specimens the number of a + t figures in 3000 mitoses was counted: since 6.75% of the treated cells were in mitosis (Table 5.16), this is equivalent to counting  $\frac{3000}{0.0675} = 44444$  cells. Of these cells 6% (i.e. 6.75 minus the control value of 0.75%) will have been blocked before fixation and must therefore be excluded from the total count in assessing the number of cells which could, if colcemid were ineffective, produce

anaphase - telophase figures: thus the equivalent of  $44444 - 2666 = 41778$  cells were counted. If colcemid is completely ineffective we would expect to count 0.12% of  $41778 = 50$  a + t figures. The observed count was 4 (Table 5.16). An estimate of the effectiveness of colcemid in arresting mitosis is therefore  $\frac{50 - 4}{50} \times 100 = 92\%$ .

This is only an approximate estimate, being based on small numbers of a + t figures in treated specimens. It is based on the untested assumption that the duration of anaphase and telophase is as long in colcemid as it is in controls, but there is no reason to expect that anaphase or telophase would be unusually rapid in the drug. This minimal estimate is sufficiently precise to indicate that colcemid acts here as an effective mitotic inhibitor after 5.5 hours of treatment. Since a + t figures were counted throughout treated dermis, this estimate applies to condensations and intercondensation regions alike. At least in intercondensation regions, the drug is probably equally effective over the period 2.25 to 6.5 hours after its addition, since the rate of accumulation of mitoses over this time is very approximately linear (Fig. 5.5).

2. Arrested mitoses and non-mitotic nuclei remain recognizable after 5.5 hours in colcemid. Estimates of the survival time of arrested mitoses have been published (Aherne and Camplejohn, 1972), but vary with tissue type from 5.5 and 6 hours to 2.6 hours. In the present study, a few degenerating mitotic figures were observed (and counted); degenerating non-mitotic nuclei were also found after 5.5 hours and, in much greater numbers, later. Since mitotic figures may be more sensitive to degradation the quoted values for the frequency of mitoses may be an underestimate, but the fact that relatively few degenerating figures were recognised and that the error is compensated to some extent by degeneration of other cells suggests that the error

will be small.

3. The drug has no effect on the passage of cells through the remainder of the cell cycle or on their entry into mitosis. Evans, Neary, and Tonkinson (1957) have noted the possibility of these effects using the related drug, colchicine, on broad bean root tips; however, in this system they found no effect in the first 6 hours of treatment.

The fact that there is no indication of a decrease in the rate of accumulation of mitoses over the first 6.5 hours in colcemid (Fig. 5.5) taken with the argument that the proliferation rate is approximately constant (see below), further supports assumptions 2 and 3.

4. The mean proliferation rate over the collection period is assumed to equal the mean rate over the period between the developmental stages represented by rows 1 and 2 (about 8 hours; see below). This holds if the system is in a steady state. The cell cycle time, averaged over the whole population in the dermis, is long, and probably only a proportion of the cells are in a mitotic cycle (see below). However, even within this subpopulation, growth does not appear to be exponential; if it were, the dividing population would expand and the frequency of mitotic figures in untreated skin would increase between rows 1 and 2 or between specimens of different age. There is no indication of a marked increase; it is therefore likely that a proportion of newly divided cells leave the mitotic cycle and that the system approximates more closely to a steady state than to exponential growth.

Additional support for this assumption comes from the fact that the sampling period occupies at least half of the interval between rows, and - since late row 1 was sampled - this falls about

the middle of the interval: even allowing some change in the rate of proliferation, it is therefore likely that the mean over the sampling period will be close to the mean over the whole interval.

5. It is necessary to assume either that colcemid is immediately effective after it is added, or that there is a lag period before mitoses accumulate. The data on mitosis in intercondensation dermis (Fig. 5.5) may be interpreted in different ways as regards this point.

On one hand, the regression line on  $\sqrt{\% \text{ mitosis}}$  versus time, for data from 2.25 to 6.5 hours in colcemid, extrapolated to time zero, gives no indication of a lag, but falls close to the control value for the frequency of mitoses in untreated skin (intercept at  $t = 0$ , 1.5%; control value, 1.1%). The rate of proliferation estimated on the assumption of no lag using the data for percent mitosis in condensations after 5.5 hours in colcemid (Table 5.17) is  $\frac{7.0}{5.5} = 1.3$  divisions per hundred cells per hour.

On the other hand, one might expect a lag period: cells already in metaphase when the drug is added may be insensitive to arrest and may thus pass unhindered through division (Aherne et al, 1977). Under these conditions arrest would begin, at the earliest, with cells entering mitosis in the presence of the drug, and the maximum intercept would be zero. Inspection of the data for the whole range from  $t = 2.25$  to 10.75 hours (Fig. 5.5) suggests a lag of around 1.5 hours. This is, of course, an approximate estimate because the data from later times are difficult to interpret (see page 200). I have used this value for the lag period to derive a second estimate of the rate of proliferation in condensations: assuming the same lag period throughout the dermis, the rate in condensations is  $\frac{7.0}{5.5 - 1.5} = 1.8$  divisions per hundred cells per hour.

These two estimates of cell proliferation rate give different estimates of the duration of mitosis, applying the equation:  $\frac{\% \text{ mitosis in untreated skin}}{\text{rate of proliferation}} = \text{duration of mitosis}$ . Taking the mean percent mitosis for condensations in rows 1 and 2 in untreated skin (1.5%) the estimated duration of mitosis is : assuming no lag, 69 minutes; assuming lag, 50 minutes. Both estimates are, however, reasonable: mitosis lasts about 1 hour in many systems (Mitchison, 1971; Prescott, 1976).

6. I have assumed that the proliferation rate is uniform throughout the condensation. The justification of this is the uniformity shown by mitotic profiles in condensations treated for 5.5 hours with colcemid (Fig. 5.4).

In summary, I have used two alternative estimates of the rate of proliferation in condensations: a minimum estimate, assuming no lag period in the action of colcemid, 1.3 divisions per hundred cells per hour; and, perhaps a more realistic estimate, assuming a lag of 1.5 hours before colcemid becomes effective, 1.8 divisions per hundred cells per hour. The fact that several of the above assumptions may only hold to a first approximation suggests that these values may underestimate the true proliferation rate, but the above arguments indicate that the error will probably be slight.

#### Time Difference between Rows 1 and 2

The time interval between rows 1 and 2 has been estimated directly from the graph of rows present versus time in culture (Fig. 3.3; page 109). The rate of formation of new rows up to 4.5 rows is approximately linear and, from the slope of the regression line over this range, has a value of 7.94 hours per row. Despite variation between individual specimens (Table 3.2; page 110), the mean values for 5 independent experiments fall close to the

regression line and this provides the justification for applying this estimate of the time between rows to the data described here which comes from 3 independent experiments made under the same conditions.

Estimate of the Contribution of Cell Proliferation to the Increase in Cell Numbers

It is first necessary to establish whether or not cells divide more than once during the 8 hour interval between stages represented by rows 1 and 2. There are no published estimates of the cell cycle time in condensations or in the surrounding dermis, and no direct estimate has been made here. It is most likely, however, that few cells divide more than once during this period. The evidence for this is as follows.

The mean cell cycle time (averaged over the whole cell population in condensations) is long and may be estimated from the equation:

$$\text{mean cycle time} = \frac{\text{duration of mitosis}}{\text{frequency of mitoses in untreated condensations}}$$

Assuming a lag period in the action of colcemid to calculate the duration of mitosis (see above), and using the mean frequency of mitoses in row 1 and row 2 condensations, the mean cycle time =  $\frac{1.15}{0.015} = 77$  hours.

The cycle time of actively proliferating cells in the skin is unlikely to be as long as this: presumably only a portion of the cells will be in a mitotic cycle. Since the proliferation rate is the same in condensation and intercondensation dermis it is likely that the cell cycle time is also approximately the same and intercondensation data is therefore relevant (Table 5.18). Although the data for times greater than 6.5 hours may be unreliable in terms of the true frequency of mitotic figures after particular times in colcemid (see Results), the figures can be used to estimate the

minimum number of cells in the mitotic cycle. For example, since about 17% mitoses have been collected by 8.25 hours and since accumulation continues after this time, at least 17% of the cells present when colcemid was added were in the mitotic cycle. A minimum estimate of the mean cycle time for cells in the mitotic cycle is thus 17% of 77 hours = 13 hours.

The variation of individual cell cycle times about the mean may span several hours (as, for example, Cairns (1977) has shown for wing-bud mesenchyme). However, the distribution is likely to approximate to a normal distribution and may be skewed towards longer times (as suggested by data published by Prescott (1976) for mammalian cells in culture). It therefore seems justifiable to assume - as I have done in the following calculations - that a negligible number of cells divide twice during the 8 hour period considered.

An estimate of the contribution of proliferation to the observed increase in cell numbers may therefore be made as follows. Let  $x$  be the number of cells in Region A at time  $t$  (hours), and  $k$  be the rate of cell proliferation (divisions per cell per hour), then  $\frac{dx}{dt} = xk$  cells per hour. (I have assumed that cell death occurs with negligible frequency: strictly, the estimate of the contribution of proliferation is influenced only if cell deaths occur with a different frequency in dividing and non-dividing populations of cells.)

A numerical approach yields an adequate approximate solution: over a small interval of time,  $\Delta t$ , the increment in  $x$ ,  $\Delta x = x k \Delta t$  where  $x$  is the number of cells at the beginning of the time interval. This quantity has been calculated for successive intervals over the total time between rows, using the cumulative total for  $x$  at each step. The total increment in cell numbers computed in this way has been expressed as a percentage of the observed increment

in cell numbers, to arrive at an estimate of the contribution of cell proliferation of the original cell population. A range of estimates have been made, using alternative data values derived as described above. Consistent values for the initial number of cells ( $7.8 \times 10^2$ ), the time between rows,  $t_r$  (7.94 hours), and the time interval for calculation,  $\Delta t$  (0.1 hours), have been used and, in most cases, I have assumed a lag in the initial effectiveness of colcemid, using the value of  $k = 0.018$  divisions per cell per hour; in estimating the minimum contribution of proliferation, I have assumed no lag and used a value of 0.013 divisions per cell per hour. The principle difference between estimates is therefore in the value chosen for the observed increment in cell numbers, which is the value about which there is most doubt,  $\Delta n$ . (Table 5.19 shows the values used and the results of the calculation.)

The best estimate of the contribution of proliferation to the increase in cell numbers in Region A is 29% (Table 5.19, column A). Although, as I have discussed above, the rate of cell proliferation may have been slightly underestimated, the increase in cell numbers is also probably slightly underestimated. These two errors will work in opposition. I conclude that proliferation of the original population of cells most probably accounts for between one-quarter and one-third of the net increase in cell numbers. It is possible (using 90% confidence limits for the increment: Table 5.15 footnote and Table 5.19, columns B and C) that proliferation yields between about one-seventh and half the net increment, while the possibility cannot be excluded that it accounts for a major part of the increment (about three-quarters; Table 5.19, column D).

Thus, cell division alone cannot account for the total increase in cell numbers in the central region of the condensation and

most probably makes a minor, but significant, contribution. The remaining cells must be added from the surrounding dermis. If  $y$  cells are added per hour,  $yt_r$  cells will be added over the total time interval. In addition, those cells which have been added, will divide (I assume they divide with the same frequency as other cells in the condensation). The number of cells created by proliferation of this incoming population may be approximated by the term  $ykt_r!$  which computes the number of cells dividing from those added at each hourly interval. Thus, the total number of cells ( $a$ ) contributed primarily or secondarily by addition to the condensation is

$$a = yt_r + ykt_r!$$

$$\therefore y = \frac{a}{t_r + kt_r!}$$

The calculated values of  $y$ ,  $yt_r$  and  $ykt_r!$  for each estimate of the contribution of cell division are shown in Table 5.19, where it can be seen that the addition of cells into this region of the condensation is probably fairly slow. The contribution by the term  $ykt_r!$  is small, and addition of new cells is the major factor.

TABLE 5.19 Estimates of the contribution of cell division and movement to the increase in cell numbers in Region A of the condensation

	Calculated Values			
	Best estimate	Alternative estimates		
	Mean	90*(max.)	90*(min.)	95*(min.)
	A	B	C	D
$x_{r1}$	$7.8 \times 10^2$	$7.8 \times 10^2$	$7.8 \times 10^2$	$7.8 \times 10^2$
$x_{r2}$	$12.2 \times 10^2$	$14.2 \times 10^2$	$10.2 \times 10^2$	$9.5 \times 10^2$
$\Delta x$	$4.4 \times 10^2$	$6.4 \times 10^2$	$2.4 \times 10^2$	$1.7 \times 10^2$
k	0.018	0.013	0.018	0.018
$t_r$	7.94	7.94	7.94	7.94
$\Delta t$	0.1	0.1	0.1	0.1
y	37	66	13	5
$yt_r$	291	521	106	41
$ykt_r!$	24	31	9	3
$\Delta x - a^{**}$	126	89	126	126
% contribution of proliferation	29%	14%	52%	74%

\* %Confidence limits for values of  $\Delta x$

\*\*  $\Delta x - a$  = increment of cells arising from proliferation of the original population in Region A.

$x_{r1}$  and  $x_{r2}$  refer to cell numbers in Region A of row 1 and 2 respectively.

See text for remaining symbols.

How do these results relate to condensation development in vivo? It is not possible to simply apply our conclusions about the proportional contributions of different processes in culture to development in vivo since the relative rates of these processes need not be the same in the two situations. This is emphasised if we consider the difference in the interval between the formation of new rows (see Chapter 3) or compare cell proliferation in cultured skin with the results reported by Wessells (1965) regarding development in vivo (reviewed in Chapter 1). Consideration of the differences, however, suggests which processes are basic and which may be secondary.

It is clear from Wessells' data on cell density that the number of cells increases at the centre of the condensation in vivo. Wessells' convincing autoradiographs show that there is virtually no proliferation in condensations of equivalent age to those examined here, which indicates that in vivo, as in culture, cell division within the centre of the condensation is not responsible for the continuous increase in cell numbers below the placode.

On autoradiographic evidence Wessells did, however, suggest that the initial core of the condensation arises by a pulse of mitotic activity and is added to by particularly active cell proliferation in the immediately surrounding dermis. The present results deal only with condensations after they become visible and it cannot therefore be excluded that initiation involves a pulse of mitotic activity. This explanation seems unlikely, however, for the formation of a recognisable condensation is rapid in culture yet no region with a high frequency of mitoses has been observed in scanning many sections of cultured specimens. Cell density data suggest an alternative mechanism: that the formation of a recognisable

condensation in culture occurs by an increase in cell volume leading to close packing. The present results also show nearly uniform proliferation throughout the condensation and neighbouring dermis, and though there is a suggestion of a small peak of mitotic activity in the dermis immediately surrounding the condensation this cannot make an appreciable contribution to the observed increase in cell numbers. It therefore seems that the addition of cells to the condensation, such as occurs in culture, is a basic part of the condensation process and that the patterns of differential proliferation, which may aid the process in vivo, are not strictly necessary for condensation development.

#### Growth of the Condensation

Since Region A includes the full depth of the dermis it is unlikely that the condensation increases in thickness simply by expanding to include the stationary cells beneath it by a change in cell phenotype: the subdermal mesenchyme in culture remains approximately constant in depth, and qualitative examination shows no especially high level of mitosis. This suggests that cells from the peripheral regions move inwards. The peripheral cell density does not decrease; in fact, peripheral regions also increase in thickness as the condensation grows in diameter and in depth. It is therefore likely that cells move into the peripheral regions from the surrounding dermis.

#### Cell Movement

How far need cells move to account for the observed increase in cell numbers? Simple calculation (assuming that cells move centripetally from all round the condensation) suggests that movement over distances in the order of only one to two cell

diameters (certainly less than 70  $\mu\text{m}$ ) would, in principle, suffice.

Three possible modes of movement into the condensation may therefore be considered:

1. Migration guided either by the substratum or by chemotaxis (Stuart et al, 1972; Garber, 1976; Ede et al, 1971).
2. Short-range random movement coupled with some mechanism for trapping or immobilizing cells which contact the periphery of the condensation; for example, cells in the condensation may be particularly adhesive.
3. Mass contraction of the tissue towards the centre of condensation (Trinkaus, 1965).

Since the first of these modes has been suggested specifically for feather condensation development it has been investigated in more detail, first, by attempting to film cells in intercondensation arrays, and second, by examining the effects of colcemid on primordium development. This work is described in the following two sections.

#### 4. TIME-LAPSE FILMING OF CELL MOVEMENT IN THE DERMIS

To investigate the nature of cell movement during condensation morphogenesis, time-lapse films of developing skin were made, using phase-contrast illumination or Nomarski optics.

##### Phase-Contrast Films

Six specimens were filmed (see Chapter 2), using X6 objective and 2 to 5 minutes lapse time. Fields were centred initially on skin immediately lateral to the most recently formed primordia.

During the course of the film, the skin developed as described in Chapter 3, at approximately the same rate as control cultures incubated routinely: primordia developed to the feather-bud stage. No streaming movements of groups of cells were observed, although in films of cell cultures, made in this laboratory (using the same magnification and lapse time), cells show marked streaming movements. However, the resolution in these films was low: in specimens which had been cultured for some time before filming, and even in skin with several rows of primordia, filmed immediately after excision, dermal arrays were not clearly distinguishable. The movement of individual fibroblastic cells could not be followed. Highly refractile, rounded cells (probably macrophages) distributed throughout the skin, could be easily followed over periods of several hours, during which they moved actively, apparently at random.

On account of the difficulty of observing the cells in the dermis at low resolution, films were made using Nomarski illumination.

Nomarski Films

Fields centred on the region between primordia of rows 1 and 2 in freshly excised skin at Stage 31 were filmed under the conditions described in Chapter 2.

Although resolution was sufficient to show dermal arrays quite clearly, the technical problems inherent in the method limited the period of filming. Two specimens were successfully filmed.

In one of these a dermal array was filmed over a period of 6 hours (x 40 objective; lapse time 2 minutes), but no gross cell movement was observed: cells lying side by side moved independently, to and fro over short distances within the array. (The optical resolution did not permit the movement of individual cells to be mapped over extended periods.) Throughout this time highly refractile cells showed a constant active movement over several cell diameters, indicating that the general conditions of culture were not limiting. The movement of these cells was accompanied by a flow of cytoplasm into their blunt pseudopodia and by rotation of the nucleus. It was not clear upon what substratum these cells moved, though they appeared to pass freely between the fibroblastic cells of the dermis, beneath and between arrays.

A lower magnification film (x 16 objective; lapse time 3 minutes) was made of the second specimen. Again, no net cell movement along the arrays was observed, though refractile cells moved actively and the condensations continued to develop over the 10 hour period of filming.

## Conclusion

Time-lapse cinematography is the ideal means of revealing pathways of cell movement in the tissue, but no net movements could be detected in the present experiments indicating that there is no marked movement of cells along arrays over the period when adjacent condensations are developing. The impression gained from the films is that arrays are rather static structures. The evidence is, however, too limited, both in resolution and in the number of films it has been possible to make, to decide if cells move along arrays over short distances sufficient to make some contribution to the increase in the number of cells in the condensation.

## 5. EXPERIMENTS USING COLCEMID

### 5.1 Introduction

The role of cell movement in the morphogenesis of condensations has been analysed using colcemid as a tool. It is well known that colcemid, in addition to its inhibitory action on mitosis, prevents the migration of fibroblastic cells in culture (Vasiliev et al, 1970; Gail and Boone, 1971): the long processes of these cells are retracted in the presence of the drug and the ruffling activity of the cell surface, which is normally confined to advancing cell processes, becomes generalised in the transient extension of numerous processes all round the cell. As a result, colcemid does not "freeze" cells, but causes very frequent changes in direction: this limits their mobility to short-range, random movements. The same effect is noted where cell orientation is

entrained by an aligned substrate or by extensive lateral contact with other cells, although, in these cases, the inhibitory action of the drug is less immediate (Vasiliev et al, 1970). Colcemid and its relative, colchicine, have been previously used to analyse the rôle of movement and changes in cell shape in morphogenesis (for example, in the development of the primary mesenchyme in Arbacia (Tilney and Gibbins, 1969), and chick blastoderm expansion (Downie, 1975). An inhibitory influence on morphogenesis is difficult to interpret in terms of cellular and sub-cellular mechanisms, for these drugs, though they are primarily disrupt microtubule systems, have wide-ranging effects on living cells (Margulis, 1973). The situation is simpler where colcemid does not inhibit morphogenesis and the involvement of cell division and migration can be ruled out. The evidence presented in this section shows that colcemid does not prevent major morphological changes in already initiated primordia: the inhibitory effect of colcemid on cell movement in the dermis has therefore been examined.

## 5.2 Observations on the Effect of Colcemid on Primordium Development

### General Observations

The effect of colcemid on primordium development was examined in 6 experiments in which specimens explanted at Stage 29+ were allowed to develop in the presence of colcemid from the beginning of culture or from a stage where several rows of primordia had formed. Specimens were treated as follows: 18 treated explants: 2 explants treated with  $5.4 \times 10^{-5}M$  colcemid, 6 with  $2.7 \times 10^{-5}M$  (2 treated 24 hours after the beginning of culture), 4 with  $5.4 \times 10^{-6}M$  (all treated 17 to 24 hours after the beginning of culture), 2 with  $5.4 \times 10^{-7}M$ , and 4 with  $1.0 \times 10^{-7}M$ ; 13 controls treated with saline.

In none of the specimens treated with colcemid at  $2.7 \times 10^{-5}$  to  $5.4 \times 10^{-7}M$  did new primordia form over a period of 2 days in the presence of the drug. In specimens in which one row was already present at explantation and to which colcemid was added from the beginning of culture, primordia showed a slight increase in opacity over the next 10 hours, but did not develop further (Fig. 5.6b). Specimens in which several rows were allowed to develop before treatment behaved differently in different concentrations of the drug. In  $2.7 \times 10^{-5}M$  colcemid, the primordia failed to elevate to form feather buds (Fig. 5.6c). In less concentrated colcemid ( $5.4 \times 10^{-6}M$ ) primordia in the medial rows acquired a sharp outline, while the opacity of the tissue between them decreased; oblique examination established that these were at the feather bud stage, and their morphology resembled that of controls. All control explants developed normally (Fig. 5.6a).

