AN EXPERIMENTAL STUDY OF WOUND HEALING IN ARION.

by

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The aim of this investigation was to study molluscan tissue regeneration, as exemplified by wound healing of the mantle edge in *Arion hortensis*, and to attempt an analysis of blastema formation.

As a necessary preliminary the structure of the mantle edge was investigated. It consists of a glandular epithelial fold within which is a zone of connective tissue composed of a dorsal pigmented region, a median vascular region through which ramifies a lattice-work of smooth muscle fibres, and a ventral region containing a mat of muscle fibres.

The structural and metabolic changes following wounding by excision were studied and where possible related. Immediately after wounding the undamaged muscle fibres adjacent to the wound contract and the meshes of the lattice close, thus temporarily constricting the blood sinuses and arresting bleeding. Wound closure is achieved initially by blood-cell agglutination and finally by coverage of the injured surface by the epithelial cells. Damaged tissue is removed from the wound by histolysis and phagocytosis. A blastema then forms at the injured surface; its cells grow, divide, and differentiate replacing the excised tissue. Differentiation begins in those regions adjacent to the uninjured tissue and extends into the rest of the blastema. Changes in the distribution of acid and alkaline phosphatase, ribonucleic acid, glycogen, and sulphydryl and disulphide groups were noted.

The method of blastema formation was analysed by the following techniques: carmine marking of epithelial cells, tissue culture of isolated blastemata, and colchicine treatment of wounded animals. It was shown that both immigrant and indigenous cells contribute to the blastema, the majority of the blastema cells being immigrant. Epithelial and connective tissue components of the regenerate arise from immigrant cells while muscle tissue develops from indigenous material.
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I. PREFACE.

Wound healing may be defined as the closure of an injured surface and the replacement of damaged tissue. The faculty of wound healing appears to be universal throughout the animal kingdom. When a wound is inflicted, either accidentally or by design, the normally protective surface layer of the body is interrupted and a variable degree of damage to the underlying tissue ensues. The damaged area allows entry of micro-organisms and egress of body fluids; the cells of the damaged tissue disintegrate liberating their contents into the wound region and affecting the healthy cells nearby. It is obviously necessary for the well-being of the animal that the wound be closed and the damaged tissue removed as quickly as possible. Wounding is a constant hazard in the animal kingdom and an individual which can perform the healing process rapidly and efficiently possesses an unquestionable selective advantage.

Wound healing may be thought of as regeneration on a small scale. Regeneration is a term used to cover all types of restoration of body form, (Needham, 1952). The degree of regenerative ability differs widely from species to species. In some fresh-water planarians a very small fragment of the body can regenerate into the whole animal, (Brøndsted, 1955), whilst in adult mammals regenerative ability is limited to wound closure, compensatory hypertrophy and physiological regeneration. The latter refers to the replacement of structures such as skin, hair, etc., lost by wear and tear, (Needham, 1952). Physiological regeneration is a chronic low-grade process which logically extends to the molecular level, that is, to the metabolic turnover of the individual cell.

Numerous investigations have been conducted into the nature and mechanism of wound healing and regeneration. In the vertebrates extensive research has been devoted to both high-grade regeneration, such as the replacement of limbs in amphibians, (Thornton, 1942;
Faber, 1960; et al.), and to regeneration of a lower grade, such as wound healing in mammals, (Billingham and Medawar, 1955; Abercrombie, 1957; Joseph and Townsend, 1961, et al.). In the invertebrates much attention has been paid to high-grade regeneration, e.g. planarian regeneration (Lender, 1950; Sengel, 1960; et al.), while lower grades such as wound healing have been somewhat neglected, particularly in molluscs. The molluscs form a highly specialised phylum, showing advanced morphological and histological features. Regenerative ability tends to decline as the complexity of the animal increases but it was recently pointed out (Vorontsova and Liosner, 1960), that though this may be true for organ regeneration it does not apply to lower grades of regeneration such as tissue replacement and wound healing.

Arion hortensis, the experimental animal selected for this investigation, forms a suitable subject for the study of wound healing and tissue regeneration in the terrestrial pulmonates. A common garden slug, it is readily available throughout the year, reaching a population maximum in October and November (Bett, 1960). If kept at a cool temperature wounds heal readily and the death rate is low. Furthermore, the absence of a shell makes the slug particularly easy to operate on. The mantle edge, which is readily accessible, was wounded by excision, using specially designed operating forceps to ensure a uniform degree of wounding throughout the investigation.