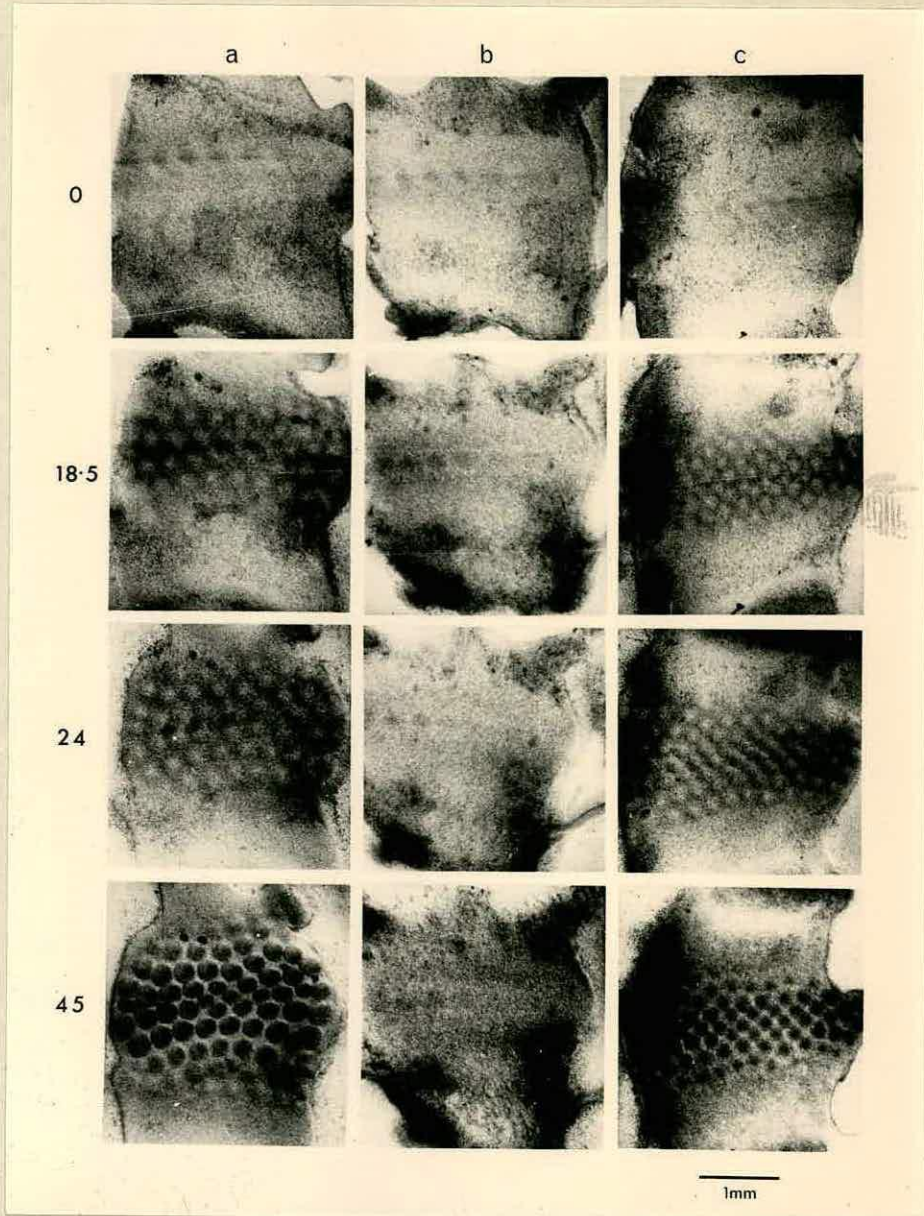


Fig. 5.6 Development of skin in the presence of colcemid.

Specimens explanted at Stage 29+, time 0. (Times in culture are shown in the left hand margin.)

a. Untreated control explant. b. Treated explant, colcemid ( $2.7 \times 10^{-5}M$ ) added at time 0. c. Treated explant, colcemid ( $2.7 \times 10^{-5}M$ ) added at 18.5 hours.

Variable results were obtained with very dilute colcemid ( $1.0 \times 10^{-7}M$ ). Of four specimens treated at Stage 29, two failed to develop primordia, one formed primordia on one side of the midline only, and the remaining specimen developed a complete covering of primordia in a manner indistinguishable from controls.

The Kinetics of Primordium Development in Colcemid

In two replicate experiments, specimens were explanted at Stage 29 to 31, and colcemid ( $5.4 \times 10^{-6}$  and  $5.4 \times 10^{-7}M$ ) was added on the second day of culture. All specimens were examined at 2 hour intervals, and treated specimens were fixed in pairs at intervals of approximately 2 to 4 hours during a period of 10.75 hours in colcemid. Controls were fixed at the beginning and end of the period of treatment. (Ten explants treated with  $5.4 \times 10^{-6}M$  colcemid, and 10 treated with  $5.4 \times 10^{-7}M$  colcemid, 6 controls treated with saline). All material was processed routinely for histology. (Data from this experiment was used in the quantitative analysis of the accumulation of mitotic figures with time, in Section 3).

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Fig. 5.7 The kinetics of primordium development in colcemid. Specimens explanted at Stage 31, time - 18 hours.

- a. Treated explant, colcemid ( $5.4 \times 10^{-6}M$ ).
- b. Untreated control explant. Colcemid was added at time 0. Times after adding colcemid are shown in the left hand margin. Lateral primordia which are just visible in a., at time 0, continued to darken in the presence of colcemid over the next 10 hours at approximately the same rate as initially equivalent primordia in the control. Medial primordia which were already opaque when colcemid was added, darkened further and acquired a sharp outline at approximately the same rate as medial primordia in controls. No new primordia formed in the presence of colcemid, whereas one new row of primordia developed in the control.

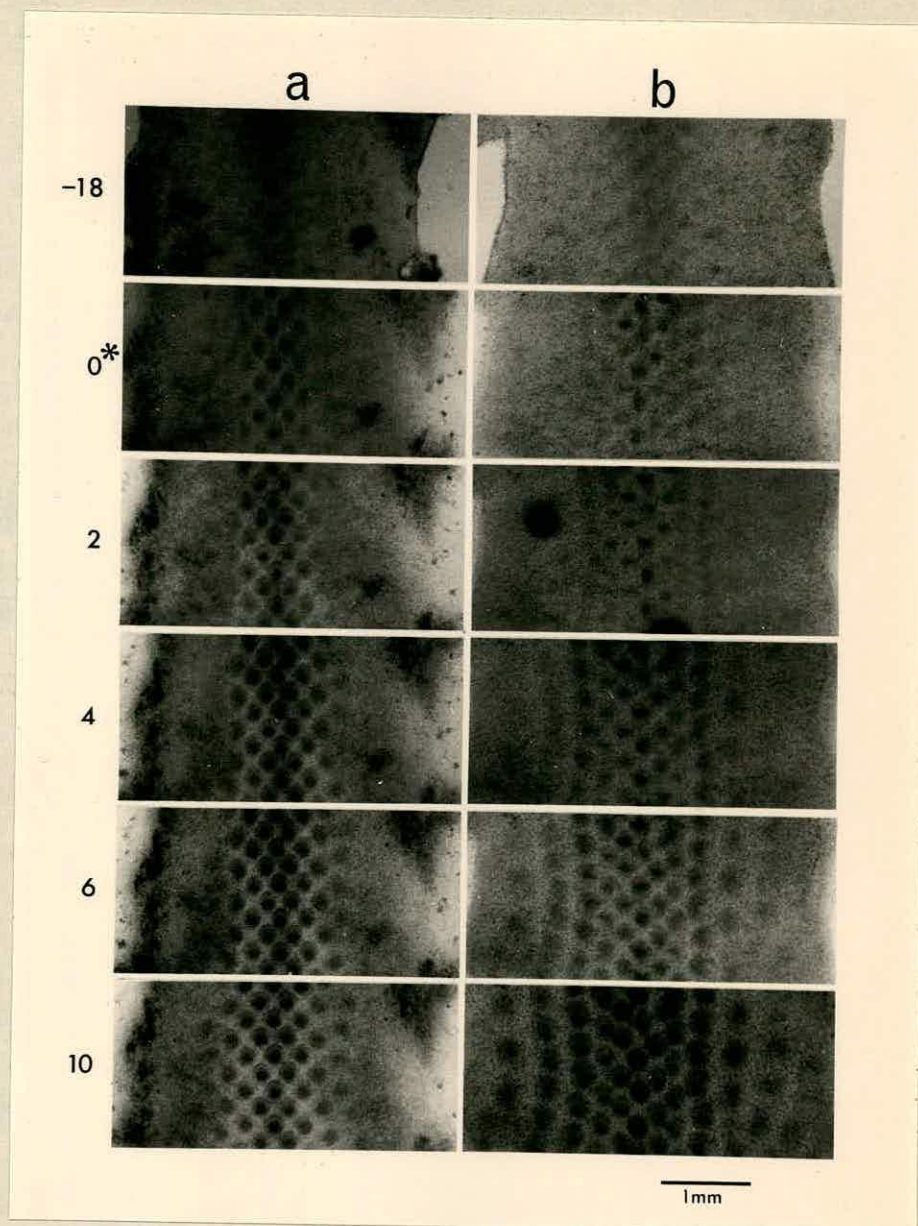


Fig. 5.7

Primordia in treated cultures developed at approximately the same rate as those in controls (and in some cases slightly more rapidly). Primordia, in the earliest visible stages of development continued to increase in opacity in the presence of colcemid; more medial primordia in several specimens developed to the feather bud stage over 10 hours in the drug. No new primordia were formed in any of the treated explants. No differences were noted between the development of explants in the different concentrations of colcemid. All controls developed in the usual manner (Fig. 5.7 shows development of a treated and a control specimen.)

Histological examination indicated a progressive disruption of the dermal structure and accumulation of mitotic figures. The data for mitosis versus time are shown in Table 5.18.

#### Histological Observations

The effects of colcemid on the histological structure of developing primordia was examined in two replicate experiments. Skin was cut transversely into 3 pieces upon explantation at Stage 29, and the pieces were grown in the same dish until several rows had formed. Piece A was fixed and the others transferred to separate dishes where one (B) was cultured in routine medium, and the other (C) in the presence of colcemid ( $5.4 \times 10^{-6}M$ ): after 10 to 10.5 hours B and C were fixed and processed together with A for routine histology. (10 specimens in all, treatments were randomly assigned to different parts of the skin in each specimen, 6 uncut controls). Serial transverse sections of each specimen were cut.

Primordia in C pieces underwent the same morphological changes, including elevation and the formation of feather buds, as were observed in B pieces and in uncut controls. In some cases, B parts of the specimen underwent slight shrinkage on transfer to the

new substratum, however, a more marked shrinkage occurred in colcemid-treated parts. (Representative explants are shown in Fig. 5.8.) Histological comparison between C and A pieces confirmed that primordia in the former had undergone elevation and development to the feather bud stage: B pieces also showed elevated primordia and feather buds (Fig. 5.9). By comparison with B, the primordia in colcemid-treated skin were more closely spaced and in several cases were smaller in diameter, though of the same height.

At the cellular level colcemid-treated explants showed marked disruption of structure (Fig. 5.10): the epidermis was convoluted and its cells were shorter than those in controls; many dermal cells were rounded-up and their outline was often poorly defined.

Fig. 5.8 (see page 227) Three explants from the same pteryla, used for histological examination of the effects of colcemid. A. Untreated control fixed at time 0. B. Untreated control transferred to routine medium at time 0. C. Treated explant transferred to medium containing colcemid ( $5.4 \times 10^{-6}M$ ), B. and C. fixed at 10.5 hours after adding colcemid. (Time after adding colcemid is shown in the right hand margin.) Lateral primordia continue to darken in B. and C. over the period examined. Primordia of the second most lateral row in both cases acquire a sharper outline.

Fig. 5.9 (see page 228) Low magnification view of transverse histological sections through the 3 explants from the same pteryla shown in Fig. 5.8. a. Untreated control fixed at time 0. b. Untreated control transferred to routine medium at time 0 and fixed 10.5 hours later. c. explant treated with colcemid ( $5.4 \times 10^{-6}M$ ) at time 0 and fixed 10.5 hours later. In a. the medial primordia are partially elevated but have not reached the feather bud stage. In b., primordia have elevated to the feather bud stage. In c., buds of similar gross morphology have also formed, but their histological structure is severely disrupted.

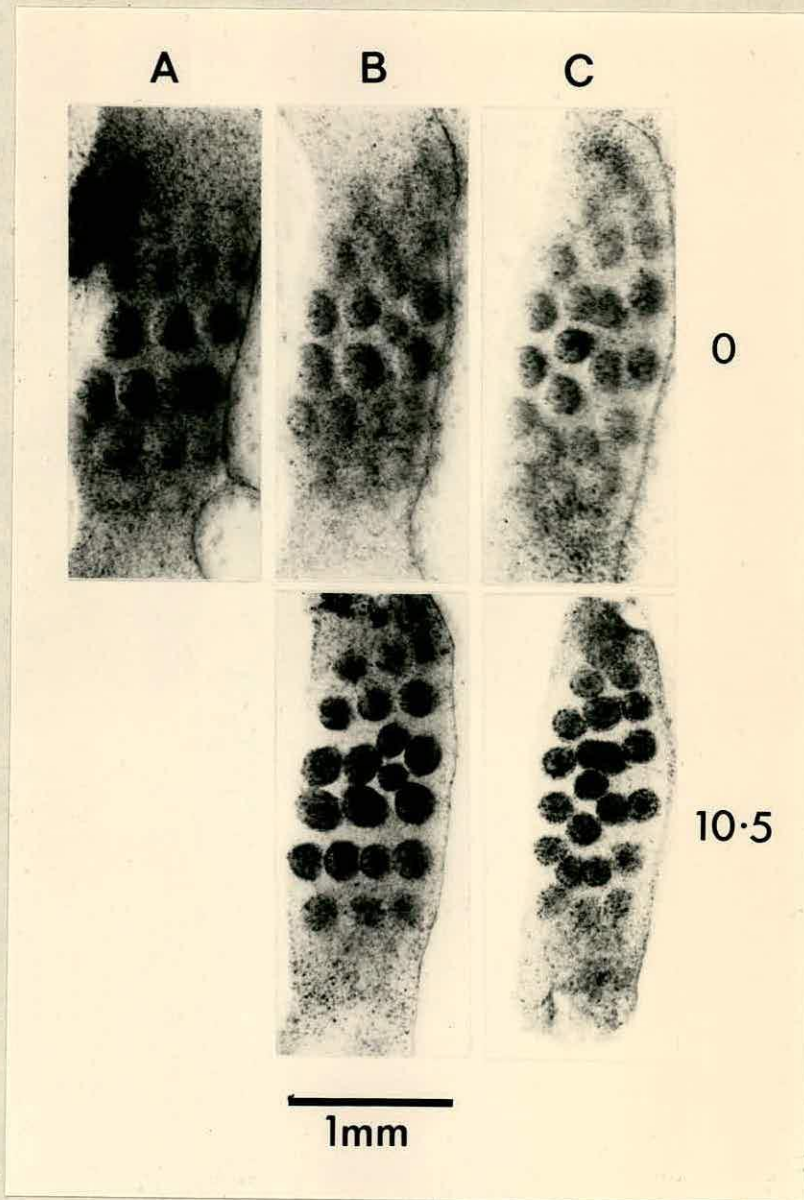


Fig. 5.8

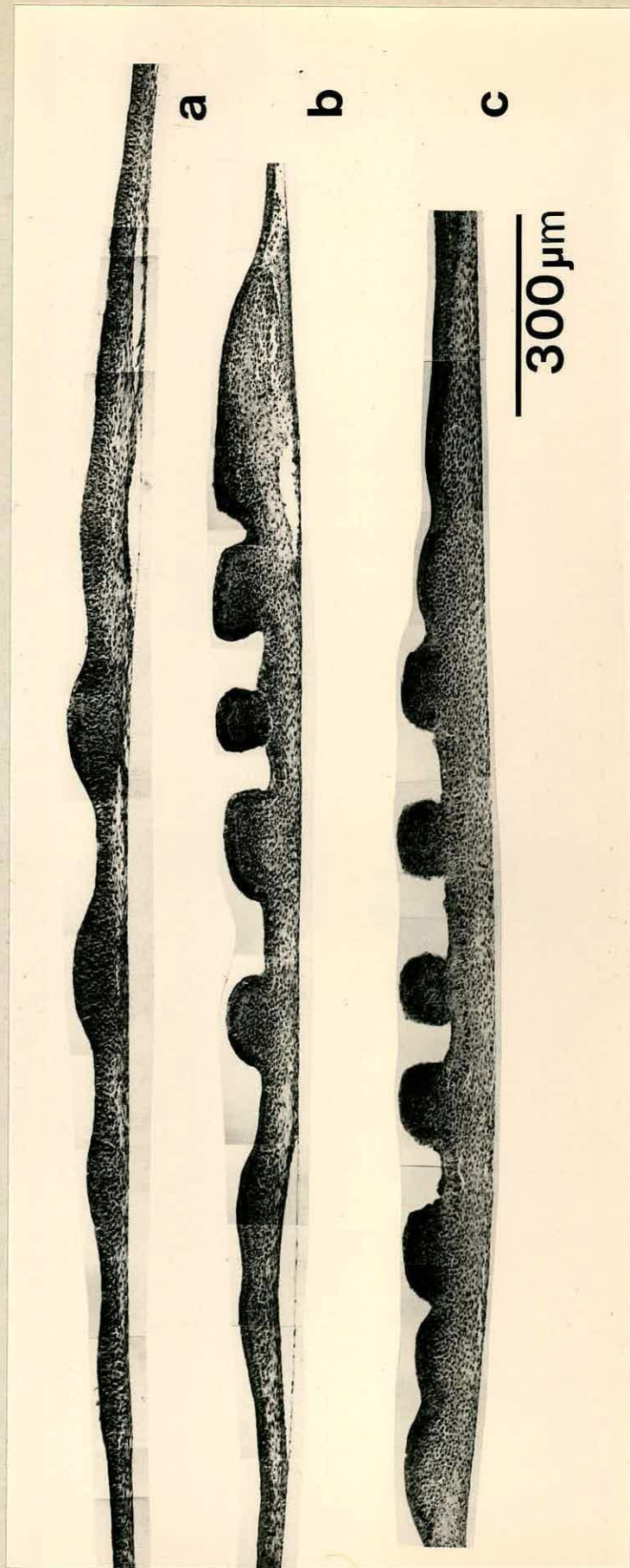


Fig. 5.9

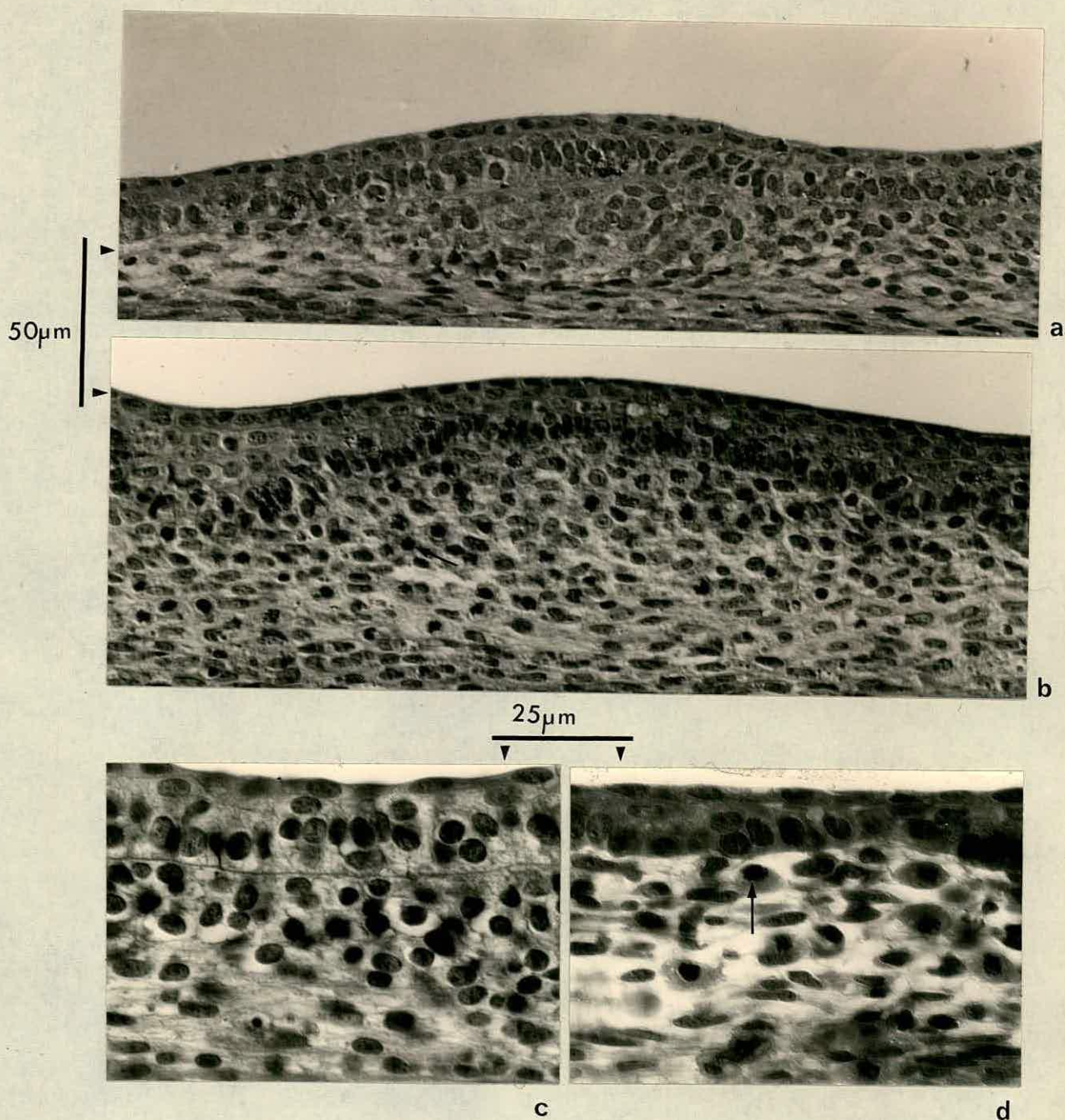


Fig. 5.10 Histological effects of colcemid treatment.

a. and c. Untreated, control skin.

b. and d. Skin treated with colcemid ( $5.4 \times 10^{-6}M$ ) for 10.5 hrs.

a. and b. General view, row 1 primordium.

c. and d. Detail of intercondensation dermis.

Arrows point to arrested mitoses in b. and d.

Large numbers of mitotic figures were distributed throughout the epidermis and dermis in treated explants.

#### Observations from Films of Colcemid-Treated Skin

Two low resolution time-lapse films were made of skin developing in culture (phase-contrast illumination, x 6 objective, lapse time 2 mins.). In each case the specimen was explanted at Stage 29 and allowed to develop until several rows had formed, then filmed for some time before treatment, after the addition of saline, and finally, after adding colcemid ( $5.4 \times 10^{-6}M$ ). The addition of saline provides a control manipulation.

Several hours of filming before adding colcemid showed that explants were developing in the usual manner. The addition of saline had no detectable effect.

After colcemid was added to the first specimen, skin over the entire field of view underwent marked shrinkage, beginning after 30 to 40 mins. of treatment and lasting for about 1 hour. Primordia which were just visible when colcemid was added increased in opacity over the following 8 hours.

In the second specimen shrinkage occurred within 1 hour of treatment and lasted about 2 hours. Primordia in the field were already becoming opaque when the drug was added and, in its presence, continued to darken over the next 8 hours and acquired a sharp outline.

In both specimens active cell motion continued apparently unabated in colcemid, but the resolution did not permit the movements of individual cells to be followed.

### 5.3 Evidence Relating to the Nature of Cell Movement in Colcemid-Treated Dermis

#### Cells in situ

The results of these experiments indicate that primordia continue to elevate to the feather bud stage if they are cultured in the presence of colcemid after their development has been initiated. In the light of the results presented in Section 3, which imply that cells move into the condensation as it thickens, it is of interest to investigate the effect of colcemid on cell movement in dermal arrays. Ideally, one would like to examine directly the movement of individual cells within the dermis in both control and treated specimens. For reasons already stated (page 96) this is not currently feasible. I have therefore used an indirect approach by comparing the changes in morphology which occur in oriented cells in situ with the changes in shape which are associated with the effect of colcemid on the movement of cells on collagen substrata, where they can be clearly seen.

The effect of colcemid on the structure of dermal arrays was examined in freshly excised skin, since the studies described in Chapter 3 have shown that these are more highly organized than arrays in cultured skin.

Freshly excised specimens with 5 to 6 rows of primordia were cultured in the presence of colcemid ( $5.4 \times 10^{-6}M$ ), for  $5\frac{1}{2}$  hours in one experiment, and 11 hours in a second experiment. Control specimens were cultured in parallel. (10 treated specimens, 3 for  $5\frac{1}{2}$  hours, 7 for 11 hours; 6 untreated controls.) Specimens were processed histologically and 7 to 10  $\mu m$  tangential sections were cut.

Control specimens showed the same structure as has already been described (Fig. 5.11 a and c). Specimens treated for  $5\frac{1}{2}$  hours in colcemid showed a striking reduction in the number of

elongated cells lying between condensations: rounded cells were commonly observed in the areas where arrays were present in control specimens (Fig. 5.11b). After 11 hours in colcemid few elongated cells remained between condensations, and condensations were generally closer together than in controls (Fig. 5.11d). Occasional remnants of arrays remained locally as small groups of aligned cells. Although both controls and treated specimens were of the same developmental age, cells within condensations of the lateral rows in treated specimens were typically rounded and showed no consistent patterns of orientation. This appearance contrasts with the radial and tangential orientation of cells in the condensations of controls.

#### Cells on Collagen Substrata

The effect of colcemid on dermal cell shape and movement was examined in detail by observing cells as they moved over the surface of, and through, the hydrated collagen lattice beyond the edge of the explant.

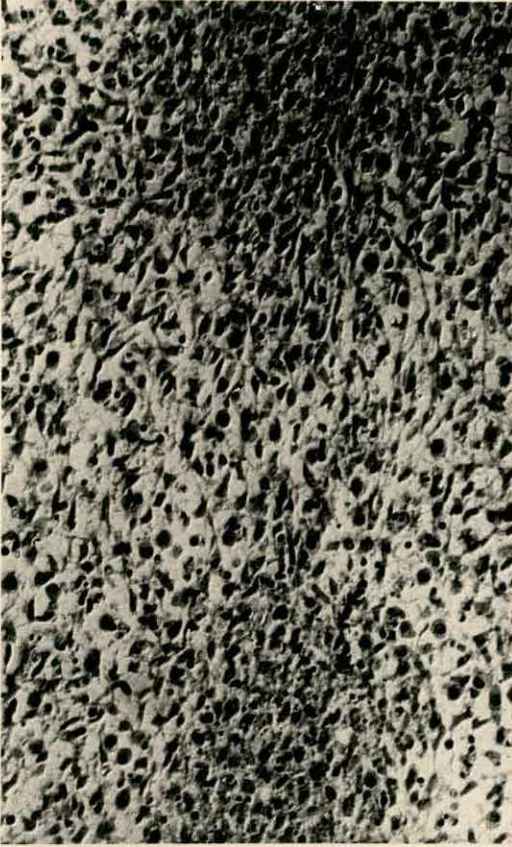
Three experiments were performed, observing cell movement both before and after adding colcemid ( $5.4 \times 10^{-6}M$ ). In one experiment photographs were taken at intervals. In the other two experiments films were made (phase-contrast, x 6 objective, lapse time 2 mins.).

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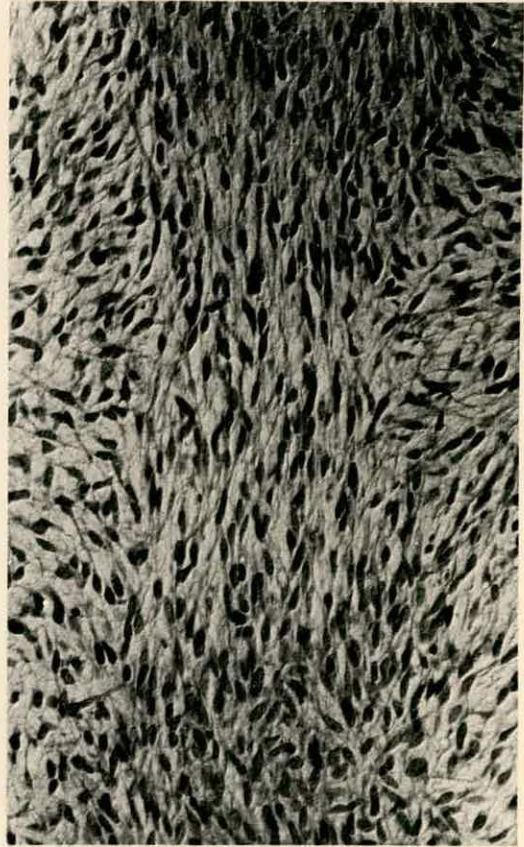
Fig. 5.11 The effect of colcemid on the structure of arrays of intercondensation cells. Tangential sections. a. and c. untreated controls, b. and d. colcemid-treated ( $5.4 \times 10^{-6}M$ ). b. treated for  $5\frac{1}{2}$  hours, d. treated for 11 hours. Note the loss of cell orientation in treated specimens. (see over)

100µm

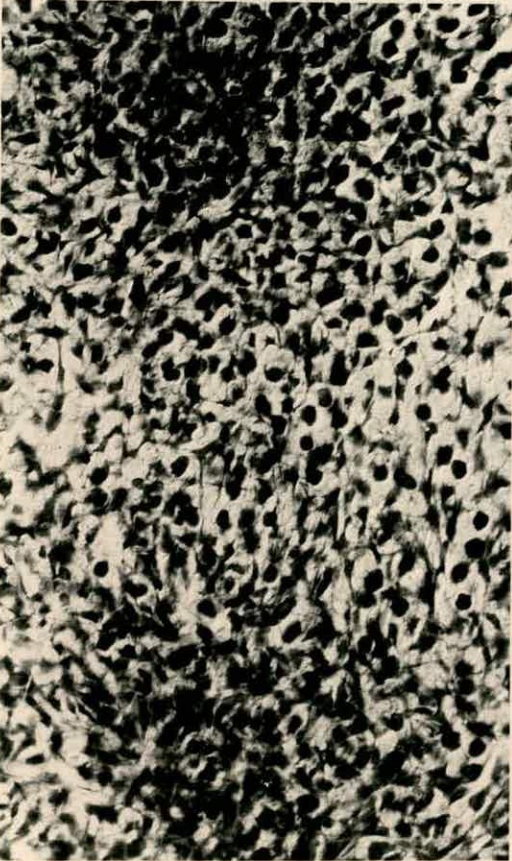
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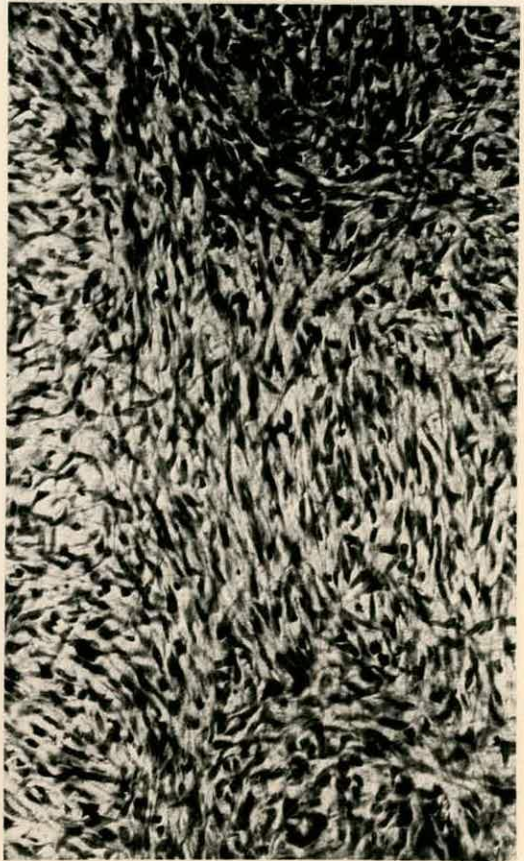
c



q



e



Periodic observations showed that untreated cells moved through the collagen matrix in their well-known bipolar spindle form (Elsdale and Bard, 1972): they were in the order of 90  $\mu\text{m}$  long. Colcemid had a rapid effect on cell shape (Fig. 5.12): within 40 mins. the longest cell processes disappeared, and though a few cells retained lengths in the order of 80  $\mu\text{m}$ , the majority became shorter (less than 60  $\mu\text{m}$  in length) and polygonal. By 1½ hours in colcemid almost all the cells had become polygonal and remained thus throughout the period of examination (over 25 hours).

Films showed that colcemid has a rapid effect on cell movement which accompanies these changes in morphology: prior to adding colcemid, elongated cells moved at approximately 15 to 20  $\mu\text{m}$  per hour (generally in the direction of outgrowth) but no cell migration occurred after adding the drug (Fig. 5.13). Over 24 hours following the addition of colcemid, the surface of many cells remained active, sending out short, transient, ruffling processes in all directions: in some cases the cell bodies were pulled to and fro by this activity within an area 60 to 90  $\mu\text{m}$  in diameter around the site of the cell before treatment. In one specimen, this movement was sufficient to bring cells into contact, but insufficient to permit escape from mutual adhesion, so that the cells slowly aggregated (Fig. 5.14).

The effect of colcemid on cells whose orientation was entrained by the substratum and by lateral contact with other cells was also examined. In two experiments dermal cells were filmed as they moved out from skin fragments explanted on aligned collagen substrata (phase contrast, x 6 objective, lapse time 2 mins.).

Aligned collagen substrata are very thin, and cells, while oriented by the collagen are in partial contact with the flat

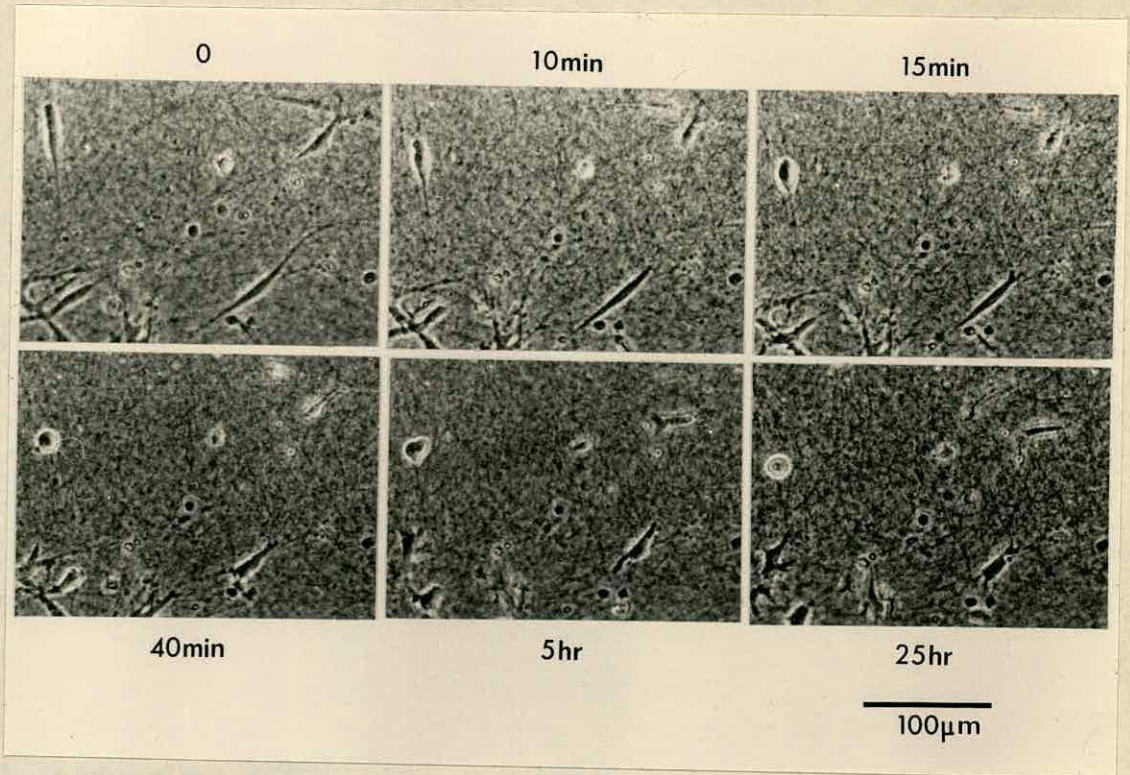


Fig. 5.12 The effect of colcemid on the morphology and movement of cells on a collagen substratum. Colcemid ( $5.4 \times 10^{-6}M$ ) added at time 0. Note the rapid retraction of the long cell processes and the rounding up of several cells in the field within the first 40 mins. Several cells, for example the one at the bottom right of each frame, continue to change their shape over the next 25 hours, but they do not move over the collagen.

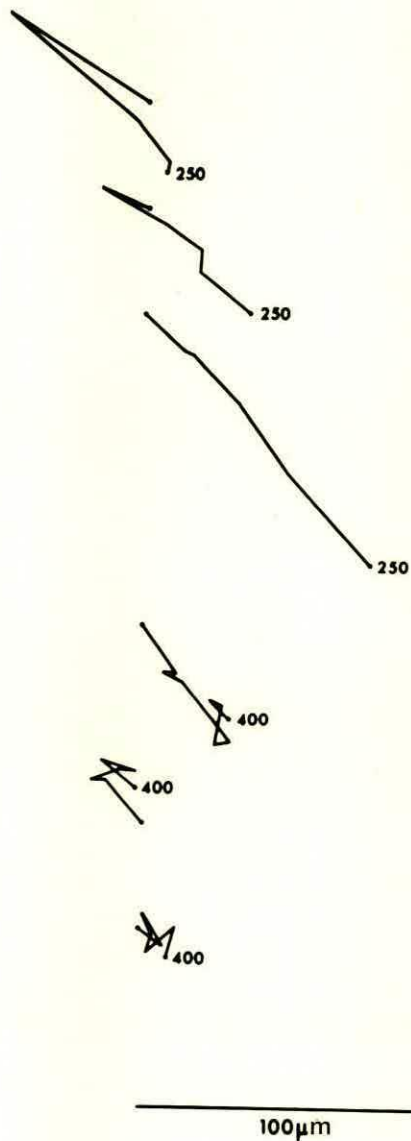


Fig. 5.13 The effect of colcemid on dermal cell movement on a hydrated collagen lattice.

These traces were made from a film by plotting the position of the cell nucleus at 50 min. intervals and joining successive positions with a line; figures show time in mins. at the end of the trace. Upper 3 traces - control: movement before the addition of colcemid.

Lower 3 traces - the movement of cells treated with colcemid ( $5.4 \times 10^{-6}M$ ), starting the trace immediately after adding the drug.

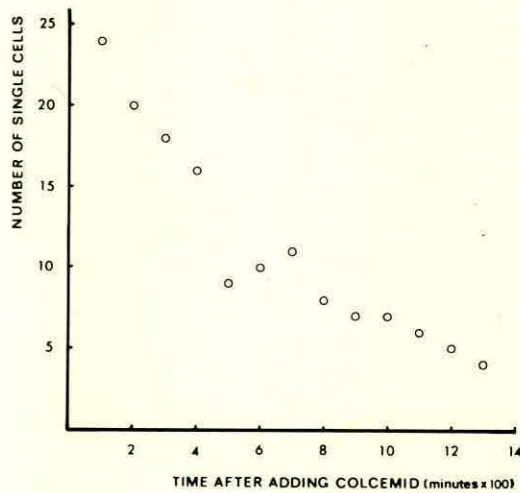


Fig. 5.14 Graph showing dermal cell aggregation in the presence of colcemid. Data was taken from a microscope field in which cells growing out from the edge of the explant in the presence of colcemid were filmed. Counts were made, at hourly intervals, of single cells (i.e. those not in clumps). As cells aggregated the number of single cells decreased.

surface of the plastic culture dish. These cells, though elongated, are flatter than those moving on the thicker 'random' collagen lattices, or those in dermal arrays. Cells were filmed before, and after, adding colcemid ( $5.4 \times 10^{-6} \text{M}$ ). Before treatment, cell movement was rapid (in the order of  $20 \mu\text{m}/\text{hour}$ ): within 45 minutes of the addition of colcemid, migration was arrested. The arrest of migration was associated with similar morphological changes to those observed in cells on thicker collagen lattices, though the cells remained flatter and more elongated for the first two or three hours of treatment.

#### 5.4 Discussion

##### Effect of Colcemid on Primordium Development

The results indicate that while the initiation of new primordia is inhibited by colcemid (except in very low concentrations where its effectiveness in arresting microtubule-dependent processes is probably impaired), the morphological development of existing primordia is not.

The first of these observations is contrary to the findings of Stuart et al, (1972) who reported that, in skin developing in the presence of colchicine, "the formation of new feather germs (i.e. primordia) was not noticeably prevented over the period of cultivation since new papillae (i.e. primordia) continued to appear". However, it is not clear from their report how long the skin was cultured in the presence of the drug, but it appears that only a 2.5 hour period may have been examined. It is difficult to interpret this inhibition (see Section 5.1): one can only note that the processes necessary for initiation may be distinguished from those necessary for continuing development of the primordium on the basis

of their colcemid sensitivity.

The development of already initiated primordia in colcemid is more interesting. Untreated primordia increase in opacity as their condensations thicken (Chapter 3). The observation that primordia exposed to colcemid from the earliest visible stages of development also continue to darken suggests that their condensations continue to increase in thickness. Histological observations further suggest that an increase in thickness occurred in treated condensations, though this evidence is less firm because tissue shrinkage and disruption obscure these early morphogenetic changes. The more marked, later elevation and formation of the feather bud clearly continue in colcemid (except in very high concentrations where disruption probably occurs to the extent that interpretation is impossible). It is difficult to assess how far cell packing and changes in cell distribution are affected by the drug. Therefore, our immediate conclusions can only apply to changes in overall morphology.

It is reasonable to suppose that the forces which are primarily responsible for the morphogenesis of primordia in colcemid are essentially the same as those which operate in untreated skin. This is supported by the fact that treated primordia develop at approximately the same rate as in controls. It is emphasised, however, that secondary aspects of development will be different in treated and untreated primordia. Forces generated by treatment - for example, tissue contraction - may aid, or modify normal morphogenesis. Moreover, several aspects of cell development will be drastically affected: division will cease, structure based on cell elongation will be lost, and ultrastructural, metabolic, and biosynthetic changes will cause a gradual deviation from the normal

course of development.

The increase in dermal thickness at the centre of an untreated condensation is associated with addition of cells to the whole condensation, probably by centripetal movement (Section 3), but may also be caused, in part, by rounding-up of the existing cell mass, perhaps in association with epidermal bending. Forces causing rounding-up may also be responsible for later elevation and bud formation. Since bud formation certainly continues in colcemid, and early thickening of the condensation probably also does, we can conclude that the forces responsible for these changes are independent of cell proliferation and oriented movement. The evidence for this is as follows.

Colcemid effectively inhibits cell proliferation in the skin (Section 3). The drug also inhibits oriented movement within the skin in a manner similar to that observed by Vasiliev et al (1970) and Gail and Boone (1971) in cells in culture. Elongated cells in situ, including those in dermal arrays, become polygonal: their morphology resembles that of cells on collagen whose migration has been arrested by the drug. Under the present conditions, movement of cells with entrained orientation in vitro is also rapidly and effectively inhibited. The effect of colcemid on cell morphology and movement was more rapid in the present experiments, both in situ and in culture, than in experiments reported by Vasiliev et al (1970) who applied lower concentrations (of the order  $10^{-7}$ M) to mammalian fibroblasts and found that morphology and movement were affected after 2 to 12 hours. The results obtained by Gail and Boone (1971), who applied colcemid (of the order  $1 \times 10^{-6}$ M) to mouse 3T3 cells and found cell morphology and movement changed after 1 to 2 hours, are more similar to those described here, where marked effects were noted

in dermal cells on collagen within one hour of treatment. Further evidence of a rapid effect on cells within the skin is the contraction of whole skin 1 to 2 hours after adding colcemid. Downie (1975) noted similar contraction of chick blastoderm 1 to 2 hours after adding colcemid. The contraction appears to be caused by shortening of the skin cells, presumably mainly dermal cells since these show greatest elongation in the plane of the skin surface: Bhisey and Freed (1971) have suggested that a microfilament system beneath the cell surface generates contractile forces which are normally opposed by colcemid sensitive microtubules.

The destabilizing effect of colcemid on the cell surface makes it unlikely that chemotaxis would continue normally in the presence of the drug: chemotaxis of neutrophil granulocyte locomotion is inhibited by anti-microtubule drugs (Bandmann, Rydgren, and Norberg, 1974). However, the possibility that fibroblast chemotaxis may be less sensitive to colcemid cannot be ruled out.

What colcemid-insensitive mechanisms are primarily responsible for primordium morphogenesis? If cell numbers in the condensation do increase as thickening proceeds in colcemid, then this is most likely accomplished by short-range, random movement combined with cell trapping: the present results indicate that these processes can, in principle, occur in colcemid (Fig. 5.14). If thickening, elevation, and bud formation depend on rounding-up of the condensation cell mass, there are three most likely mechanisms: first, an increase in cell surface contact, due to cell-cell adhesiveness being greater in the condensation than in the surrounding dermis; second, differential contraction of the surfaces of condensation cells exposed to the surrounding dermis; third, epidermal folding due, probably, to differences in apical and basal contraction of cells in the sheet

(while epidermal structure is gradually disrupted in colcemid, it may be maintained sufficiently long for such a mechanism to operate).

The present experiments do not distinguish between these mechanisms.

These possibilities are discussed further in Section 7.

In conclusion, whatever mechanisms do operate, the present results imply that the overall changes in shape which occur during primordium morphogenesis are governed by the non-proliferative activities of cells at the primordium site and not by patterns of cell orientation in the surrounding dermis.