Study of the structure of the mantle edge was followed by experimental work involving:

(i) a survey of normal regeneration of the mantle edge after experimental injury;

(ii) a more detailed investigation of certain features noted in the above survey, namely the method, rate and control of epithelial cell movement, the method of blastema formation and the origin of gland cells in the regenerate.
II. MAINTENANCE OF EXPERIMENTAL ANIMALS.

The specimens of *Arion hortensis* used in this research were collected from gardens in the central London area, Cornwall, Cheltenham and the West Riding of Yorkshire. They were maintained in a constant temperature room at 15°C and illuminated by a tungsten filament lamp throughout the investigation.

Stock animals were kept in wide-mouthed glass jars, 20 cms. tall with a diameter of 15 cms., which had been fitted with finely perforated plastic lids and lined with filter paper moistened with tap water. 40 specimens were kept in each jar. The slugs were fed twice weekly on raw potato and carrot, coated with a 1:1 mixture of Bemax and Complan. They were transferred to clean jars each week. The animals bred successfully under these conditions.

All eggs laid were removed from the stock jars and placed on moist filter paper in covered crystallizing dishes where they hatched within 21 days. Newly hatched slugs were kept in small specimen tubes lined with moist filter paper. Handling of the young animals was reduced to a minimum. The specimen tubes were each fitted with a perforated cork through which a glass tube, drawn out at both ends into a fine capillary, was passed. This permitted the entry of air but minimised water loss (Stephenson, 1962). The young animals were fed on the same diet as the adults.

The experimental animals were kept at 15°C because it was found that healing proceeded at a convenient rate at that temperature whilst infection was reduced. In trial experiments conducted at 11°C, 15°C, and 25°C respectively, it was found that although the rate of healing increased with the temperature the number of infected animals also increased.
III. STRUCTURE OF THE MANTLE EDGE.

INTRODUCTION.

The mantle is the epithelium covering the visceral hump of molluscs. In the gastropods it extends outwards as a fold at the junction of the visceral mass with the head and foot. Where it overhangs the mantle cavity or lung it is termed the mantle skirt, (Fretter and Graham, 1962). In slugs that part of the mantle anterior to the pneumostome extends forwards as a fold of skin beneath which the head can be retracted (Fig. 1a, 1b). It is with this skin-fold and the tissues it encloses, here termed the 'mantle edge', that the present investigation is concerned.

Although the mantle and adjacent tissues of shell-bearing molluscs have been examined by many workers (de Villepoix, 1892; Hayasi, 1938; Bevelander and Benzer, 1948; Heaysman, 1951; Guarabassi and Piacenza, 1958; et al.), those of the slugs, which are either shell-less or have a reduced shell, have been somewhat neglected. The only previous work traced is that of Barr who, in her paper of 1928, described the "mucous and skin glands" of Arion ater var. castagnea mentioning those of the mantle in her work.

The mantle edge of Arion has not, as far as can be determined, been studied before. Information is available, however, on molluscan cell-types similar to those of which it is composed. A resume of this information is given below.

Glands.

Scientific study of the cutaneous glands of molluscs was commenced in the late nineteenth century. In 1876 Leydig published a paper in which their origin and development were discussed. He distinguished between mucous, protein and calcium glands. In 1885 Barfurth, writing on the histochemical study of glycogen, mentioned molluscan mucous glands though he did not give a detailed description of them. de Villepoix (1892) described the calcium glands of
**Fig. 1a.** Anterior region of Arion hortensis showing the mantle edge.

**Fig. 1b.** Sagittal section through mantle edge and head.

- **d.e.h.** dorsal epithelium of head; **d.e.m.** dorsal epithelium of mantle edge; **e.f.** epithelium of foot; **j.** jaw; **m.c.** mantle cavity; **m.e.(1)**, mantle edge reflected dorsally; **m.e.(2)**, mantle edge in normal position; **n.p.z.** non-pigmented zone; **o.** odontophore; **v.e.m.** ventral epithelium of mantle edge.
molluscs. Herfs (1922) referred in his work to clusters of mucous cells which he termed "Paketdrüsen". In 1923 Cretin described large cells found in molluscan blood as mucous cells. His interest lay, however, primarily with the blood and he did not describe the cutaneous glands. Barr (1928) described three types of cutaneous gland:

(a) unicellular mucous glands;
(b) calcic glands, (containing calcareous material);
(c) pigmentary glands, producing black granules.