## 6. EVIDENCE FROM DISSECTION EXPERIMENTS

### 6.1 Introduction

Preliminary observations indicated that the strength of adhesion between dermis and epidermis is greatest in areas of skin which contain primordia. This suggested that the tissues might form an especially strong attachment within individual primordia. It was thought that this, in turn, might reflect the formation of strong adhesions by dermal cells in the condensation and that these could play a part in cell aggregation and in the morphogenesis of the primordium. The relative strength of attachment of dermal cells to the basement membrane and to one another over the surface of the dermis has therefore been investigated. This has been done by looking at the way the skin comes apart during gentle dissection. The results of dissection have been examined using scanning electron microscopy because this provides an ideal means of viewing large areas of dissected tissue interface at high resolution.

## 6.2 Method

Skin was explanted at Stage 29 to 31 and dissected on the second day of culture. In one series of experiments, skin was dissected without pretreatment; in a second series, a mild trypsin-treatment was applied before dissection so that the basement membrane could be peeled from the dermis and its undersurface exposed to view.

Dissection without the aid of typrsin: Specimens were rinsed twice with Dulbeccos saline then dissected under saline: using fine forceps, the epidermis was carefully lifted free from one end of the skin and gently peeled back from the dermis which remained anchored to the substratum. Immediately after dissection, the dermis was removed from the substratum, mounted on filter-paper, fixed and processed for SEM. In some specimens, only the dermis was examined in the SEM. In others, the epidermis, too, was examined: in these specimens, the epidermis was left partly attached to the skin, because it is very thin and difficult to handle on its own. In one experiment, 3 specimens were also processed histologically. (10 specimens examined in the SEM; 3 independent experiments.)

Dissection with the aid of trypsin: Each specimen was rinsed twice with Dulbeccos saline then submerged in cold trypsin (see Chapter 2) and incubated at 4°C for a period of 40 minutes to 2 hours. At the end of this time the skin was rinsed with culture medium to arrest the action of the enzyme, then rinsed twice with saline, submerged in saline and dissected as described above, leaving the epidermis attached over part of the skin. (15 specimens, 5 independent experiments.)

### 6.3 Results

#### Observations Made During Dissection

In every case, a continuous, thin sheet of tissue was removed from the dermis. However, when specimens which contained more than about 6 rows of primordia were dissected without the aid of trypsin, this sheet split parallel to its surface and, while the upper part of the sheet was removed intact, the lower part remained attached to the dermis in areas where condensations were present. This was visible, under the dissecting microscope, as a thin veil of material lying over the dermis. When younger specimens were dissected without pre-treatment, it was clear that adhesion between the tissues was especially strong at individual primordium sites: the epidermal sheet peeled smoothly free over most of the skin, but remained temporarily attached, then jerked free, at each condensation site.

In specimens dissected after tyrrpsin treatment, the ease with which the tissues could be separated varied with the length of treatment: specimens treated for less than 1 hour gave the same results as untreated specimens; 1 to  $1\frac{3}{4}$  hours in the presence of the enzyme allowed the sheet to be removed without splitting (except where about 10 or more rows were present, when the sheet split over the medial area), but it did jerk free from condensations as it was peeled back; after 2 hours treatment the tissues could invariably be separated freely and uniformly over the skin.

#### Observations on Fixed Material

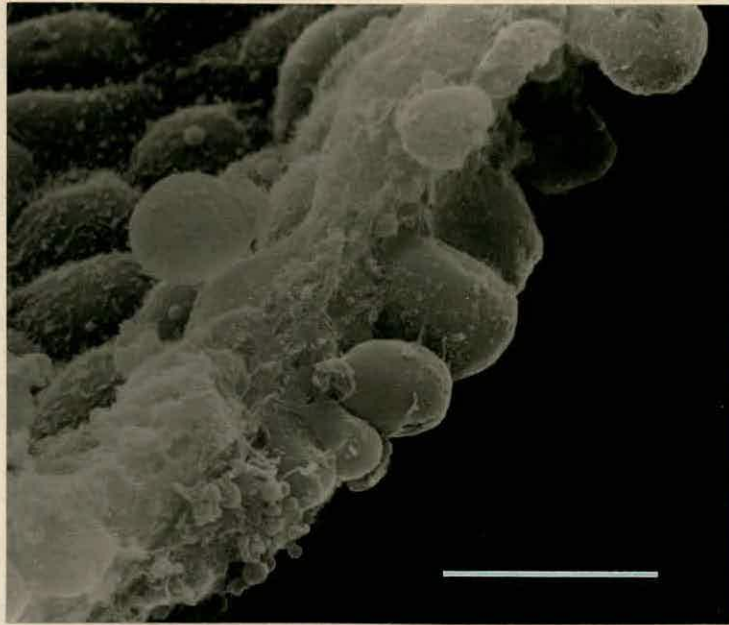
In those specimens dissected without pretreatment, in which the epidermal sheet had split, SEM revealed that the periderm had been removed intact but its lower surface was covered with debris

from the broken epidermis, and a layer of epidermal debris lay immediately over the medial dermis where primordia were present. (Fig. 5.15). Histological examination confirmed that the dermis was covered by broken epidermis. In 2 specimens where the epidermis had been removed intact, there was some evidence of localized strong attachment between the tissues within individual primordia. The dermis was covered with a thin film of amorphous material, but precisely over condensations in the second and third most lateral rows, the dermal surface was broken to form 'craters' (Fig. 5.16) from which material had apparently been removed with the epidermis. In the same two specimens, small areas of broken epidermis were observed over individual condensations in the medial row.

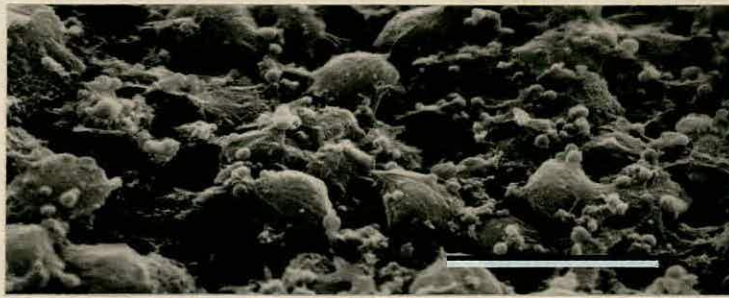
In specimens treated with trypsin for 1 hour or less the lateral dermis was exposed, but large areas containing condensations were covered with a layer of broken epidermis. Longer treatments had completely removed the epidermis and basement membrane over almost all the dermis (Fig. 5.17, see page 248): skin treated for 2 hours showed large areas of basement membrane with few adhering cells; however, skin dissected after  $1\frac{1}{2}$  to  $1\frac{3}{4}$  hours in trypsin

Fig. 5.15 Scanning electron micrographs of skin dissected without the aid of trypsin.

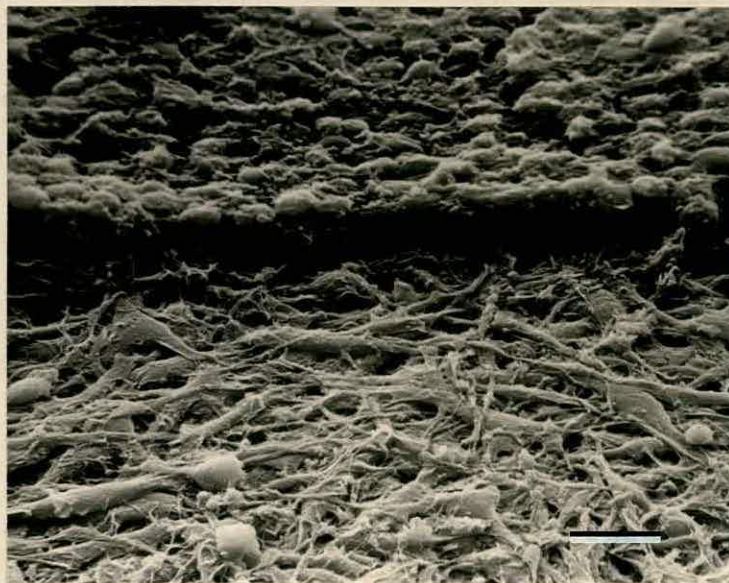
- a. Structure of the sheet of tissue removed from the dermis: periderm (upper left shows peridermal surface) with broken epidermis beneath it. b. Broken epidermal material remaining on the dermis after stripping. c. The edge of broken epidermis showing that this material lies on the dermis, which is exposed in the lower part of the picture. Scale bars for a, b and c represent 10  $\mu$ m. Tilt: a -, b  $45^\circ$ , c  $30^\circ$ . (Tilt is given for this, and subsequent micrographs only where the field of view lay parallel to the surface of the specimen mounting stub.)



a



b



c

Fig. 5.15



Fig. 5.16 The surface of dermis from which epidermis has been removed without the aid of trypsin.

The centre of the picture shows the site of a condensation from which material appears to have been removed with the epidermis. Scale bar represents 30  $\mu\text{m}$ .

showed dermal cells adhering to the membrane in a striking pattern.

In these latter specimens cells were attached to the membrane in discrete groups, about 100 - 170  $\mu\text{m}$  in diameter, each group lying in a shallow depression formed by the undersurface of an epidermal placode, in precise correspondence with the position of a condensation in the dermis (see for example, Fig. 5.20). In most cases these groups of cells were circular, though in a few cases they were ring- or crescent-, shaped.

The overall distribution of cells on the basement membrane in these specimens provides evidence of the changes which occur in cell adhesion during the development of condensations. The

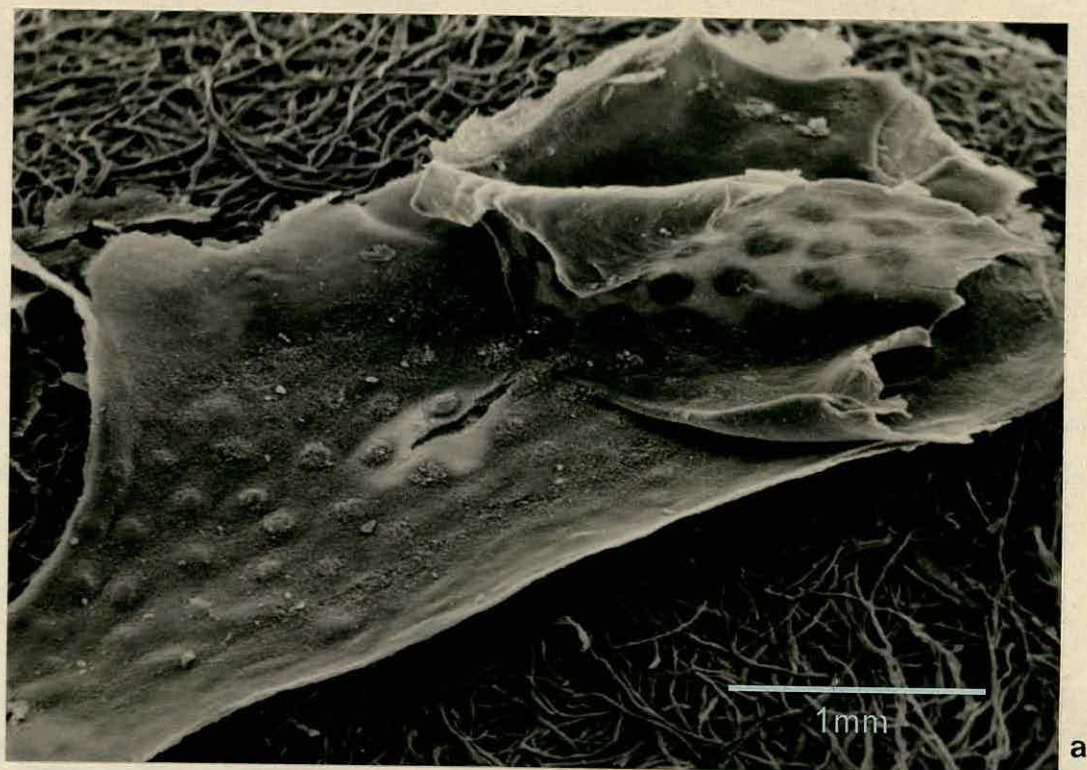


Fig. 5.17 Scanning electron micrograph of skin dissected after  $1\frac{3}{4}$  hours trypsin treatment.

a. General view: exposed dermis is shown left of the picture, epidermis has been peeled off towards the right exposing its undersurface. Beyond the peeled-off epidermis the upper surface of intact skin can be seen.

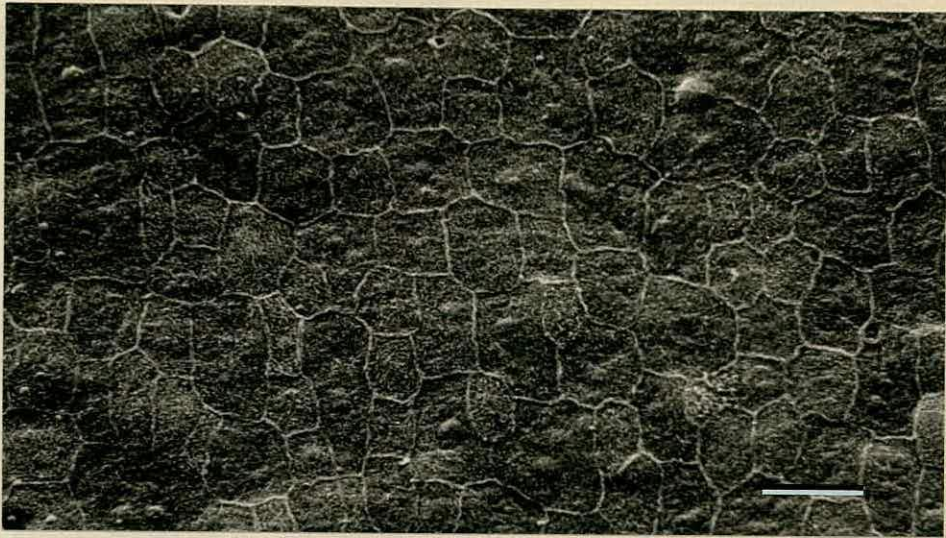
(b., c. and d. see over)

b. Surface of intact skin: periderm.

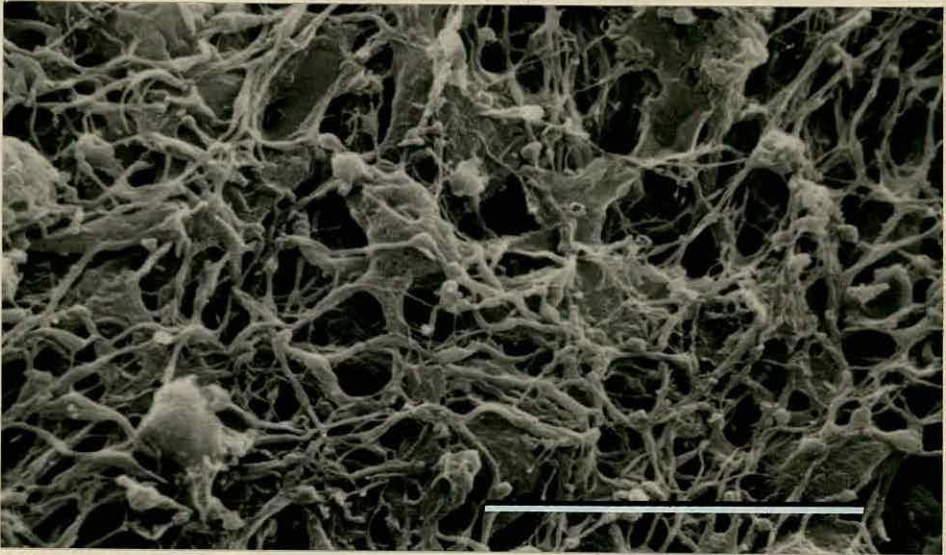
c. The surface of the dermis exposed after dissection.

d. The undersurface of the epidermis removed from the dermis by dissection. The epidermis is covered with an amorphous material, which by its position and appearance can be identified as basement membrane. This picture shows a tear in the membrane through which epidermal cells can be seen.

Scale bars b, c and d represent 10  $\mu$ m.  
a, b and c 30° tilt.



b



c



d

Fig. 5.17b, c and d

distribution will be described in lateral to medial sequence following the course of development. (This is summarised in Fig. 5.22).

The basement membrane was bare in areas stripped from lateral, unpatterned dermis. However, in about half the specimens examined, scattered cells were attached to the membrane in a continuous strip about 110  $\mu\text{m}$  wide (Fig. 5.18a), in the region equivalent to the row of initiating condensations. Many cells in this dermal region had a rounded shape in contrast to the flat cells which predominated in the lateral, and intercondensation, dermis (Fig. 5.19a-c). The cells adhering to the basement membrane were attached by fine processes similar to those which have been described in SEM studies of fibroblastic cells on substrata in culture (Fig. 5.18b) (Vasiliev and Gelfand, 1973; Revel, Hoch and Ho, 1974). Over the surrounding basement membrane, as over all areas of the membrane free of cells, the surface was littered with short broken fibres or cell processes.

Placodes stripped from the most lateral row of definitive condensations were visible as shallow depressions in the undersurface of the epidermis, but these were free from adhering dermal cells (Fig. 5.20). Cells at the surface of the condensations themselves were typically spindle-shaped or spherical, but were not closely packed (Fig. 5.19d).

More medial rows of placodes showed groups of adhering cells which increased in diameter and depth medially, but only a few dermal cells were attached to the basement membrane between placodes (Fig. 5.20); in the position of the most medial rows of condensations the epidermis had split as in untreated specimens. Comparison between specimens showed that the size of these groups of cells decreased with increasing duration of trypsin treatment. However, within each specimen groups in the same row were of approximately

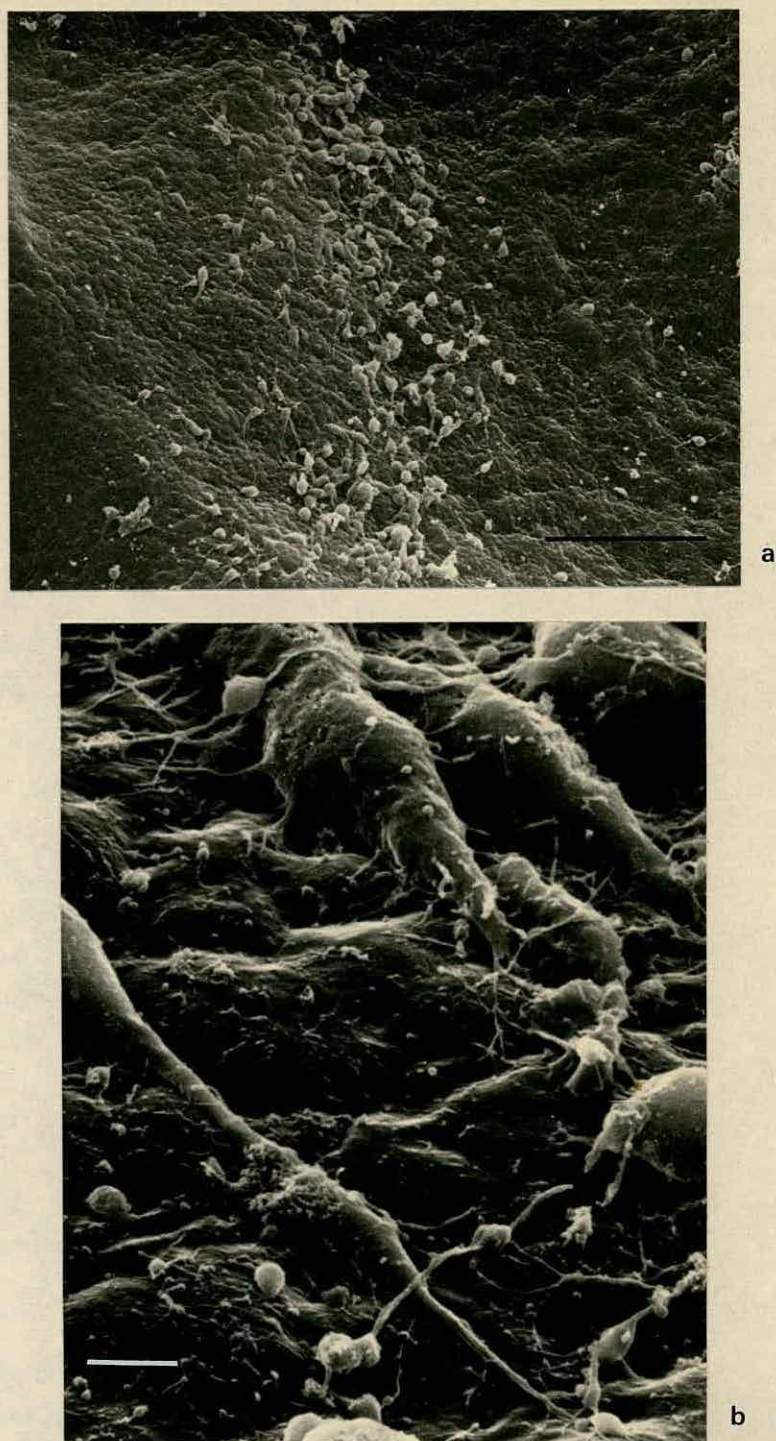


Fig. 5.18 A band of cells adhering to the basement membrane stripped from the site of initiating condensations in skin dissected after trypsin treatment for  $1\frac{1}{2}$  hours.  
a. Low magnification view. b. Detail of cell processes attached to the membrane.  
Scale bars a - 100  $\mu\text{m}$ , b - 3  $\mu\text{m}$ .

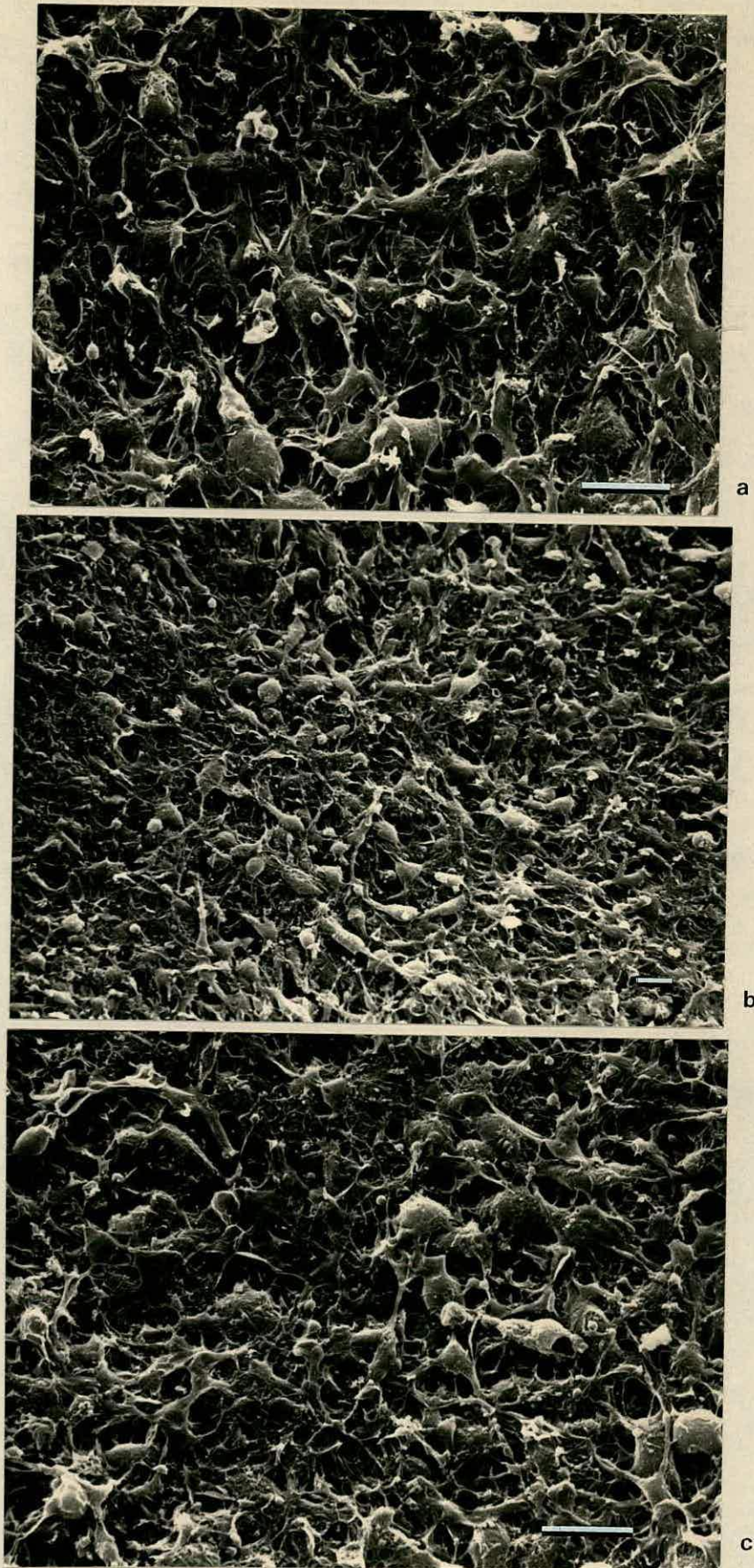


Fig. 5.19 (for legend see over)

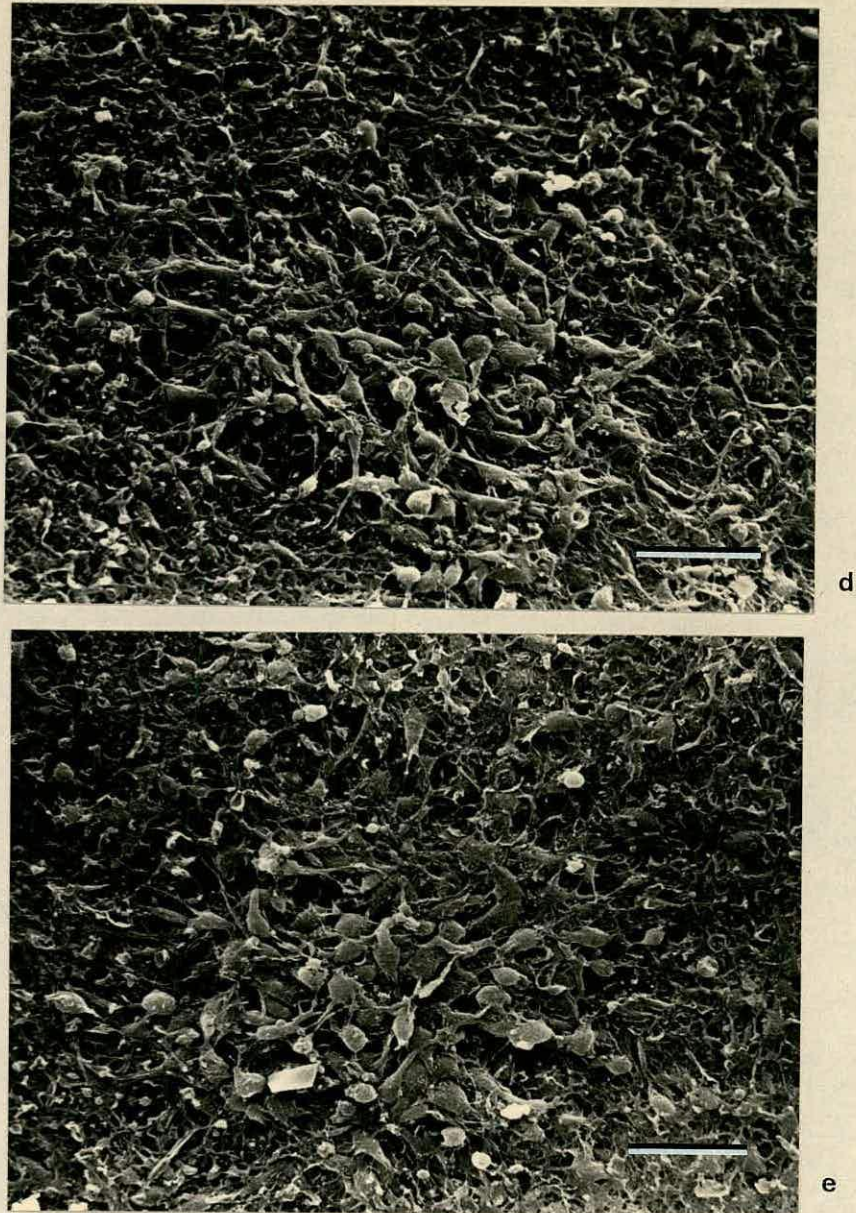


Fig. 5.19 The structure of the dermal surface from which epidermis has been removed after  $1\frac{1}{2}$  hours trypsin treatment.

a. Lateral dermis showing flat cells with lamellapodia.  
 b. Dermis in the position of an initiating condensation. Note the high proportion of rounded cells, though the cells are not closely packed. c. Intercondensation dermis between 2 definitive condensations in the most lateral row. d. Definitive condensation in the most lateral row. Spherical or spindle-shaped cells predominate but these are not tightly packed. e. Condensation in the second most lateral row. Cells are more tightly packed than in condensations in the previous row. Scale bars a, b and c represent 10  $\mu\text{m}$ ; d and e, 30  $\mu\text{m}$ . Tilt a to d,  $30^\circ$ ; e,  $0^\circ$ .

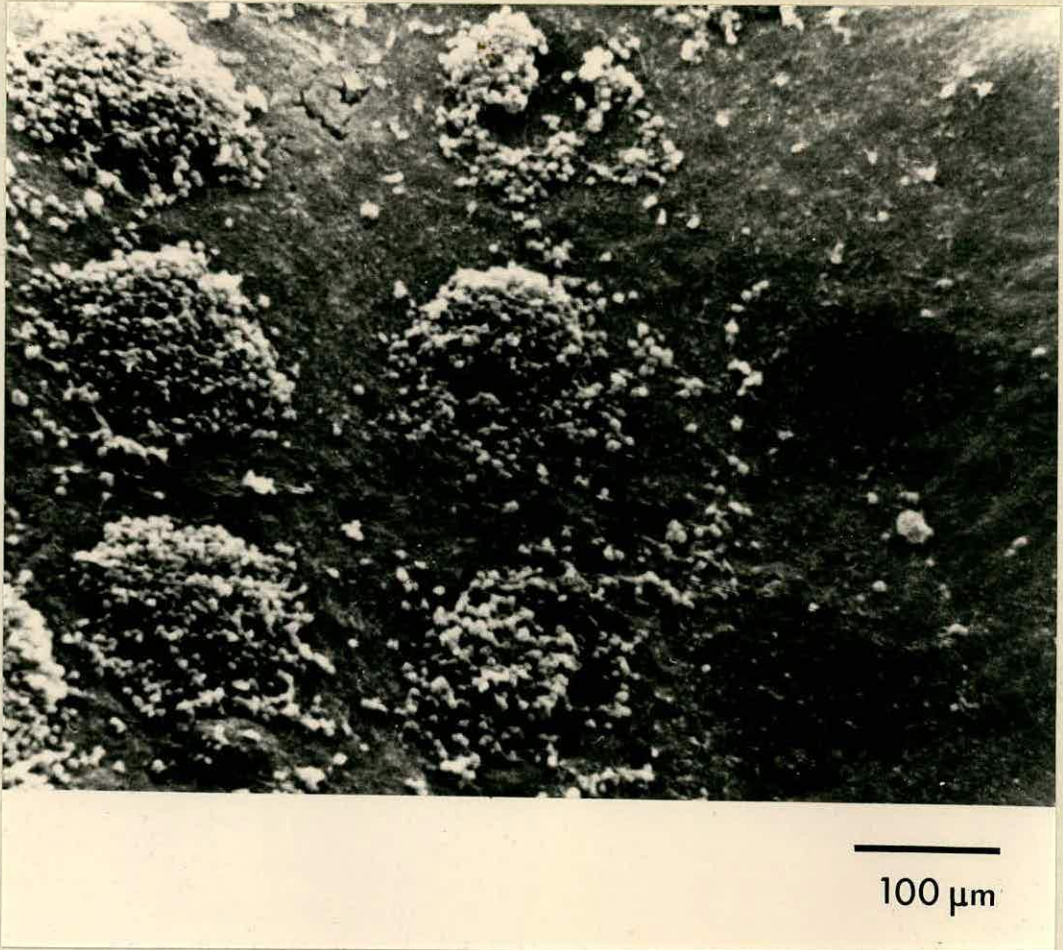
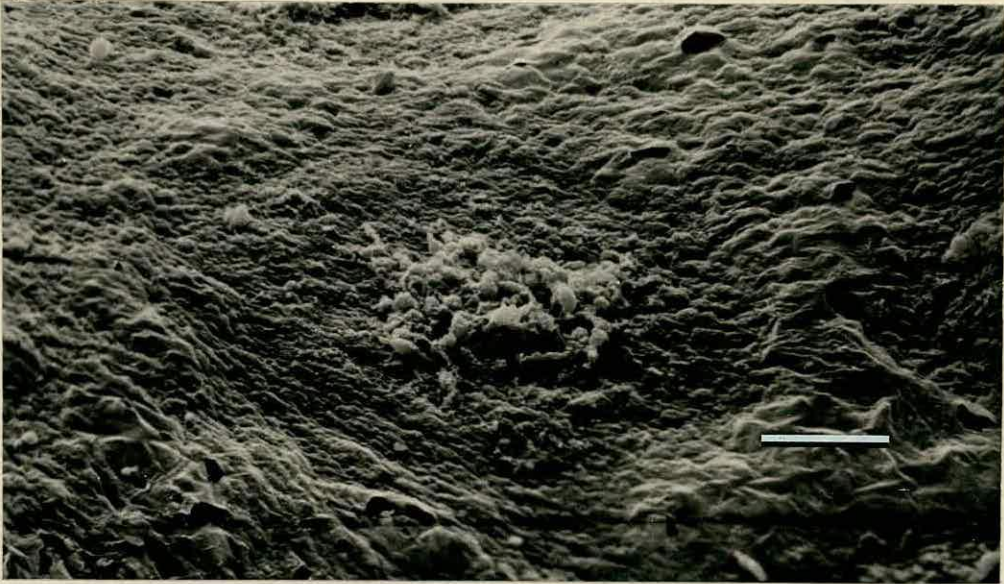


Fig. 5.20 Distribution of cells on the basement membrane in skin dissected after  $1\frac{1}{2}$  hours trypsin treatment. Lateral is towards the right, where the most lateral placode depressions are seen as dark areas free from cells. In the centre is shown three placodes with adhering groups of cells, and left, three slightly larger groups of cells associated with placodes of the next row.

uniform size including those very close to the edge of the epidermal sheet, suggesting that variation between rows is not caused by a reduction in the effectiveness of trypsin with distance from the edge of the skin. Small groups of cells (Fig. 5.21 and 5.23) lay near the centre of the placode undersurface, forming a layer one cell thick, each cell being attached to the basement membrane by processes similar to those described above. Larger groups, however, comprised several layers of almost spherical cells, many of which appeared to be attached only to their neighbours and not directly to the basement membrane (Fig. 5.24). Cells at the surface of the corresponding condensations were packed in a similar manner.

At the junction between the dermis and the separated layer of epidermis it was possible to observe the way cells adhering to the basement membrane had been lifted free from the dermis: elongated cells lay stretched between small areas of attachment to the condensation and basement membrane (Fig. 5.25). The region of strong attachment was strictly confined to the condensation: over the surface of the surrounding dermis, few cells or cell processes were attached to the membrane. No structures were observed at the junction between dermis and epidermis which could be identified as anchor filament bundles though these would be difficult to distinguish from cell processes in the SEM.



5.21

A group of cells lying in the centre of a depression corresponding to the undersurface of an epidermal placode. Scale bar represents 30  $\mu\text{m}$ .

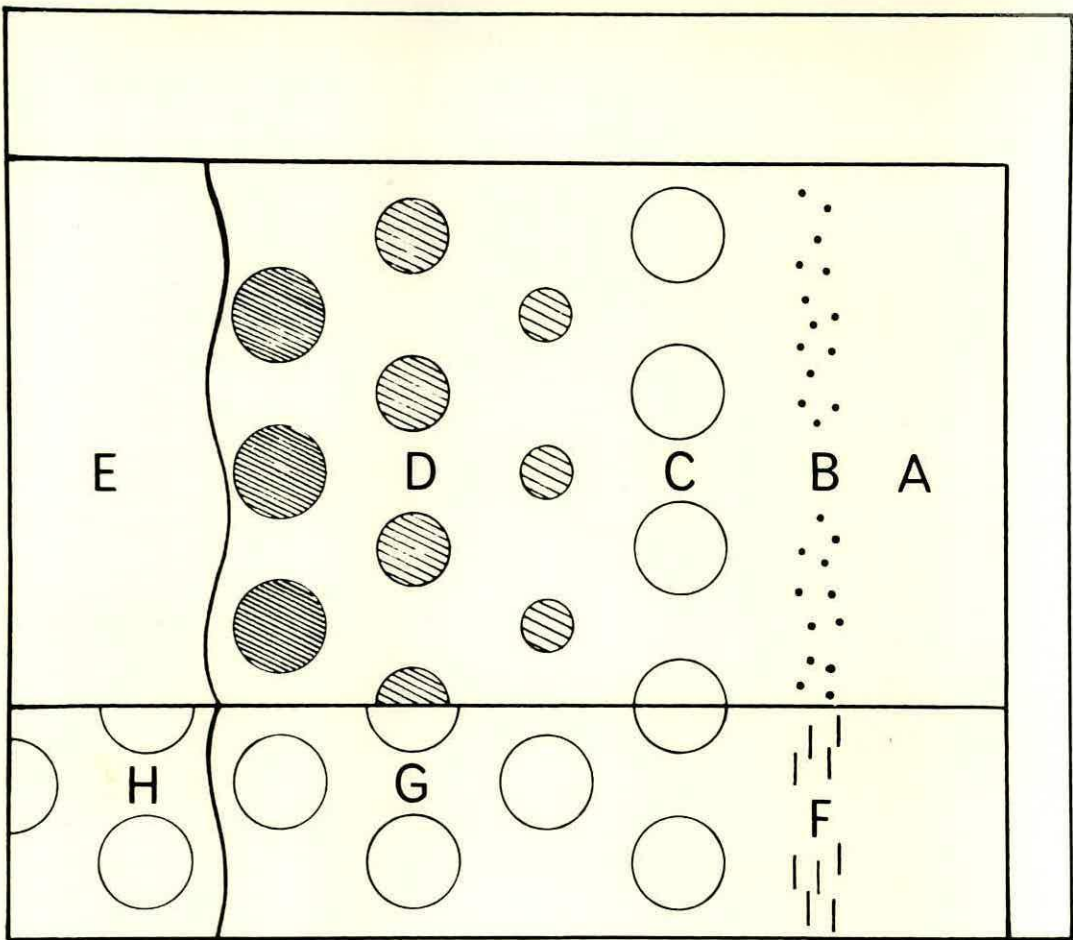


Fig. 5.22 Diagram to show distribution of dermal cells adhering to the basement membrane after the membrane had been peeled free from the dermis with the aid of mild trypsin treatment. A - basement membrane free of cells, B - strip of scattered cells adhering to the membrane, C - underside of placodes without adhering cells, D - rows of groups of dermal cells adhering to the basement membrane below placodes, groups increasing in diameter and thickness medially, E - broken epidermis where the basement membrane has remained attached to the dermis over the most medial condensations, F - strip of dermis comprising predominantly spindle-shaped cells, G - rows of condensations, H - dermis covered by basement membrane and broken epidermis.

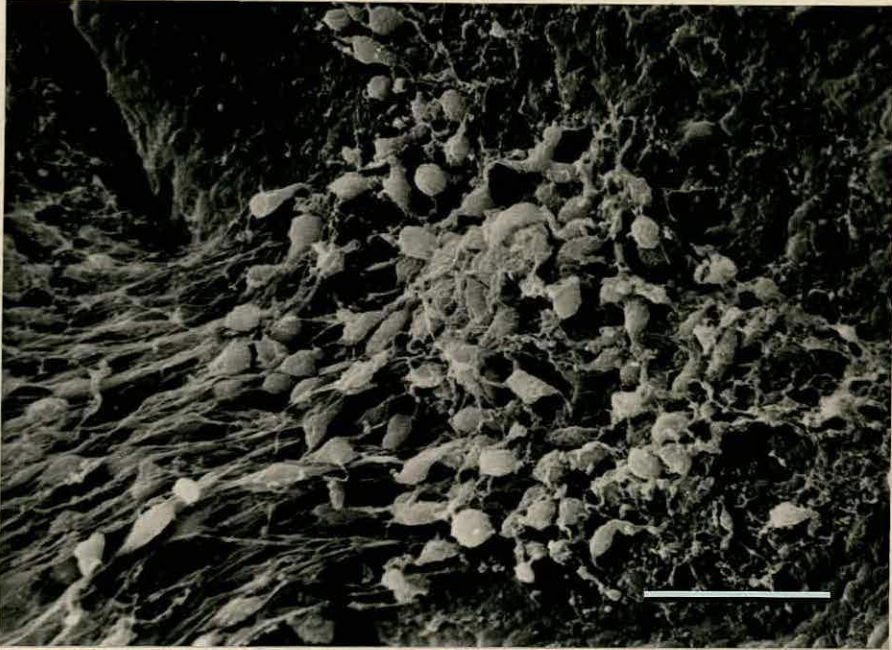


Fig. 5.25 Cells stretched between a condensation (lower left) and a group on the basement membrane (centre right) where the epidermis and dermis are being separated. Cells are not stretched between the tissues in intercondensation areas. Scale bar represents 30  $\mu\text{m}$ .

## Discussion

These results provide evidence of relatively strong adhesion of cells in condensations to the basement membrane and to one-another. What is strong adhesion in the present context?

In both series of experiments, the method of detecting strong adhesions is to reveal the weakest interfaces along which the tissue breaks. Of the several possible interpretations of the results of each experiment, consideration of the combined results yields the following conclusion: over most of the skin the weakest interface is between dermis and epidermis, but in primordia attachment between the tissues is especially strong; this reflects increased strength of attachment between dermis and basement membrane. It is particularly important to note that cells are not lifted free from the condensation because adhesions are weak in this region of the dermis: observations on the junction of separating dermis and epidermis show that cells are strongly attached to the dermis in the condensation and are torn free rather than merely lifted out, whereas there are no signs of strong attachment of cell processes to the basement membrane in intercondensation regions. (In the region immediately lateral to formed condensations, where a band of scattered cells remains adherent to the basement membrane, the situation may be different. The appearance of these cells suggests that they may not be tightly bound to their neighbours. Thus, though it is quite possible that they are bound especially strongly to the basement membrane, it is equally possible that they are lifted free from relatively non-cohesive dermis.) Since, in the larger groups of cells on the basement membrane, many cells are attached only to their neighbours it appears that cell-cell attachment is also locally strong within

condensations.

Are these interfaces of strong attachment really sites of strong cell adhesion? The first point to make here is that adhesion between basement membrane and condensation is locally strong even in the absence of trypsin. The groups of cells attached to the basement membrane in trypsin experiments therefore do not merely reflect a lack of penetration of the enzyme into the dense condensation tissue or qualitative regional differences in enzyme-sensitivity of attachment. It is certainly possible that differences in the rate of penetration of trypsin between loosely, and closely, apposed surfaces retards its action in the condensation, thus accentuating patterns of adhesion in the dermis. This effect may even be responsible for the observed 'strong adhesion' within large groups of cells on the basement membrane, for strong cell-cell adhesions have only been demonstrated after applying trypsin. On one hand, there is evidence against this interpretation. The results of in vitro studies suggest that trypsin permeates cells quite freely and acts on the cytoskeleton, causing the cells to round-up, rather than acting directly on adhesive contacts (Revel, Hoch, and Ho, 1974; Whur, Koppel, Urquhart, and Williams, 1977; Rees, Lloyd, and Thom, 1977). SEM observations suggest that intercondensation cells are indeed more rounded after the trypsin treatment used here than cells in untreated specimens (compare Figs. 5.15c and 5.19); however, most cells do not round-up completely, but remain quite flat or elongated, suggesting that the effects of treatment are mild. These arguments would suggest that the patterns of strong attachment between cells, observed in trypsin-treated specimens truly represent differences in adhesion. On the other hand, this line of argument does not immediately explain why the tissues are more easily

separated after trypsin treatment. Perhaps connections between cell body and adhesive sites are made more tenuous by enzyme treatment and are thus more easily broken. Whether, or not, the susceptibility of cell-cell attachments in condensation and intercondensation dermis differ merely because trypsin more rapidly makes these connections tenuous in the latter than in the former, and whether, or not, this bears a meaningful relation to the ease with which adhesions are broken naturally between mobile cells, remains to be firmly settled. Some insight into this point, and into the more important question of how frequent are adhesive contacts in the different regions of the dermis, might be gained by TEM examination of early stages of condensation in specimens treated with trypsin and in untreated controls.

There is no indication in these experiments of any mesh of fibres and cell processes between cells and the basement membrane such as is observed in histological sections (Section 2). It is not clear whether, or not, this material has been removed by the trypsin. Cells in condensations appear to be attached directly to the amorphous membrane. The observation that cells are attached to the membrane by processes similar to those observed in monolayer culture suggests that they actively attach to the membrane and are not merely passively packed against it.

The strength of adhesion between dermis and basement membrane in the absence of trypsin, taken with the greater density of cells at the surface of the dermis in intercondensation regions than in condensations in cultured skin, implies that the number of attachments per cell, or the strength of each attachment, to the basement membrane is greater in the condensation than in intercondensation dermis. The close contacts between dermal cells and

the basement membrane, which ultrastructural studies of in vivo material show are most frequent in condensations (Kischer, 1968; Sengel and Rusaouen, 1969), presumably reflect these adhesions. This evidence would suggest that the number of adhesions per cell is greatest in the condensation.

Since adhesions are demonstrated in the present experiments by separation of existing structures the results provide no information on the possible adhesiveness of individual cells as they come together during the formation of the condensation (the differences between adhesion resisting separation and initial adhesive interactions have recently been discussed in a review by Grinnell (1978)).