She also reported red granules scattered amongst the dermal mucous glands.

Roth's studies (1929) on the epithelium of *Helix pomatia* revealed for the first time credible evidence that two types of mucous gland might be present in the integument. One of the two cell-types described produced a foamy secretion, while the second contained fine mucous threads or even refractory granules. Roth thought it possible that the granules had developed from the foamy secretion and that the second type of cell was simply a later developmental stage of the first. Calcium glands and four types of protein gland, the latter possibly being stages in the development of a single gland, were also described. Roth admitted that differential staining of the calcium glands was difficult.

Interest in the molluscan cutaneous glands was revived in the nineteen fifties. Heaysman (1951) described the calcium glands in some detail, and suggested that calcium carbonate was deposited not in cells but in spaces in the haemocoele close to the epithelium. She also claimed that the calcium carbonate entered into solution before being discharged. This theory was disputed by Campion (1957) who claimed to have seen nuclei within the calcium cells, and also calcium carbonate leaving the cells in granular form. Campion also reported finding fat, protein, and pigment glands, as well as four types of mucus-secreting cell. The presence of fat is of interest, for it had not been previously demonstrated in the skin.
of *Helix aspersa*, on which most of Campion's work was based. Campion regarded all the glands as merocrine, the cell body remaining intact when the secretion was expelled. She also ascribed special functions to the different glandular types.

It is now generally accepted that there are four categories of molluscan cutaneous glands, all unicellular:

(a) mucous glands,
(b) protein glands,
(c) calcium glands,
(d) pigment glands.

There is some doubt as to whether or not these categories should be further subdivided.

Comparatively little research has been done on the development of the glands. Roth (1929) thought that developmental stages of the glands were found only before hatching or in the very young animal. It is now known that developmental stages do occur in adult animals. There is some doubt as to the origin of the cutaneous glands. Campion (1957), working with adult animals, believed that they arose from connective tissue, while Roth (1929), who used embryos and hatchlings, thought they were epithelial in origin. Further work is obviously indicated.

**Vascular system.**

There appears to be some doubt as to whether or not the blood spaces have a cellular lining. McGee-Russell (1954) suggested that true blood vessels, lined with round cells, are often present in molluscs and that the walls of these vessels might be strengthened with fibrous tissue. He used the term "haemocoel cells" for the lining cells and believed them to be equivalent to the "Leydig cells" of Cuenot (1892) and the "amoebocytes B" of Wagge (1951). He also stated that the haemocoel cells were not amoebocyte but were held together firmly by an intercellular cement.
substance. He admitted, however, that the vessels do break down in the pulmonary region, where the giant veins are essentially lacunae in the muscular connective tissue.

**Blood.**

The blood of gastropod molluscs, and in particular the blood cells, has been investigated by Cuenot (1891, 1914), Kollmann, (1908), and by George and Ferguson (1950). Cuenot reported that only one type of cell was present in the blood, an amoeboid cell containing granules probably of an enzymatic nature. Kollmann, however, believed that granular cells were uncommon in the blood of gastropod molluscs though he had seen such cells in the blood of Viviparus viviparus. More recent work on molluscan blood, that of George and Ferguson, has shown that in stenoglossan molluscs there are three types of blood cell:

(a) amoeboid lymphocytes, usually with clear basophilic cytoplasm though occasionally containing granules;

(b) granular phagocytes, with vacuolated basophilic cytoplasm;

(c) granular amoebocytes containing eosinophilic granules.

The origin of the blood cells has not been established.

**Muscle cells.**

According to Grassé (1960) the muscles below the molluscan integument are smooth, and are composed of unicellular fibres with a contractile sheath and a central zone of cytoplasm containing a nucleus. Each muscle cell or fibre contains contractile fibrils, arranged in a helical manner in many cases, and there is no obvious cross-striation.