In conclusion, the evidence presented here indicates that during the morphogenesis of the condensation cells become strongly adherent to the basement membrane and possibly also to one-another. These strong adhesions are first detectible only after the condensation has begun to form, though the possibility remains that more sensitive methods would reveal adhesions between cells in smaller groups at earlier stages. Evidence on the variation in the size of groups of cells remaining on the membrane after dissection suggests that strong adhesions are confined at first to cells at the centre of the condensation, but with time are formed over a greater area of the basement membrane and perhaps a greater depth in the condensation.

The rôle of these strong adhesions in primordium morphogenesis is discussed in Section 7 in the light of the other results presented in this chapter.

The purpose of this section is to draw together conclusions which arise from the foregoing parallel studies and to briefly discuss these with specific reference to the feather system. These conclusions are discussed in a wider perspective in relation to the development of other organ primordia in Chapter 6.

Two main mechanisms have been proposed to account for the development of dermal condensations: a local increase in cell proliferation (Wessells, 1965), and matrix-directed migration of cells within intercondensation arrays into condensations (Stuart et al, 1972; Garber, 1976). The present evidence suggests that neither of these mechanisms is primarily responsible for condensation development. Quantitative results indicate that the development of recognisable condensations in culture is not dependent on differences in the rate of cell proliferation and strongly suggest that proliferation alone plays a minor part in accounting for the increase in cell numbers in the condensation. Experiments using colcemid indicate that proliferation is not necessary for overall changes in primordium shape during elevation and feather-bud formation. Evidence that movement of cells along arrays is not a major mechanism in the development of the condensation is as follows. The morphology of arrays at their intersections and their relations to condensations in vivo suggest that they play a structural rôle rather than act as pathways of cell movement. Evidence from films also suggests that cells in arrays are not migratory. Experiments using colcemid indicate that elevation and probably thickening of condensations occurs in the absence of arrays. The fact that well defined condensations formed in culture in a few specimens where no arrays were

observed (Chapter 3) bears further evidence against the essential involvement of these cell patterns. These conclusions are in accord with the published evidence reviewed in Chapter 1.

The present evidence is too limited to allow an unambiguous interpretation of condensation morphogenesis in terms of proven cellular changes. However, discussion of the available evidence highlights some of the problems and leads to useful hypotheses within which to frame questions for future investigation.

The results presented in this thesis indicate that cells move into condensations from the surrounding dermis. Why do cells move into foci of condensation? Ede, Hinchliffe, and Mees (1971) suggested a chemotactic mechanism. Wolpert (1969) has suggested, in more general terms, that cells 'interpret' their position within a continuous spatial gradient of some parameter (positional information; hence, p.i.), and that cell movement is a response to p.i. which leads the cell to a place appropriate to its specific prospective position (Wolpert, 1971). Either of these two mechanisms might operate by eliciting preferential extension of locomotory cell processes in the direction of the condensation or increasing their strength of adhesion to the substratum or their force of contraction in this direction. The present evidence does not exclude these possibilities.

The results presented here do, however, suggest that short-range centripetal movement (over 1 to 2 cell lengths) would, in principle, account for the increase in cell numbers in condensations in cultured skin. There is no good evidence that longer-range migration occurs. It is therefore suggested that the development of a condensation depends primarily on changes in the non-proliferative behaviour of cells at and close to the condensation site.

The main possibilities under this hypothesis are based on acquisition of new contractile or adhesive properties by cells, first at the centre of the condensation site, then over a radially increasing area. One possibility is that contraction of cell processes may pull cells together. It is noteworthy that the earliest cellular change at the centre of the condensation is rounding-up of a small cluster of cells. Whether, or not, this change in cell shape is associated with active filopodial contraction remains to be established.

A second possibility is that cells at the condensation site become more adhesive to one another than to cells of the surrounding dermis. Stuart et al (1972) suggested that specific cell-cell adhesion might play a part in cell aggregation at the condensation site and that this might be similar to the tissue-specific adhesion which has been studied in chick neural retina cells by Moscona's group. Moscona and co-workers have subsequently identified particular cell surface protein(s) by electrophoresis and have implicated these in retina-specific cell adhesion using immunological blocking methods (McClay and Moscona, 1974; Moscona 1977) while others have studied their activation and interaction (Rutishauser, Thiery, Brackenbury, Sela, and Edelman, 1976). The view taken here differs from that of Stuart et al in that local adhesion is considered sufficient to account for all the cell movement involved.

If cell aggregation does occur by adhesion, whether specific or not, close packing of the cells might suggest that adhesion is quantitatively increased in the condensation. Ultrastructural evidence (Kischer, 1968; Sengel and Rusaouën, 1969) which indicates that cells in mature condensations in vivo are in

contact over a much greater area of their surface than are cells in the surrounding dermis suggests that at least some of this increase in contact may be due to an increase in the adhesive area of the cell surface.

Steinberg's differential adhesion hypothesis (DAH) (reviewed by Steinberg, 1975) would therefore seem appropriate to explain aggregation. This hypothesis is based on the apparent similarity of tissue masses to liquid phases (isolated pieces of tissue round-up within a few hours; cells sort-out from mixed aggregates like separating liquid phases). The DAH proposes that the mutual adhesiveness of homotypic cells varies quantitatively between different cell types and is a fairly homogeneous property of the cell surface. It proposes that adhesive interaction can be treated as a reversible process in thermodynamic terms, so that aggregation of homotypic cells is viewed as the displacement of less stable adhesions by more stable ones, leading to minimisation of the interfacial free energy over the whole cell pattern. The hypothesis has been applied primarily to cell sorting-out in experimental, mixed aggregates, but is also intended to account for in vivo cell movements.

Harris (1976) has argued, however, that several assumptions of the DAH are unlikely to be met: two points made by Harris are of particular interest here. First, cell adhesions to planar substrata in vitro are confined to small areas of the cell surface (Harris, 1973b) and may be mobile. How far evidence relating to cells moving continuously over flat, artificial substrata can be extrapolated, without modification, to explain the interaction between cells in aggregates may be questioned. The relation of localized, morphologically distinct junctions to the initial adhesive interaction between aggregating cells is complex and not at present

clear (Overton, 1975, 1977). Nevertheless, it is fair to say that specialized junctions, such as desmosomes, do contribute to cell adhesion and Harris's argument that cell aggregation is not merely a consequence of an increase in the total area of cell surface contact is worth considering. Harris suggests that aggregation could, for example, depend on controlling the distribution and mobility of adhesive sites. Willingham, Yamada, Yamada, Pouysegur and Pastan (1977) have shown that cell spreading and adhesion on in vitro substrata, in a variety of cell types and after a range of treatments affecting cell behaviour, consistently correlate with the development of a system of microfilament bundles in the cell. Trotter, Foerder and Keller (1978) showed that sites of cell substratum adhesion in vitro are situated where microfilament bundles meet the sub-surface layer of filaments. This and other evidence supports the view put forward by several workers (for example, by Rees et al, 1977) that the distribution of adhesive sites may be controlled by the cytoskeleton, and that this control plays an important part in determining the adhesive properties of the cell. The cytoskeleton comprises microtubules, actin-containing microfilaments, and 10 nm filaments, which appear to form a closely integrated system responsible for the development and support of cell shape and probably play a part in contraction and possibly in determining the distribution of organelles and the position of the nucleus (Trotter et al, 1978, provide a useful review of the most recent evidence in this field). Thus, the effective adhesiveness of the cell may be closely related to its contractile properties and shape through the organisation of the cytoskeleton.

The second point raised by Harris is that breakage of adhesions is not merely the reverse of the process of the initial

adhesive contact between cells. For example, adhesions may be secondarily stabilized covalently so that the work of de-adhesion may not be equivalent to the work of adhesion. Thus displacement of one adhesion by another would not simply depend on the relative initial adhesiveness of the cells. If aggregation involves repeated making and breaking of adhesions, the strength of secondary adhesion will be important. In fact, the direction of movement does seem to be determined by competition between contractile cell processes which extend in different directions and adhere to the neighbouring cells of the matrix. Evidence for this mode of cell movement comes both from detailed in vitro studies of cells on substrata of variable adhesiveness (Harris, 1973a) and from morphological observations of the dynamics of cell movement in vivo (Gustafson and Wolpert, 1961; Trinkaus, 1973; Izzard, 1974; Bard and Hay, 1975). The direction of net cell movement may thus depend either on the relative strength of adhesion of different filopodia to their respective substrata or on the differential contraction of filopodia in the direction of the condensation.

Harris (1976) also suggested that differential cell surface contraction may account for cell behaviour in mixed aggregates, but, as an explanation of the aggregation of cells into feather condensations, this seems unlikely on many grounds: for example, it is difficult to explain the pattern of cells at the periphery of the condensation in this way.

These considerations illustrate the difficulties of formulating specific hypotheses which are based on differences in cell adhesiveness or contraction to explain the condensation process in the feather system. All that can be said is that these aspects of cell behaviour seem likely to be involved in cell aggregation and

packing. Direct evidence of changes in adhesiveness or in the contractile properties of the cells is lacking. As a guide to further work, however, several general hypotheses might be tested: a) cell-cell adhesiveness at the prospective condensation site may increase leading to trapping of moving cells; b) cell-basement membrane adhesiveness may be locally strong at the prospective condensation site leading to cell trapping during the initial stages of condensation; c) changes in cell behaviour may begin in a small group of cells at the centre of the condensation site, immediately below the basement membrane, and spread radially as development proceeds; since these changes need occur only over a limited area, the acquisition of new properties may be transmitted by cell contact; d) cell packing may be a consequence of adhesion, either generalized over the whole cell surface, or in localized junctions. To emphasize the preliminary nature of these hypotheses involving adhesion it is worth noting that, for example, cells may alternatively become packed in the condensation because they increase in volume (Section 3) and because the population is expanding by proliferation. Changes in the intercellular matrix might also be invoked as a primary cause of condensation.

The crucial point regarding hypotheses (a) and (d) is whether properties of the free cell surface change to make it potentially more adhesive as a prelude to condensation, or strong adhesions form only after the cells are packed (Section 6). Further evidence on this point may be gained by assaying the adhesiveness of dissociated cells using methods such as those of Roth, McGuire and Roseman (1971); Hornby (1973); Walther, Öhman and Roseman (1973). However, the qualities assayed by these tests are not necessarily those of prime importance in morphogenesis

in vivo. These assays should therefore be combined with studies on the behaviour of the cells, for example, migrating from pieces of condensation and intercondensation tissue in vitro, by a method analogous to that used by Cairns (1975) investigating limb-bud mesenchyme. Ultrastructural evidence may be sought regarding the area of surface contact and the frequency of specialized junctions between non-packed cells near the periphery of very early condensations to assess whether adhesion or packing is responsible for close surface apposition. Cell adhesion to the basement membrane (hypothesis (b)) may be a reflection of specific or non-specific cell adhesiveness, or may depend on specific properties of the basement membrane. To test the adhesive properties of the basement membrane cells might be seeded onto the exposed dermal surface of the membrane and their patterns of movement observed. The adhesive properties of the membrane may explain the ability of well-formed placodes to organize dermis into condensations in experimental tissue combinations (Sengel, 1958; Linsenmayer, 1972; Novel, 1973). To test if the process of condensation depends on cells adhering to the membrane the morphological development of condensations may be examined after the epidermis and basement membrane have been removed. Preliminary experiments indicate that this is feasible and suggest that condensations continue to develop for up to 12 hours. Finally, the hypothesis that changes in behaviour are mediated by cell contact might be investigated by cutting skin to expose condensations and the surrounding dermis and then explanting the skin onto a sub-confluent monolayer of dermal cells and filming the reaction of individual cells to contact with those of the condensation or intercondensation dermis.

Cells may move individually into the condensation or the tissue may show mass contraction towards the focus of condensation. Since most of the cells are in extensive contact with their neighbours (Wessells and Evans, 1968) one might expect the phenomenon of contact inhibition of movement (CIM) to severely restrict the movement of individual cells. Abercrombie and Heaysman (1952) showed that the speed of movement of chick heart fibroblasts in vitro is reduced by a contact interaction between the cells. CIM has since been studied in detail in vitro (reviewed by Abercrombie, 1970; and Martz and Steinberg, 1973), and has been demonstrated in vivo in mesenchymal cells colonizing the chick corneal stroma (Bard and Hay, 1975). Abercrombie (1970) has pointed out that, in fibroblast cell networks, the movement of individual cells is probably subject to CIM, whereas mass contraction of the tissue is not. However, caution must be exercised in extrapolating to unknown situations in vivo. Moreover, evidence from studies on the intermingling of cells from experimentally fused tissue masses suggest that, in some cases at least, mesenchymal cells translocate over moderate distances through fairly dense tissue (Armstrong and Armstrong, 1973) although this may not be a general rule (Weston and Abercrombie, 1967). Direct evidence for this point may be obtainable in the future by using improved methods of filming condensation development (Chapter 2). If, as suggested above, aggregation occurs over only short distances, and is contact-mediated, then the movement of individual cells could probably still account for condensation over the time observed, despite the operation of CIM.

Either mass tissue contraction or individual cell movement towards the focus of condensation might be expected to set up tensions in the dermis. Studies of cell movement in vivo

(Izzard, 1974; Trinkaus, 1973) have shown that, in many cases, when a cell moves in one direction by the contraction of its filopodia, adhesions to neighbouring cells and matrix in the opposite direction are not released smoothly but tend to pull the tissue in the direction of movement. It seems possible, therefore, that movement into the condensation will create tensions in the matrix which might help to explain patterns of cell orientation in the surrounding dermis as argued in Section 2. As the embryo grows, the skin may stretch and separation of condensations will tend to enhance these contraction-generated tensions. This would be expected to increase the degree of cell orientation between condensations. The fact that arrays are less well organized in culture than in vivo may be partly due to the absence of growth. Difficulties in the tension hypothesis as a complete explanation (Section 2) suggest that other factors may play a rôle in organizing arrays.

Why are dermal condensations stable? Though it has been known for some time that cells are not lost to the surrounding dermis, nor do surrounding cells infiltrate the condensation core, (Wessells, 1965) the cause of stability has received no attention. Evidence from films (Section 4) suggests that cells in the condensation are not paralysed. The fact that the shape of the condensed cell mass changes during morphogenesis without apparent deformation of the cells suggests that cells might slip past one another rather freely. That cells can move in tissues where the density is intermediate between that found in condensations and in inter-condensation dermis is suggested by the experiments of Armstrong and Armstrong (1973). If cells are free to jostle and slip past one another why do they not disperse from the condensation? Of the several possible explanations a quantitative increase in cell-cell

and cell-basement membrane adhesiveness is the simplest, and is the only one for which there is positive evidence (Section 6).

The forces responsible for the change in shape of the primordium have also received little attention in the feather system. From the point of view of changes in shape of the dermal condensation this represents a partial rounding up of the cell mass. This may be analogous to the rounding up of pieces of tissue or cell aggregates in isolation or in contact with heterotypic tissue which has been attributed to adhesive forces tending to maximise the area of homotypic cell surface contact (Steinberg, 1975) or to contraction of the cell surface at the periphery of the aggregate which is in contact with heterotypic tissue or the surrounding medium (Harris, 1976). Either of these general hypotheses might account for the morphogenesis of the feather condensation. It is also possible that the shape of the condensation may be moulded by the folding epidermal sheet; cell-basement membrane adhesion may play a part in this process. This latter possibility may be tested by examining the morphogenesis of condensations in dermis from which the epidermis and basement membrane have been removed.

How does the development of the condensation relate to primordium morphogenesis as a whole? Apart from the direct physical interactions between dermis and basement membrane discussed above, the present experiments provide no evidence about the mechanisms of tissue interaction. However, interaction with the epidermis is required for condensation formation (as indicated by the failure of development in the scaleless mutant, see Chapter 1 for review) and the epidermis is, at least in part, responsible for the synthesis of the basement membrane (Dodson, 1967). Development of the dermis may thus be causally linked to the activity of the epidermis through the adhesion of dermal cells to epidermis-controlled

components of the basement membrane. The observation that dermis-basement membrane adhesion is unusually low in tarsometatarsal skin of the scaleless mutant during the period shortly after the scale ridge is initiated in controls (Sawyer and Abbott, 1972) supports this hypothesis.

Whatever the mechanisms of tissue interaction it is interesting to note the similarity between changes in cell behaviour in the dermis and epidermis in the developing primordium. In both cases, there is evidence of a transient cessation of mitotic activity and DNA synthesis, an increase in cell volume, and an increase in the area of cell-surface contact associated with changes in cell shape which are initially most marked near the centre of the primordium. One might speculate that these changes reflect similar alterations in cell structure and behaviour in the two tissues, for example, changes in cell surface structure leading directly to an increase in the area of cell surface contact. In the two-dimensional epithelial sheet this would lead to cell elongation and the formation of a placode, in the three-dimensional network of the mesenchyme the same changes would lead to the formation of a condensation. If these properties were acquired by cells first at the centre of the primordium site in the epidermis, then transmitted radially through the epidermis, and if similar changes were subsequently induced in the dermis beginning below the centre of the placode and again extending radially to other cells, this simple pattern would account for the sequence of morphological development and the overall form of the primordium. This scheme is also in accord with the suggestion, arising from evidence on the feather pattern, that primordium centres are defined first. Whether, or

not, such a simple scheme plays a part in the undoubtedly complex process of primordium morphogenesis remains to be investigated.

CHAPTER 6

GENERAL DISCUSSION

The results presented in Chapter 4 suggest that the onset of morphogenesis is under the control of a locally set timing mechanism at each medio-lateral position throughout the pteryla so that the sequence of morphogenesis may be thought of as controlled by a time map. In this section the properties of this time map and its relation to timing mechanisms which operate in other systems are briefly discussed.

The precision with which the time of morphogenesis of each row has been measured is not greater than the time between the formation of successive rows. Moreover, the time of completion of the whole pteryla has only been measured approximately in vivo and in culture. An estimate of the actual precision of the time map itself must therefore await further investigation.

The results suggest that the time map is continuous (within the limits of resolution of the results): that is, primordia can form at any position on a continuous gradient of time in space. This is indicated by the fact that primordia form at regular, but different, intervals in vivo and in culture after the map is set.

Since the map is set over most of the pteryla before Stage 29+, and since the skin grows between this time and the completion of morphogenesis in the pteryla, the gradient of timing must also grow. Since the skin expands by interstitial growth, this implies that the time map is complete, so that the time at which the lateral primordia will form is already pre-set in the lateral regions of the pteryla at Stage 29. Thus, in culture, where no lateral growth occurs, primordia should initiate more slowly than in vivo and the most lateral primordia should form at

approximately the same time as the most lateral primordia in vivo. It will be important to test these predictions, because the present results do not completely rule out other interpretations (for example, that regulation and subsequent propagation of competence occurred lateral to the cut in the present experiments).

The results described in Chapter 3 indicate that development of successive rows in culture certainly takes longer than in vivo. A rough test of the above predictions, based on the present data, suggests that the pterygia is complete both in vivo and in culture around 45 to 50 hours after Stage 29. This could, of course, be a coincidence unrelated to the time map and merely reflecting the less than ideal conditions pertaining in culture. More precise information will be required to establish this point. The temperature dependence of the time of morphogenesis in vivo and in culture may provide a simple initial test of whether the coincidence depends only on culture conditions since it is unlikely that limiting deficiencies in the culture system will result in the same temperature coefficients as the normal time map.

A time map which is complete from a relatively early stage in development would have the interesting property that, once the map is set, the relative timing of morphogenesis would be independent of variations in growth rate.

The present results provide no definite indication of when the time map is set nor do they indicate how this is accomplished. The simplest possibility is that the map is set sequentially in a medio-lateral direction, but it is alternatively possible that the time is set simultaneously over the whole pterygia against a background of some pre-existing graded property.

How is the time kept? The time assayed in these experiments is, of course, not absolute, but is measured by comparison of the progress of development in different parts of the pteryla. Moreover, since the setting-up time is not known, we have no information regarding how long the local time is kept. This latter factor may determine the precision of local timing relative to time in neighbouring regions. Local variations may lead to irregularities in the time gradient, though it is probable that these would be smoothed out by local interactions between neighbouring regions.

One can imagine four broad categories of timing mechanism: first, an oscillator linked to a counter; second, a regular, continuous degradation process starting with a fixed amount of substance and running to a threshold or zero concentration; third, a similar accumulation process running to a threshold concentration; and finally, a linear sequence of dependent events. There is no evidence to favour any of these possibilities and indeed they will be difficult to distinguish experimentally. The possibility of an oscillator might be tested by attempts to perturb or entrain it (Winfree, 1975) using brief or periodic temperature treatments.

At the end of the time period competence to form feathers would be triggered or simply attained. According to the evidence discussed in Chapter 1 the attainment of competence is presumably associated with the formation of dense dermis and may arise primarily through the attainment of a sufficient cell population density. Possibly, the completion of the time period results directly in the division of a proportion of the cells.

Temporal control is obviously an important phenomenon throughout development and it is likely that the timing of many key developmental events is quite closely controlled. However,

the control of timing is less obvious for single events than for repeated ones, for example the formation of successive, equivalent organ primordia. One example of a well-defined single event which has attracted interest in the timing mechanism is the necrosis of tissue in the superficial mesoderm at the pre- and post-axial borders of the chick limb-bud and between the digits. Fallon and Saunders (1968) studied the control of cell death in the mesoderm from the posterior necrotic zone (PNZ) of the limb-bud. Cell death occurs in PNZ mesoderm in culture, synchronously with control mesoderm in the chick at Stage 24, after it is removed from the chick at any time between Stage 17 and 23. Fallon and Saunders explored the effects of temperature and association with wing mesoderm on the time of necrosis, but their results do not allow one to distinguish between the different possible physiological timing mechanisms. They do, however, show that the timing mechanism is intrinsic to small pieces of tissue and perhaps to individual cells.

One of the most thoroughly studied examples of a repeated event in development under close temporal control is the formation of somites in the paraxial mesoderm. Cooke and Zeeman (1976) have reviewed the relevant literature relating to this system and the evidence need not, therefore, be described in detail here. The main points are that the number of somites is maintained by embryonic regulation after early disturbances such as tissue excision, but that later in development the number of somites and the time of their formation are fixed. Large and small embryos develop somites synchronously over each proportion of their length, and morphogenesis proceeds with normal timing beyond a cut made relatively late in development. Thus, there appears to be a complete time map for morphogenesis similar to that suggested here for the feather system.

Cooke and Zeeman proposed a model to account for the development of repeated structures in animal morphogenesis where the number of structures is controlled independently of the size of the organism. The essential features of their model are worth considering here because this system shows striking similarities with, and crucial differences from, the feather system. Cooke and Zeeman's model comprises two main components. First, a time map or gradient which controls the sequential attainment of competence to form somites antero-posteriorly along the axis. This is set early in development by direct relation to a gradient of positional information which has the regulative properties attributed to positional information gradients by Wolpert (1969). After the time map is set, timing runs out at successive positions down the axis initiating a rapid cellular change associated with competence to form somites. This rapid change is thought of as a 'wavefront' passing down the embryo. To explain the precise periodicity of somite formation, all the cells in the paraxial mesoderm are supposed to share a synchronous oscillation. The oscillator in each cell interacts with the passing wavefront in such a way that the passage of the wavefront is alternately slowed and accelerated, thus gating the possibility of rapid cellular change. Thus, groups of cells attain competence nearly synchronously and sufficiently separate in time from neighbouring groups so that discrete somites are formed. There is, therefore, no direct interaction between formed and incipient somites. Although the dependence on a gradient of positional information gives the system the capacity to regulate, once the time gradient is set the position of each somite is predictable.

One basic difference between somite and feather systems is that the position of feathers is not determined until some time after the timing mechanism is set. Moreover, the positions of feather primordia appear to depend, in part, on local interactions between adjacent primordia. Thus, there is no indication that periodicity depends on an oscillator in feather pattern formation.

However, the similarity of temporal controls in the two systems raises the question of whether a common type of timing mechanism exists for the control of these, and similar, developmental processes, which may be independent of the processes themselves, or time keeping is merely a consequence of close control of the developmental sequence unique to each system. Analysis of time control in other systems may help resolve this question. The development of the vertebrate dentition pattern (Edmund, 1964) may be a good candidate for further examination.

## 2. THE POSITIONS OF FEATHER PRIMORDIA

### 2.1 Introduction

The results presented in Chapter 4 suggest that the positions of primordia may be influenced, on one hand, by organisation which predisposes the direction or position of chevrons and, on the other hand, by local interaction between primordium sites close to the time of morphogenesis. Evidence for the former control is less strong than for the latter.

In this section these contributing factors are discussed in relation to the evidence reviewed in Chapter 1 and to published studies on the patterns of other integumental appendages.

### 2.2 Anterio-Posterior Positions of Initial Primordia and Chevrons

Most of the evidence relates to the spinal pteryla and discussion will be confined, at first, to this region. It is generally argued that the position of the first primordium in the pteryla (or the first in each of the larger individual tracts) is defined by a special, but unknown, mechanism. Several authors have suggested that the position of subsequent primordia is determined autonomously in the skin by some mechanism which spaces out repetitive units in initially bland tissue (Ede, 1972; Novel, 1973; Sengel, 1976b). This view is to be contrasted with the possibility that the position of each initial primordium is individually determined in direct relation to the axial segmentation of the embryo. These two extreme views form the framework for discussion in the present section.

The only direct investigation of the influence of axial segmentation on the position and number of primordia in the initial

row was made by Mauger and Sengel (1970) who concluded, from their fate-maps of the spinal pterygia, that the pattern of mesenchymal cells of different somitic origin is so irregular along the spinal integument that regularity in the feather pattern must arise secondarily. Mauger and Sengel reported a one-to-one relation between primordia in the initial row and the somites in the lumbo-sacro-caudal region. However, these authors concluded, on the basis of their mapping experiments and the fact that a one-to-one relation does not pertain in other regions, that the correspondence between somites and primordia in the posterior tracts of the pterygia is "fortuitous". Direct inductive influence of the underlying embryonic structures on the positions of primordia is ruled out in the case of the thigh pterygia (Linsenmayer, 1972), though evidence for the same conclusion in relation to the posterior regions of the spinal pterygia (Novel, 1973) is less strong. These lines of evidence are reviewed in detail in Chapter 1. On the basis of a simple packing mechanism proposed for pattern formation in the lateral parts of the pterygia it seemed unnecessary to invoke special mechanisms for spacing primordia in the initial row.

There remains, however, the problem of how the position of the first primordium is defined. Moreover, the evidence that the antero-posterior position of primordia is defined entirely by a simple packing mechanism in the skin is weak. The fate-map data appears to be contradictory (as reviewed in Chapter 1). The method of carbon-particle marking is notoriously imprecise when applied over long developmental periods (see, for example, Summerbell and Lewis, 1975). The present results lead to the suggestion that positions of primordia in lateral areas of the pterygia are not defined by a simple packing mechanism alone. The possibility that

the positions of chevrons, and thus of initial primordia, might be partly determined by pre-existing organisation suggests that there is a need to re-examine the interpretation proposed by Mauger and Sengel. Evidence on related integumental patterns, which have not been previously considered in the literature on the feather pattern, is relevant here.

Is the one-to-one correspondence between initial primordia and somites fortuitous? The number of primordia involved, and the precision of correspondence makes it unlikely that this relationship arises by chance. Examination of the careful drawings made by Lucas and Stettenheim (1972) of pterylae in several other species of fowl suggest that a one-to-one relationship could only hold, at most, over only limited areas of the dorsal skin, thus allowing the possibility that any such relationship is fortuitous. However, examination of ancestral birds provides good evidence to the contrary.

Archaeopteryx, which is the earliest known ancestral bird (Heilmann, 1926) most probably evolved from small, swiftly-moving dinosaurs (Ostrom, 1976). The bird possessed a long Reptilian-type tail which has been lost in the evolution of present-day birds, probably by fusion of most of the original post-pelvic vertebrae (Heilmann, 1926). A one-to-one relation holds between successive pairs of para-medialfeathers and the vertebrae over most segments of the tail in these primitive birds. This was noted in 'Archaeornis' by Heilmann (1926) and is also clear from examination of a cast, in the Royal Scottish Museum, of the British Museum (National History) specimen of Archaeopteryx: each of the seven most posterior vertebrae is associated with a pair of feathers.

Feathers evolved from Reptilian corneoscutes and at least some aspects of the pattern-forming mechanism have apparently been

conserved in evolution (Chapter 1). It is therefore relevant to consider the positional relation of corneoscutes to vertebrae. This provides additional evidence for a common one-to-one correlation. Of all extant reptiles, the Crocodylia bear the closest skeletal resemblance to birds (Walker, 1972; Regal, 1975). Several Crocodylian reptiles show a strict one-to-one correspondence between vertebrae and corneoscutes over much of their body length (Romer, 1956). The same is true of several species of lizard (Romer, 1956).

This evidence makes it clear that correspondence between vertebrae and integumental appendages is common in the evolutionarily related species and is therefore almost certainly not fortuitous in the chicken. Instead, it seems that the mechanism governing the relationship between these structures has the potential for precise control of their register over many successive body segments. The evidence does not, however, prove a causal relation between the development of individual feathers (or corneoscutes) and somites. A reasonable alternative explanation is that the strict correlation is maintained through close control of autonomous mechanisms of appendage pattern formation in the skin, without causal links to the development of each body segment. It is conceivable that such a mechanism is the feather system is a legacy from the correlation between corneoscutes, osteoscutes and vertebrae found in several reptiles (Romer, 1956). Osteoscutes, or 'bony scales', are more or less massive membrane-bones in the dermis which provide armour in many Reptilian groups, and such a correlation would have adaptive value in giving strength and support to the integumental armour.

There is no direct evidence to distinguish critically between these explanations. Experiments on the feather system, described in Chapter 1, in which the embryonic axis is irradiated

close to the time of somitogenesis or lengths of somitic mesoderm are replaced with other tissue, create transverse strips of bare skin in the spinal pterygia. However, they do not give sufficiently precise results to determine whether or not the number of primordia which are absent bears a constant relation to the number of somites destroyed. Moreover, in this type of experiment, the absence of feathers probably reflects the lack of competent feather-forming material rather than specifically indicating the absence of information which might determine the formation of primordia in particular positions. Yntema (1970) has made similar experiments to examine the effect of excising dermatomal material from successive groups of somites (including neural tube and neural crest material in the excision) on the development of the horny epidermal scales in the carapace (dorsal shell) of the turtle Chelydra serpentina. These experiments gave similar results to those made on the feather system, except that the somitic material adjacent to the operated region failed to regulate as it does in the chick (Chapter 1, page 18). They share the problems of interpretation described above.

It is therefore necessary to resort to indirect evidence in examining the relative plausibility of causal or adaptive relationships between epidermal appendage position and axial segmentation.

Since the corneoscute and feather patterns are defined by the dermis (Chapter 1) it is probably relevant to consider the pattern of dermal osteoscutes and its relation to axial segmentation. There is evidence of a strict one-to-one relation between dorsal, medial, osteoscutes and vertebrae in a large number of present-day and fossil Reptilian groups (Romer, 1956). One of the best examples is Nodosaurus (Fig. 6.1) where the relation holds true over extensive

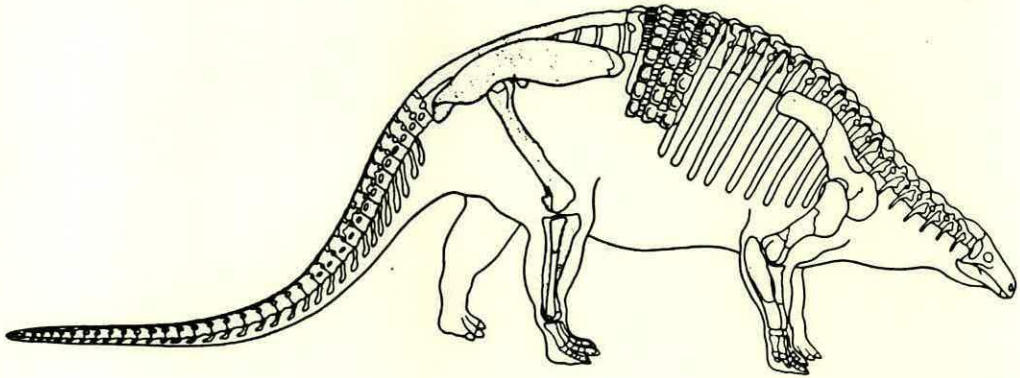


Fig. 6.1 Nodosaurus (From Romer (1966) after Lull)

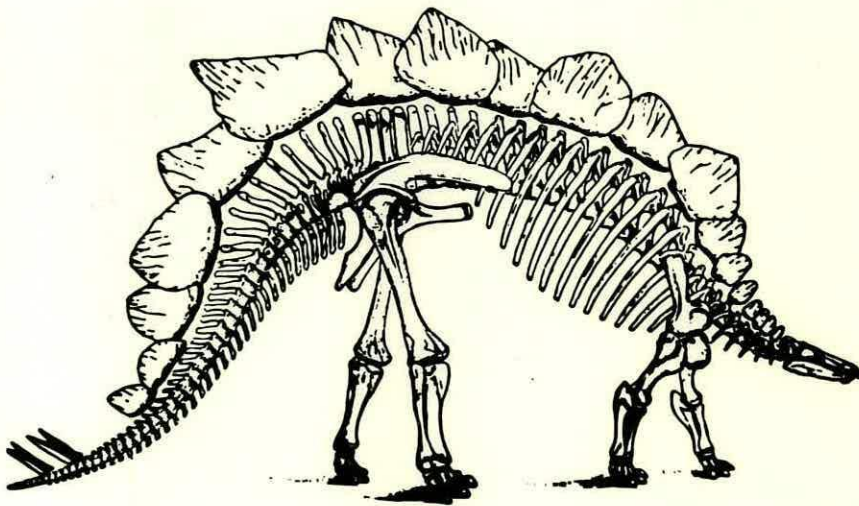


Fig. 6.2 Stegosaurus (From Romer (1966) after Marsh and Gilmore)

transverse rows of scutes. A one-to-one relation is by no means universal: the number of scutes is frequently reduced, for example in Stegosaurus (Fig. 6.2). However, most of the very primitive reptiles bore segmental osteoscutes (for example, Kotlassia (Romer, 1956)); moreover, on comparative palaeontological evidence, Zangerl (1969) has suggested that segmental osteoscutes were the primitive condition in turtles. Thus, such a relation may be the primitive condition in reptiles. This conclusion allows, but does not prove, that the relationship between segments and osteoscutes is a direct, causal one.

Zangerl (1969) has suggested that axial segmentation governs the position of medial osteoscutes in the central part of the turtle carapace. This interpretation is based mainly on the close morphological association in this region, between the ribs and initial centres of ossification of the forming bony plates (Kalin, 1945). However, in turtles, and other reptiles where osteoscutes form an intimate physical relationship with the axial skeleton, the pattern-determining mechanism may be a direct physical one rather than the sort which might be of relevance to consideration of the corneoscute pattern.

It is clear that, even where a strict one-to-one relation holds in the general pattern, variation can occur. Zangerl (1969) has noted the existence of repetitive variants in the turtle osteoscuta pattern. These are particular variants of local aspects of the pattern which are found with a frequency characteristic of the species and genus. Repetitive variants of the medial osteoscuta pattern occur with especially low frequency over the central region where the dermis is closely associated with the axial skeleton. The low frequency of these variants lends support to the

suggestion that here the osteoscutate pattern is under tight control. Nevertheless, the fact that variation is found in this region is telling. Variation in the one-to-one relation between osteoscutates and vertebrae has also been reported in detailed accounts of individual fossil specimens of other reptiles: for example, in Euparkeria the number of para-medial osteoscutates can be locally unequal to the number of vertebrae, although they show approximately equal spacing (Ewer, 1965). The existence of variants clearly suggests that even if there is segmental control it is secondary to mechanisms responsible for the periodic nature of the pattern and the approximate spacing of the osteoscutates.

Even if a relevant causal relation between osteoscutate positions and segmentation were found to hold, we would still like to know if corneoscutate and osteoscutate patterns were related. Limited evidence on this score comes from the correlation of corneoscutate and osteoscutate patterns in the turtle. Parker (1901) was the first to emphasise a possible correlation between the mechanisms responsible for these two patterns in his report of two abnormal specimens of the sculptured tortoise Chelopus insculptus. This evidence is not, however, unambiguous. Zangerl (1969) has suggested that the two patterns are independently controlled, but it seems wise to reserve judgement till more direct, experimental evidence is available.

One further line of approach provides a little more evidence. This derives from Zangerl's analysis of repetitive variants of the corneoscutate pattern in turtles based on his examination of 4050 individual animals. These variants comprise deletions, duplications, and additions of horny scales. The number of specimens

of each variant is limited even in this large sample, but in spite of this the data provides a rich source of information on the mechanisms of epidermal appendage formation.

Although corneoscutes of the turtle carapace are larger than the osteoscutes and do not show a one-to-one relation with the vertebrae, they do have a consistent and well-defined positional relation to both vertebrae and osteoscutes, despite differences in relative size between species (Zangerl, 1969). It is therefore relevant to ask if their position is influenced by the segmental organisation of the embryo. One might expect the most stringent influence on the corneoscute pattern where axial segmentation most influences the dermal osteoscute pattern: that is, over the eight segments most centrally situated beneath carapace. Inspection of the data on corneoscute pattern variants (Zangerl, 1969, Table 1) shows that variants are, indeed, less frequent over the central segments. This evidence does not, of course, prove that segmentation controls the corneoscute pattern. It does, however, suggest that the same local, tight control may be imposed on both osteoscute and corneoscute patterns. The apparently segmental nature of the organisation governing the osteoscute pattern leads to the tentative suggestion that the dermal organisation governing corneoscute position is also under the influence of axial segmentation. The fact that corneoscutes form well before the morphological development of osteoscutes and even before the growing ribs become closely associated with the dermis (Kálin, 1945; Zangerl, 1969) suggests that corneoscute position is not influenced by simple physical interaction with the axial skeleton. There is thus no reason to expect that control of the corneoscute pattern in turtles differs essentially from the control of corneoscute patterns in other

reptiles and it seems possible, on the basis of other similarities between corneoscute and feather pattern-forming mechanisms, that a similar control might operate in the feather system.

From the above arguments we may make the following conclusions. Correlation between appendage patterns and axial segmentation is almost certainly not fortuitous, but reflects a fairly stringent control which can maintain a one-to-one correspondence over many successive body segments. Such control may vary locally in its stringency and appears to be secondary to a mechanism which accounts for the essentially periodic nature of the pattern and for the approximate spacing of primordia. Several lines of evidence from Reptilian integumental patterns lead one to suspect that the relationship between appendage positions and body segments is a causal rather than adaptive one (in the sense defined above). The tenuous nature of the evidence for a causal relationship must, however, be emphasised: we cannot, at present, decide critically between the two possibilities.

Evidence presented in Chapter 4 on the curvature of newly formed chevrons suggests that the positions of primordia in lateral parts of the pteryla cannot be accounted for by a simple packing mechanism.

On the available evidence it is therefore worth taking seriously the possibility that two levels of organisation interact to determine the feather pattern. The first of these is the simple spacing mechanism based on local interactions which has been discussed widely in the literature. Coupled with the timing mechanism discussed in Section 1, this will account for a packing pattern which, in practice, would be a more or less regular diamond arrangement. It is suggested that this level of organisation is

coupled to a second which is laid down prior to the establishment of individual primordium sites and which influences the position of primordia more or less stringently. The available evidence says very little about this level of organisation. Only experimental analysis will establish firmly if such organisation exists and explore its nature.

At this stage, specific hypotheses are needed for experimental test. Two hypothesis may be considered; first, that the positions of primordia are influenced by the pattern in which cells from differently determined mesodermal regions colonise the integument; second, that in the spinal pteryla primordium sites are directly related to axial segmentation. The first hypothesis arises mainly from the need to explain chevron curves, the second from the need to account for the correspondence between vertebræ and initial primordia. The two are obviously related. It should be noted, however, that there is no apriori reason why chevron curvature is controlled by the same organisation as governs the positions of initial primordia, nor is there any specific evidence that regions of determination in the paraxial mesoderm co-map with somites.

The kind of effect envisaged in making the first hypothesis is that boundaries between differently determined cell populations, or geographical heterogeneity within populations (for example variation in competence) would influence the positions in which primordia form. For the former possibility, the boundaries would probably have to be quite sharp: this is discussed below. Chevron curves would be defined by the regional characteristics of dermatomal cell migration and subsequent integumental growth which produced curved boundaries, or simply a curved 'grain', in the prospective dermis in lateral parts of the pteryla. Regional

differences in the mesoderm have been shown to determine the size and spacing of the individual primordia, the size and shape of the pteryla, and possibly the direction of the chevrons (Mauger, 1972b), but the possible effects of mesodermal regionalisation on the positions of primordia have not been critically examined.

According to the second hypothesis, the position of the first primordia in the pteryla would not be determined by a special mechanism: instead, primordium spacing would be coupled to axial segmentation over several successive body segments.

The second of these hypotheses has already been considered by Mauger and Sengel (1970) who objected that an explanation of the precise position of primordia based on axial segmentation cannot explain the development of other pterylae. The first hypothesis allows this objection to be circumvented, for regionalisation does not depend on segmentation per se, though it may be linked to segmentation in the dorsal region: non-somitic mesoderm is also regionalised (Mauger, 1972b), and regionalisation precedes overt segmentation in the paraxial mesoderm (Mauger, 1972b). These hypotheses are consistent with the current evidence, particularly if one accepts the limitations of the published fate maps.

Investigation of the first hypothesis would involve construction of more precise fate maps (based on the origin of cells which normally contribute to different areas of the integument) using improved marking methods (for example, using tritiated thymidine or quail-chick heterospecific transplantation) and perhaps using data from heat shock experiments (see below). In particular, we need to know more about the geographical relation of the differently determined mesodermal regions to the well-defined regions of the feather pattern; we need to know how sharply demarcated and how

small and homogenous are the mesodermal regions.

A direct experimental test of the second hypothesis would ideally be to disrupt axial segmentation without destroying presumptive dermal material and to look for departures from the normal feather pattern. A suitable means of doing this may be to apply a brief temperature shock during somitogenesis. Experiments using Rana and Xenopus have shown that treatment with a brief heat shock prior to somite formation induces local failure of proper segmentation without affecting the ability of the cells to differentiate (Dr. T. Elsdale, personal communication).

A brief and highly speculative discussion of the significance of mesodermal regionalisation may help to place these ideas in a wider perspective.

Regional differences in the determination of the mesoderm are not, of course, confined to those which directly influence the feather pattern. Experiments by Sengel's group (reviewed briefly by Sengel, 1976b) show that mesoderm is also regionalised with respect to the development of the ribs and axial skeleton. We may therefore ask if signs of regional determination are expressed by other components of the skin. Diamond, Cooper and MacIntyre (1976) have suggested, on the basis of experiments on nerve sprouting in the amphibian limb, that nerves colonise non-overlapping territories in the skin. These territories are not bounded by obvious structural features, nor are they defined in the skin alone, but are apparently determined with reference to an overall body space (rotation of a piece of skin makes no difference to the actual outline of the territory). (Whether or not this space definition may be usefully thought of in terms of positional information (Wolpert, 1969) remains to be examined). One might speculate that differently

determined populations of mesodermal cells are restricted in their movement and growth in relation to the same system of space definition. It would be of interest to know if nerve territories exist in chicken skin and if these coincide with regional boundaries in the feather pattern.

Diamond et al suggest that the spatial restriction of nerve sprouting may be related to the phenomenon of compartmentalisation in insects which has been revealed by clonal analysis using somatic recombination methods. A compartment is a geographical space in an organism to which a population of cells, descended from a small group early in development, is restricted; cells in different compartments are differently determined, and determination appears to occur by successive binary commitments involving the activation of specific homeotic genes (these aspects of compartmentalisation are reviewed by Morata and Lawrence, 1977; and Kauffman, Shymko and Trabert, 1978). Compartmental boundaries show no obvious physical barriers. Certainly compartmentalisation of differently determined cells may be extreme in the insect cuticle, and caution must be exercised in extending these principles to vertebrate development. However, we might expect that such a basic aspect of development, though extreme in insects, will not prove exceptional. There are some parallels between the progressive determination of the presumptive dermis and the development of the insect compartment. However, it is not yet clear if skin determination is a series of binary commitments or how strictly determination is guarded through successive divisions in the cell lineage. Most significant for the present discussion, it is not clear how sharply defined are the boundaries between mesodermal regions, and we do not know if cells with different regional determination can participate in the formation

of the same primordium and thus if sharp boundaries between regions would influence the position of primordia.

Clearly, these ideas overstep the evidence. They do, however, point out that further investigation of these aspects of feather pattern formation enters an area of potentially much wider and deeper interest than the study of the development of feathers themselves.

### 2.3 Local Interactions Governing the Positions of Primordia

It was pointed out in Chapter 1 that the results obtained by Linsenmayer (1972) suggest that the positions of primordia may be influenced by early-determined organisation in the skin of the thigh. In as much as the present results indicate the importance of pre-existing organisation, they confirm this possibility. However, it is not clear that organisation which may influence the antero-posterior positions of primordia, as discussed above, can push the system towards an increased number of feather primordia per unit area of skin when the pattern forms without the normal degree of growth. Nor is it clear if further pre-existing organisation which is not revealed in this study influences primordium position. Although in the present study the density of primordia is approximately the same in cultured and freshly excised skin, the possibility that the feather density is slightly higher in the former remains to be critically examined.