Although the ultra-structure of striped muscle has been thoroughly investigated since the advent of electron microscopy that of smooth muscle, especially of invertebrates, has attracted less attention. Hanson and Lowy (1957) have, however, published work on the smooth
Muscle of the squid, the pharyngeal retractor muscles of *Helix pomatia*, and the adductor muscle of the oyster. The muscle fibres usually contain two types of myofilament, composed of myosin and paramyosin respectively. In *Helix* the myofilaments are irregularly arranged, while in the squid they are grouped into myofibrils separated by sarcoplasm with a complex reticular system. The structure of smooth muscle fibres is not universally constant. In the earthworm, for example, the contractile myofibrils are central, and the nucleus and sarcoplasm peripheral, (Hanson, 1957). Smooth muscle fibres are traditionally thought of as being unicellular though intercellular bridges, resulting in the formation of partial syncitia, have been reported in the smooth muscle of the mammalian uterus, (Mark, 1956). Where two fibres touch, interdigitation of the cytolemmae is sometimes found, (Caesar, Edwards and Ruska, 1957, studying smooth muscle of the mouse). The nature of the junction of smooth muscle cells has not been investigated in molluscs, attention being directed more to the structure of the myofilaments and their inter-relationships. It is important, however, that information be obtained on this point, for the nature of the intercellular connections must affect the transport of metabolites between the cells and also their mode of action.

**Pigment.**

Pigments are common in the molluscan integument. Kennedy, (1959) has shown that both melanins and uroporphyrins are present in the integument of *Arion ater* and in "certain other coloured forms of *Arion"*. He has suggested that there is a direct relationship between the amount of uroporphyrin, a photosensitive pigment, and the degree of melanism in the specimens he studied. To my knowledge no identification of the pigments in the integument of *Arion hortensis* has yet been made.
METHODS USED.

The animals used for histological work were maintained under the conditions described on p. 9, at a constant temperature of 15°C.

Optical and electron microscopy were used in the investigation and an attempt was also made to identify the pigments present in the mantle edge.

1. Optical microscopy.
(a) Animals were selected at random and killed by immersion in one of the fixatives listed in Table 1. The material was embedded in 54°C paraffin wax to which a trace of cerein had been added. Sections were cut at either 5μ or 8μ.

(b) Fresh material was frozen onto a sledge microtome fitted with knife and stage Frigistor cooling units, sectioned at thicknesses ranging from 4μ to 10μ and then fixed.

Material prepared by either of the above methods was stained by one of the techniques listed in Table 2. Sections were mounted in D.P.X., Canada Balsam, or Farrant’s Medium.

2. Electron microscopy.

The methods of tissue preparation were taken from the Servall handbook (1959).

One of two fixatives was used:
(a) 1% buffered osmium tetroxide, (Palade, 1952).
The buffer was prepared from a 0.28 M. stock solution, composed of 2.88 g. sodium veronal and 1.15 g. sodium acetate in 100 ml. water. The pH of the buffer was adjusted to 7.0 by adding 0.1 W. hydrochloric acid drop by drop to the buffer stock solution. The neutralised buffer solution was mixed with an equal volume of 2% osmium tetroxide in water to produce the fixative used. The neutralised buffer was found to be unstable, crystallizing out on standing. It was therefore freshly prepared from the stock buffer solution.
as required.

(b) Permanganate fixative, (Luft, 1956).

This was prepared as required from equal parts of a 1.2% stock solution of potassium permanganate and neutralised acetate-veronal buffer.

After treatment with either of the above fixatives the material was carefully dehydrated, embedded in a mixture of 9 parts methyl methacrylate to 1 part n-butyl methacrylate and sectioned with a Cambridge ultramicrotome.

Some of the sectioned material was impregnated with lead hydroxide using the procedure of Millonig, (1961). Grids supporting sections were floated in drops of the staining solution, washed in distilled water, and allowed to dry in air before examination. The sections prepared were examined with a modified Siemens electron microscope and photographed.

3. Identification of pigments.

A modification of the method of Kennedy (1959) was used to determine the presence of porphyrins.

10 large specimens of Arion hortensis were killed by brief immersion in ethanol and their mantle edges removed. The tissue was cut up into small pieces and extracted overnight at room temperature in the dark with 25 ml. of a mixture of 19 parts methanol to one part of sulphuric acid. The residue was filtered off and re-extracted with a further 25 ml. of the acidified methanol solution for 4 hours. The two extracts were mixed, diluted with an equal quantity of water, cooled to 5°C and extracted with chloroform. Both the chloroform extracts and the hypophases were examined in ultraviolet light and any fluorescence noted.

The tissue was treated as follows to determine the nature of the dark granules it was known to contain. A further sample of 10 mantle edges was taken and extracted first with water, then with
acetone, and lastly with a 1% solution of sodium hydroxide. The colour of the extracts was noted. Sections of the tissue were treated with hydrogen peroxide and with ammoniacal solutions of silver and any reactions observed.
<table>
<thead>
<tr>
<th>TABLE 1. FIXATIVES.</th>
<th>FIXATIVE</th>
<th>PURPOSE</th>
<th>REFERENCE</th>
</tr>
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<tbody>
<tr>
<td>2. 5-amino acridine hydrochloride.</td>
<td>Acid mucopolysaccharide fixation.</td>
<td>William and Jackson (1956).</td>
<td></td>
</tr>
<tr>
<td>STAIN</td>
<td>FOR IDENTIFICATION OF:</td>
<td>STAINING TECHNIQUES</td>
<td>REFERENCE</td>
</tr>
<tr>
<td>-------</td>
<td>------------------------</td>
<td>---------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>19. Toluidine blue.</td>
<td>y-metachromasia with acid mucopolysaccharides; also stains nuclei.</td>
<td>Pearse (1960).</td>
<td></td>
</tr>
</tbody>
</table>
OBSERVATIONS.

A transverse section of the mantle edge shows it to be composed of:
1) a dorsal epithelium, partly glandular, consisting of a single layer of cells;
2) a central zone of connective tissue, which can be subdivided into:
   (a) a dorsal pigmented region;
   (b) a median, highly vascular region, containing a lattice-work of muscle fibres;
   (c) a ventral region containing a mat of horizontally arranged muscle fibres;
3) a ventral epithelium, also partly glandular, consisting of a single layer of cells, (Fig. 2a).

The dorsal surface of the mantle edge is curved and the ventral surface flat. The dorsal epithelium is continuous with the ventral epithelium, which it meets peripherally at an acute angle. The ventral epithelium is also continuous with the epithelium of the head, (Figs. 1a, 1b).

1. Dorsal epithelium.

The dorsal epithelium is composed of two cell-types: glandular and non-glandular cells. The non-glandular cells account for about 60% of the total number of cells.

A. Non-glandular cells.

These cells are cubical to low columnar in shape. The height of the cells is $15 \mu \pm 0.6 \mu$. These figures represent the mean and standard error for 10 cells from Bouin-fixed material. Although other fixatives were used, (see Table 1), all the dimensions recorded in this investigation were taken from Bouin-fixed material to facilitate comparison, unless otherwise stated. Each cell has a central nucleus, usually ovoid in shape, and of length $5 \mu \pm 0.5 \mu$. 
Fig. 2a. Transverse section of mantle edge to show gross histology.

b.s., blood spaces; d.e.h., dorsal epithelium of head; g.d.e., glandular dorsal epithelium; g.v.e., glandular ventral epithelium; m.c.t., median connective tissue, with blood spaces and muscle lattice; p.c.t., pigmented dorsal connective tissue; v.c.t., ventral connective tissue containing muscle mat.
The nucleus usually contains one nucleolus, which may be distinguished from the rest of the chromatic material by using the methyl green/ pyronin Y method of Kurnick (1955) by which the nucleolus stains red and the chromosomes green. The nucleolus is difficult to distinguish after routine staining methods. The epithelial cells are non-ciliated, except around the pneumostome, where cilia of length $4\mu \pm 0.4\mu$ are found on their free surface, (Fig. 3).

The epithelial cells rest on a thin basement membrane, which stains blue with Azan and gives a faint pink coloration with periodic acid/ Schiff. It appears to be continuous and passes around the base of the glandular cells. It is sometimes masked by the pigment granules of the superficial connective tissue, (Fig. 2b).

B. Glands.

The cutaneous glands are all unicellular and usually extend into the sub-epithelial connective tissue, opening to the surface between the non-glandular cells. The glands are of three types:

(i) mucous glands;
(ii) protein glands;
(iii) calcium glands.

(i) **Mucous glands**.

There appear to be three distinct types of unicellular mucous gland associated with the dorsal epithelium. It is possible, however, that these are all developmental stages of a single type. The evidence for this is discussed later.