We may draw together in summary conclusions from the results presented in Chapter 4. It is clear that the positions in which primordia will form can be influenced if the available space is restricted shortly before morphogenesis. Adjacent sites interact. It is assumed that similar interactions play a part in defining the

feather pattern. Neither diagonal extension of dermal arrays nor steric interactions between sites appear to determine the pattern and, since these possibilities are also disfavoured by the arguments presented in Chapter 1, the most likely mechanism of pattern formation appears to be based on chemical inhibition or competition along the lines proposed by Ede (1972). However, there is as yet no positive evidence to support this mechanism. Moreover, Ede's actual scheme must be considerably modified, in that competence is not attained by diffusion, but is pre-programmed as discussed in Section 1. Finally, the results suggest that the centre of the primordium site is defined first and its diameter established secondarily.

The most important conclusion to be drawn from these results is that the production of the feather pattern does not necessarily depend on the unique features of the feather system. There is a little experimental evidence that the features of the feather pattern-forming mechanism described above are, in fact, common to related patterns of serially repeated units. This evidence comes from the study of scleral ossicles which form in a ring around the eye in reptiles and many birds giving it skeletal support. Their number and shape vary greatly in different reptiles (Romer, 1956), but a particular number is modal in each species. Like feathers, their number does not show regulation under experimental manipulation and can be reduced in the chick by draining the eye and thus reducing the diameter to which the eye grows (this is analogous to culture of dorsal skin) (Coulombre, Coulombre and Mehta, 1962). Coulombre et al also noted that interaction between adjacent ossicles can modify the shape of the developing ossicle without primordia coming into apparent physical contact. This may

be similar to the interaction which limits the diameter of the feather primordium forming near a cut in the skin. Since it is possible that scleral ossicles are evolutionarily related to integumental appendages, this evidence lends support to the argument that the pattern-forming mechanism may be common to integumental appendages in general and that investigation of the feather system may therefore be of potentially wide relevance.

### 3. PERSPECTIVE VIEW OF FEATHER CONDENSATION DEVELOPMENT

#### 3.1 Introduction

The results presented in this thesis suggest that feather condensations form by short-range, associative cell movements, and that local changes in cell behaviour at the primordium site are responsible for the formation of the condensation. The particular hypotheses outlined in Chapter 5 (Section 7) are discussed here in the light of what is known about the morphogenesis of other organ primordia.

#### 3.2 Mechanisms

##### Diversity

There is great diversity in the development of mesenchymal condensations, or of mesenchymal regions where cell numbers increase without an increase in population density (e.g. in the emerging limb-bud). For example, there is wide variation in the relative contributions of cell proliferation and movement (Chapter 1, page 79). Nevertheless, the developmental programmes for many different organ primordia probably share common elements. For example, even where local proliferation is the major cause of an increase in cell numbers, additional changes in the properties of the mesenchymal population of the primordium - in cell-cell adhesiveness, etc. - must be invoked to explain the stability and change in shape of the primordium: this has been pointed out, with reference to limb-bud, by Searls and Janners (1971). Diverse primordia may be usefully compared in this light.

##### Short-Range Cell Movement

There is strong evidence that short-range movements, similar to those proposed here for the development of feather

condensations, operate in other systems. Two of the most thoroughly studied organ primordia form condensations in this way.

First, mouse metanephrogenic cells cultured transfilter to inducing tissue have been shown, using time-lapse filming, to form condensations by short-range, apparently random, movements combined with a trapping mechanism (Saxén et al, 1968). This system admittedly shows several important differences from the feather system, for the aggregates later develop into secretory kidney tubules.

The second example, the development of chick limb pre-cartilage condensations, may be more closely comparable with the feather system. Cell population density increases by 60% in condensations in vivo (about the same proportional increase as in Stage 30 feather condensations (Wessells, 1965)), and the cells pack with large areas of closely apposed cell surface (Thorogood and Hinchliffe, 1975). Condensations do not appear to form by local proliferation: quantitative mitotic data suggest that the rate of cell proliferation decreases within pre-cartilage condensations as they form (Ede, Flint, and Teague, 1975), though a detailed study of mitoses in forming condensations and the surrounding mesenchyme has not been made. Cells taken from limbs just before condensations develop will form aggregates in vitro which are morphologically and biochemically similar to pre-cartilage tissue in vivo (Ede and Flint, 1972; Ahrens, Solursh, and Reiter, 1977; Lewis, Pratt, Pennypacker, and Hassell, 1978). Careful filming of cultures indicates that, as aggregates begin to form, cells at the focus of condensation move more rapidly than before and become rounded while peripheral cells move centripetally over distances in the order of 30  $\mu\text{m}$  in 30 hours (Ede, Flint, Wilby, and Colquhoun, 1977).

Analysis of cell polarity (by the relative positions of golgi and nucleus, see Chapter 1) in condensations in vivo suggests that similar movements occur in vivo and that, in addition, some cells move centrifugally and circumferentially (Ede, et al, 1977).

The argument (Chapter 5, Section 7) that feather condensations are centres of tissue contraction would seem, at first, to apply with equal force to pre-cartilage condensations in the limb-bud where cells are in contact with their neighbours all around (Ede, Bellairs, and Bancroft, 1974; Thorogood and Hinchliffe, 1975; Kelly and Fallon, 1978). Why then are there no striking patterns of radically oriented cells, or of cells aligned between cartilage condensations? It is certainly arguable that this is evidence against the hypothesis that arrays arise from tensions generated by condensation in the skin, but it is, perhaps, more likely that the difference between the two tissues reflects a difference in the organisation of the extracellular matrix. In skin, long bundles of fibres lie approximately parallel to the skin surface (Overton and Collins, 1976; see also Chapter 5, Section 2), and these are well suited to alignment under stress, whereas condensations in the limb-bud are embedded in a tissue in which there is no evidence, from the abovementioned TEM and SEM studies, of large numbers of long matrix fibres.

Why do cells aggregate into the condensation? The answer is not known for any organ primordium. However, while deeper analysis might be expected to uncover considerable diversity of actual mechanisms, published observations and experimental evidence on a wide variety of systems have suggested the same preliminary hypotheses as arise from the present work on the feather system. A brief, selective discussion of this evidence will illuminate the

possibilities and problems inherent in the hypotheses proposed in Chapter 5 and will show where further exploration of the feather system may aid an understanding of general principles.

In Chapter 5, both differential contraction and differential adhesion were suggested as forces underlying aggregation. Mechanisms based on cell-surface contraction are, in the first instances, more complex to the intuitive imagination and more difficult to test experimentally than those based on adhesion. Probably for these reasons the rôle of cell contraction in mesenchymal systems in vivo has received very little experimental attention. Investigation of the mechanics and control of cell movement and the precise significance of cytoskeletal elements detectable by electron microscopy and immunological labelling (Wessells et al, 1971; Willingham, Yamada, Yamada, Pouysegur and Pastan, 1977; Trotter et al, 1978) is the most promising approach to understanding the rôle of cell contraction in morphogenesis. This may allow a dynamic interpretation of static histological and ultrastructural evidence, and point the way to direct investigation of factors controlling local contraction.

Most of the evidence, therefore, relates to cell adhesion. The evidence will be discussed under two headings: first, close-range associative movements due to cell-cell adhesion; second, cell migration guided by cell-matrix interactions.

#### A. Cell-Cell Adhesion

To account for kidney pre-tubule cell aggregation, Saxén and Wartiovaara (1966) offered a speculative hypothesis based on filming observations of cells in culture and other, circumstantial, evidence. They suggested that a group of mesenchymal cells gradually increases in adhesiveness under the inductive

influence of the tip of an overlying, ectoderm-derived, ureteric bud. The acquisition of increased adhesiveness spreads from cell to cell in the mesenchyme, becoming less intense with distance from the inducing tip of the bud, thus ensuring that moving cells are trapped and gathered towards a central focus. Circumstantial evidence points tentatively to a similar mechanism in the feather system. Strong evidence for such a mechanism would be the demonstration of increased adhesiveness in cells undergoing condensation. In few systems, however, is there any positive evidence of this. Consideration of evidence from studies on somitogenesis illustrates the problems raised by deeper analysis.

Bellairs and Portch (1977) and Bellairs, Curtis, and Sanders (1978) showed that dissociated, chick somitic mesoderm cells are more mutually adhesive than presomitic cells. Bellairs et al (1978) suggested that this increase in adhesiveness caused cell-packing in the somite and that the side of the cell facing the centre of aggregation may initially be more adhesive than the opposite side. However, the evidence does not show whether the cells become adhesive before or after they pack, so we do not know if adhesiveness is responsible for packing: the problem is thus the same as met here in the feather system. Would further experiments on dissociated cells approach this problem? A complication concerning this is illustrated by the careful work of Flint (1977) who showed that, in mouse somitogenesis, the absolute area of mutual cell surface contact remains constant, but, as cells pack, their morphology changes towards a more rounded form and their total surface area decreases. Thus, the proportion of each cell's surface which is in contact with other cells increases. Contacts become redistributed as the cells pack. There is no information on changes in cell adhesiveness in the mouse

somite system. However, if the distribution of adhesive sites is important in determining the local adhesiveness of the cell surface, in vivo, this may not be detectible in trypsin-dissociated cells where the distribution of sites may be more uniform (Rees, et al, 1977) and where cell volume may be changed. This emphasises the need to complement experimental studies on dissociated cells - from forming somites or feather condensations - with examination of the actual relations of cells in situ, and the need to arrive at a better understanding of the significance, for adhesion, of the various structural specialisations of apposed cell surfaces (Overton, 1975, 1977) and surface enzymes such as glycosyltransferases (Shur, 1977a and b).

#### B. Cell-Matrix Interactions

Close apposition of mesenchymal cells to the undersurface of an epithelium is common to many organ primordia and, in some cases, there is evidence of adhesion between the tissues, for example between tissues forming chicken scales (Sawyer and Abbott, 1972), mammalian teeth (Kollar, 1972), and mouse pancreas (Wessells and Cohen, 1967): in each case the tissues are difficult to separate. Only in the last example, however, has locally high adhesion within the primordium been demonstrated: trypsin-pancreatin-aided dissection leaves mesenchymal cells attached to the undersurface of the endothelium specifically in the prospective position of the pancreas. The similarity with the patterns of strong attachment of dermal cells to the basement membrane is striking, especially since, in both cases, the phenomenon is observed during a crucial phase of development when the primordium begins to take shape, the mesenchyme bulging outward beneath a concave epithelium, and when inductive tissue interactions may also be occurring. This encourages the idea

that further study of mesenchymal cell attachment to the basement membrane in the feather system may be of general interest.

The importance of the surface characteristics, including adhesiveness, of the basement membrane or the underside of an epithelial sheet, in directing migration and trapping cells has been suggested by observations and experiments on a wide variety of systems: examples are, Sea Urchin gastrulation (Gustafson and Wolpert, 1961), early chick embryo morphogenesis (Trelstad, Hay, and Revel, 1967), chick heart development (De Haan, 1964) and rabbit tooth primordium formation (Slavkin, Bringas, Cameron, Le Baron, and Bavetta, 1969). A common assumption of the hypotheses offered to explain directional movement and aggregation of cells in these cases is that the filopodia of mesenchymal cells explore the characteristics of their substratum and react selectively to initiate movement in a particular direction governed by these characteristics. Studies of cell behaviour in vitro, on substrata with patterns of differential, non-specific, adhesiveness, confirm that cells can behave in this way (Harris, 1973a; Albrecht-Buehler, 1976). However, there is, as yet, no positive evidence that differences in substratum adhesiveness guide cell movements in vivo. Experiments on the feather system suggested in Chapter 5 (page 271) may provide an opportunity to explore these possibilities further.

These ideas emphasise the importance of investigating regional chemical differences in the basement membrane. Since the interaction of cells with the intercellular matrix (including non-adhesive interactions) may also be important in governing morphogenetic movements, it is also relevant to consider briefly the chemical composition of the intercellular matrix, its significance for cell movement, and its relation to the basement membrane.

Present histochemical, autoradiographic, and extraction methods reveal the synthesis and composition of the matrix in quite crude terms, measuring the abundance of general classes of molecule. It is not surprising, therefore, that, as Manasek (1975) reviewing the importance matrix has concluded, qualitatively similar molecules are synthesised in a wide range of situations while marked spatial and temporal differences in matrix composition are quantitative. Evidence of regional differences in the basement membrane (Bernfield, Banerjee, and Cohn, 1972; Cuhna, 1976; Cohn, Banerjee, and Bernfield, 1977) and in the intercellular mesenchymal matrix (Pratt, Larsen, and Johnston, 1975; Morriss and Solursh, 1978) has been obtained in a variety of systems in crucial phases of development.

Although the significance of these compositional differences for cell behaviour is not clearly understood, some general points can be made. Glycosaminoglycans, in particular hyaluronate which has a large volume when hydrated, are important in the creation of intercellular spaces (Hay and Meier, 1973; Trelstad, Hayashi, and Toole, 1974). Synthesis of hyaluronate correlates, in some cases, with cell migration. For example, Pratt et al, (1975) showed that hyaluronate deposition precedes chick neural crest cell migration and that the cells may use this matrix as a substratum. These authors also suggested that removal of hyaluronate may cause cell aggregation during development. Cell population density is inversely correlated with the amount of hyaluronate and chondroitin sulphate in the matrix of neural fold and primitive streak regions of the rat embryo (Fisher and Solursh, 1977; Morriss and Solursh, 1978). Morphological continuity of stainable components of the basement membrane, intercellular matrix, and cell surface coat in some mesenchymal tissues (Kelley, 1977; Morriss and Solursh, 1978)

emphasises that local changes in matrix chemistry may have ramifications, not only for cell movement through the tissue, but for correlated physical interaction of cells with the basement membrane, cell-cell interaction, and chemical interaction between cells and the highly charged, complex matrix molecules.

These lines of evidence from other systems point to important gaps in our knowledge of feather primordium development. Histochemical studies on the feather system (Chapter 1) are not of sufficient detail to determine, for example, whether changes in the hyaluronate content of the matrix occur during condensation formation. In the light of the present emphasis on short-range cell aggregation, it may be more informative to test the hypothesis that hyaluronate concentration decreases at the condensation site than to investigate the orientation of collagen in intercondensation regions. Moreover, by a correlative study of cell movement and basement membrane biochemistry in the feather system - either on membrane exposed by trypsin-aided dissection, or in intact skin using improved filming methods - it may be possible to explore the physico-chemical basis of the striking patterns of differential cell attachment to areas of the membrane.

Lastly, concern with the rôle and mechanism of cell movement in the formation of condensations should not obscure the need to investigate the crucial, and difficult, question of how patterns of cell behaviour or matrix arise.

### 3.3 The Rôle of Close Apposition in Cell and Tissue Interactions

Although close-range associations have been discussed above in terms of their possible direct physical rôle in modulating cell movement, they may also mediate biochemical interactions between cells and tissues which invoke specific metabolic responses and influence the course of cytodifferentiation.

In terms of intra-condensation interactions, the chick pre-cartilage condensation is the best studied example. Initially, molecular expression of the chondrogenic phenotype is reversible and dependent on aggregation (Abbott and Holtzer, 1966).

Chondrogenesis, in terms of the secretion of histochemically detectable sulphated glycosaminoglycans, may depend on an increase in intracellular cAMP levels in response to aggregation (Ahrens et al, 1977). However, after a certain developmental age (Stage 25 in wing buds), expression of the chondrogenic phenotype in culture becomes independent of cell aggregation (Ahrens et al, 1977). Whether this transient dependence of biosynthesis on the formation of a condensation is a general phenomenon, and whether high intracellular levels of cAMP are a general result of cell-cell interaction in aggregates, remains to be examined.

Study of the interaction between tissues separated by nucleopore filters of varying porosity have implicated direct cell-cell or cell-matrix contact in inductive interactions in several systems (Saxén, 1977); for example, in kidney tubulogenesis (Nordling et al, 1977) and in somite chondrogenesis (Lash and Vasan, 1978).

The suitability of the skin system for experiments on tissue combinations and the large amount of information which has accumulated from such studies make the skin one of the most

intensively investigated systems from the point of view of tissue interaction. However, the feather system lags behind others in our knowledge of the immediate metabolic and biosynthetic consequences of cell and tissue interaction. This is due, in part, to the absence of any large scale synthesis of specific chemical markers of appendage differentiation before 12 days development when feather-keratins begin to be synthesised in the epidermis in amounts easily detectable by TEM or quantitative gel electrophoresis (although electrophoresis shows trace amounts at least one day earlier) (Kemp, Dwyer, and Rogers, 1974). Very recently, patterns of keratin synthesis have been assayed electrophoretically in tissue combination experiments by Dhouailly, Rogers, and Sengel (1978). The results show that biochemical differentiation parallels morphogenesis of feathers or scales in reciprocal combinations of dorsal and tarsometatarsal tissues. Moreover, it seems that feather or scale patterns may be 'triggered' en bloc, for combinations of dorsal epidermis from the chick with mouse plantar dermis form foot pads with a chick scale keratin pattern. There is an urgent need to extend these investigations to the biochemical changes which occur in both epidermis and dermis during, and immediately after, primordium morphogenesis. The system provides sufficient material for basic biochemical investigations (by gel electrophoresis, and autoradiography) and this is clearly a first step in obtaining markers of early differentiation which might be used to test the molecular consequences of morphogenesis in experimental situations.

### 3.4 Evolutionary Relationships

The relationship between evolution and development is of crucial importance, but has received little attention in the literature on developmental mechanisms. Although, as a number of authors (for example, Regal, 1975) have pointed out, evolution acts on developmental mechanisms, the inverse relation is probably also important in the sense that evolutionary responses may be guided by what kind of structures it is possible to develop. In this latter sense both overt developmental programmes and perhaps those which may be latent in the genetic organization will help determine the evolution of new structures.

The skin system is ideally suited to a study of the relation between development and evolution of organ primordia since a variety of primordia are formed whose evolutionary relationship is fairly well known.

The mechanism of appendage primordium morphogenesis, unlike that of appendage formation (Chapter 1), has not been conserved in evolution. Thus, the primordia of Reptilian scales form neither true placodes nor dermal condensations (Maderson, 1965), those of Avian scales form placodes but only small dermal condensations at the upper, anterior and posterior folds of the scale ridge (Sawyer, 1972a), while those of the feather and hair form both placodes and dermal condensations. There are considerable difficulties in drawing immediate parallels between the development of evolutionary related primordia such as these. Without more detailed developmental and evolutionary evidence it is impossible to say how far resemblances are due to conservation or convergence of developmental mechanisms. However, it is worth pointing briefly

to some of the interesting possibilities and questions which further analysis of the developmental mechanisms may help us understand.

Similarities between the developmental programmes for dermal condensation in scale, feather, and hair primordia are tentatively suggested by the evidence from recombination experiments. Mouse dermal cells will form condensations under the influence of feather epidermis and can play a part in the preliminary stages of feather development (Dhouailly, 1973, 1978). Chick tarsometatarsal dermis (up to 12 days old) will form feathers in combination with dorsal epidermis. With tarsometatarsal dermis from 13 day-old chicks scales develop in combinations with feather epidermis, but feather structures form at the tips of the scales (Rawles, 1963). Sawyer (1972a) has noted that chick scale primordia form dermal condensations at about 12 days development, but lose these at about 13 days. On the basis of this temporal correlation, Sawyer speculates that the ability to form feather condensations in tarsometatarsal dermis is related to the ability of this tissue to form scale condensations. The fact that feathers form at the tips of scales in experimental combinations suggests a spatial correlation between the formation of the two types of condensation. These findings emphasise the possible similarity between the mechanisms of development of contemporary feather and scale condensations. These developmental relationships may stem from an evolutionary relationship if Maderson's hypothesis that feathers evolved by progressive elongation of the tips Archosaurian scales is valid.

The present results throw a little more light on the relationship between the condensations of the different appendages in that they emphasise that dermal arrays, which are found only in the feather system, are of secondary importance in the development

of feather condensations. This supports the possibility that the mechanisms of condensation in the different appendages are essentially the same.

On the basis of this evidence it is interesting to look further back in evolution and to ask, for example, if the dermal condensations in feathers, hairs and Avian scales are related to the development of dermal ossicles in the early reptiles. (Dermal ossicles are membrane bones and those of the early reptiles presumably passed, like membrane bones in modern reptiles and in other animals, through a stage of mesenchymal condensation.) It might be, for example, that condensation formation is an inherent property of the dermis. The association between dermal and epidermal development, which is observed in some cases in the Reptilia, but is not essential for Reptilian epidermal corneoscute formation, may have become more tight in the evolution of Avian scales and feathers and (probably independently (Spearman, 1964)) in the evolution of mammalian hairs. The dermal condensation part of the programme for ossicle development may have evolved to play a subsidiary, but necessary, rôle in epidermal appendage morphogenesis. In this light a comparison between condensation development in scleral ossicles of the chick (perhaps representing the development of more primitive reptilian ossicles) and in feather primordia may be interesting. The extent to which cells may aggregate into scleral ossicles is not strictly known, but Hale (1956) has shown that cell proliferation (as assayed from percent mitosis data) may be able to account for the observed increase in cell numbers. Thus, the balance of cell division versus movement is probably different in the primordia of ossicles and feathers. It may be that this

balance has shifted during evolution of the feather primordium and that a more primitive property than aggregative movement is that which is shared with the condensation of the ossicle primordium, the capacity to pack tightly and to maintain a stable condensation structure.

These considerations are largely speculative, but immediately suggest the importance of examining the relationship between the development of evolutionary related appendages. For example, if qualitative differences in adhesion or cell surface antigenicity are found between the feather condensation and surrounding dermis, it will be important to test the mutual adhesiveness of condensation cells from the appendages of different zoological Classes and to compare their immunological cross reactivity. This type of approach may illuminate the evolutionary relationship of the skin appendages and open the way to a broader understanding of the interplay between development and evolution.

The main implication of the work presented in this thesis is that the development of feather primordia has more in common with the development of other organ primordia than has previously been emphasised in the literature. Thus, the study of feather primordium development may provide more immediate insights into the general principles of primordium development than have been sought in this system.

Three main, broad conclusions arise from the results and point to important areas for future study:

1. Pre-established conditions play a part in governing the overall pattern of morphogenesis:

a) the time of onset of morphogenesis is pre-set over most of the width of the skin before, or at, the time of morphogenesis of the first row in the pteryla;

b) the evidence suggests - but does not prove - that the antero-posterior position of the initial primordia and the position of chevrons depends partly on pre-existing organization.

2. The position of each primordium is partly established by local interactions close to the time of morphogenesis. The evidence suggests that these interactions do not depend on the extension of dermal arrays or on a physical packing mechanism, but that a small group of dermal cells at the centre of the primordium site may initiate morphogenesis in response to local conditions. The most suitable model to explain the generation of these local conditions appears to be a modification of that proposed by Ede (1972).

3. The morphogenesis of individual condensations probably depends on changes in the local non-proliferative behaviour of dermal cells at the prospective condensation site which causes their movement over short distances into the condensation. Changes in the adhesive and contractile properties of the cells seem the most likely possibilities for future investigation.

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