**Type I.** This type of mucous gland is large and ovoid to club-shaped, opening onto the epithelial surface by means of a short, slender neck. After Bouin fixation the contents of the gland appear reticular or bubbly. The nucleus is peripherally placed and baso-lateral in position. The height of the cell is $95\mu \pm 4\mu$, and the greatest width $44\mu \pm 3\mu$. The appearance of the cell is shown in Fig. 2b.
**Fig. 2b.** Transverse section of mantle and underlying tissue showing certain cutaneous glands.

- m.c., mantle cavity; n.g.e.c., non-glandular epithelial cells;
- p.g., pigment granules; pr., protein gland; s.pr., secretion of protein gland; s.T.I., secretion of Type I gland; s.T.II., secretion of Type II gland; T.I., Type I mucous gland; T.II., Type II mucous gland.

Fixative: Bouin.
Stain: Steedman's alcian blue, eosin, and Delafield's haematoxylin.
Fig. 3. Ciliated epithelial cells from pneumostome area.

c., cilia;
c.g., calcium gland;
e.n., epithelial cell nucleus.
Histochemical studies of the secretion of the Type I cell show it to be acid mucopolysaccharide in nature, as the following staining reactions indicate.

<table>
<thead>
<tr>
<th>TEST</th>
<th>RESPONSE</th>
<th>CONCLUSIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Periodic acid/ Schiff</td>
<td>Faintly positive</td>
<td>The test depends upon the oxidative cleavage of ( \alpha )-glycol groups, and is often used as a generic test for carbohydrates. It is also given by ( \alpha )-ketones, ( \alpha )-hydroxyaldehydes, ( \alpha )-ketonaldehydes, ( \alpha )-diketones, or by amino derivatives of any of the above compounds. The secretions is probably carbohydrate. The faintness of the reaction may be due to substitution of some of the reactive groups (Meyer and Odier, 1946).</td>
</tr>
<tr>
<td>(P.A/S.)</td>
<td></td>
<td>This indicates the presence of phenolic compounds (if not doubly substituted in the ortho- and meta-positions), and amongst the amino acids, of tyrosine, (Casselman, 1962).</td>
</tr>
<tr>
<td>2. Millon's reaction</td>
<td>Positive</td>
<td>The secretion is non-lipid. The test was made because glycolipids give a positive response with P.A/S., (Casselman, 1962).</td>
</tr>
<tr>
<td>3. Sudan black B.</td>
<td>Negative</td>
<td>Neither starch nor glycogen are present in observable quantities, (Bernfield, 1951).</td>
</tr>
<tr>
<td>4. Amylase treatment</td>
<td>Secretion not removed.</td>
<td>The secretion is thus neither a muco- nor a glyco- protein, both of</td>
</tr>
<tr>
<td>5. Toluidine blue</td>
<td>( \gamma )-metachromasia.</td>
<td></td>
</tr>
</tbody>
</table>
TEST.  RESPONSE.  CONCLUSIONS.


7. Hyaluronidase.  Still stained with S.A.B.  This reaction is given by acid mucopolysaccharides and by hyaluro sulphate, (Pearse, 1960).

It has also been observed that the secretion does not stain with Heidenhain's haematoxylin, though it does stain with Ehrlich's haematoxylin and neutral red. These responses help in the location of Type I mucous glands in routine histological sections.

The omission of Hale's test (Hale, 1946), once suggested as a method of revealing acid polysaccharides, is deliberate. The method, which involves the adsorption of colloidal iron oxide by acidic tissue constituents and the subsequent demonstration of the iron by the prussian blue reaction, is now known not to be specific for acid polysaccharides, nor for any chemically definable group of substances, (Casselman, 1962).

**Type II.** This mucous gland is similar in shape and size to the Type I gland described above. It, also, has bubbly contents, a peripheral, baso-lateral nucleus, and a short, slender neck. The average height of such a gland is $95\mu \pm 6\mu$ and the greatest width $42\mu \pm 4\mu$.

Type I and Type II mucous glands can be distinguished by their staining reactions. Type II cells stain only faintly with Steedman's alcian blue; Type I cells stain much more deeply, (Fig. 2b). No cells with an intermediate affinity for the dye have been found.
The secretion of the Type II glands reacts to the other histochemical tests applied in the same manner as the Type I glands, and is therefore an acid mucopolysaccharide. The difference noted with Steedman's alcian blue may indicate either:

(i) that the secretions of the two glands are chemically distinct;

or (ii) that the amounts of reacting substances differ.

Type III. A mucous gland of this type is small, (height $14\mu \pm 2\mu$, greatest width $7\mu \pm 1\mu$), ovoid, and superficially placed. It has a short neck and contains a bubbly secretion which reacts to all the histochemical tests applied in the same manner as the Type I glands. The secretion is thus an acid mucopolysaccharide. It is probable that the Type III glands are an early stage in the development of the Type I glands (p. 23). Type III glands are shown in Figs. 2a and 4.

Optical microscopy reveals little of the structure of the mucous glands. Electron microscopy shows that the secretion is contained in vesicles and that there are many internal cell membranes (Fig. 5). A similar arrangement is found in cells which synthesize and secrete protein (Birbeck and Mercer, 1961). In Fig. 5, the appearance of the secretion differs from one part of the cell to another; in other cells the secretion appears to be homogenous.

(ii) Protein glands.

These are unicellular structures, ribbon-shaped and twisted in form, with a basal nucleus, (Fig. 6). Since the gland is approximately the same width throughout there is no distinct neck region. The depth of penetration of the glands into the connective tissue is $134\mu \pm 2\mu$, and their width $13\mu \pm 2\mu$. The glandular secretion is finely granular and normally uniformly distributed throughout the cell, though it tends to shrink away from the cell membrane in Bouin-fixed material.
Fig. 4. Dorso-lateral region of mantle edge showing certain cutaneous glands.

d.c.g., duct of calcium gland;
pr., protein gland;
T.I., Type I mucous gland;
T.III., Type III mucous glands.
**Fig. 5.** Electron micrograph of mucus gland.

- **c.**, chromatin; **n.**, nucleus; **n.m.**, nuclear membrane;
- **v.(a)** and **v.(b)**, vesicles containing secretions of different appearance.

**Fixative:** 1% osmium tetroxide.

**Stain:** Lead hydroxide.
Fig. 6. Transverse section of mantle edge showing protein glands.

Fixative: Bouin.
Stain: Heidenhain's haematoxylin and orange G.

d.e., dorsal epithelium; m.g., mucous gland; pr.g., protein glands.
The results of histochemical studies on the secretion of the protein glands are given below.

<table>
<thead>
<tr>
<th>TEST</th>
<th>RESPONSE</th>
<th>CONCLUSIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. P.A./S.</td>
<td>Negative</td>
<td>The secretion is not carbohydrate in form.</td>
</tr>
<tr>
<td>2. Sudan black B.</td>
<td>Negative</td>
<td>No lipids are present.</td>
</tr>
<tr>
<td>3. Millon's reagent.</td>
<td>Positive</td>
<td>The secretion contains phenolic compounds, such as the amino acid tyrosine. The test is generally considered to indicate the presence of protein.</td>
</tr>
<tr>
<td>4. Ferric ferricyanide treatment</td>
<td>Positive</td>
<td>Treatment with ferric ferricyanide is a test for SS and SH groups, giving a precipitate of Prussian blue in their presence, whilst n-ethyl maleimide causes specific blocking of protein bound SH end groups (Pearse, 1960). The amino acids containing such groups are cysteine, cystine, and methionine, (Chevremont and Fréderic, 1943), and one or more of these acids is therefore present.</td>
</tr>
<tr>
<td>5. Blocking of SH end groups by n-ethyl maleimide, followed by (4).</td>
<td>Negative</td>
<td></td>
</tr>
</tbody>
</table>

The secretion is thus proteinaceous, containing tyrosine and one or more of the following: cysteine, cystine, and methionine. It has not been shown if the protein is simple or conjugated.

The secretion stains black with Heidenhain's haematoxylin, bright red with eosin, and orange-red with Mallory's triple stain. These reactions are of use in the identification of protein glands in routine histological sections.
(iii) Calcium glands.

These, like the rest of the cutaneous glands, are unicellular. They probably originate in the connective tissue, (see pp.123, 242), but since they are so closely associated with the epithelium they are considered here. The calcium glands are club-shaped and somewhat elongated. Their outer margin takes on an irregular shape after fixation in neutral formalin. The position of the ovoid nucleus is variable. It is usually embedded in the lining cytoplasm of the gland, towards the mid-point of its length. The nucleus is often obscured by granules of the calcium salt contained within the gland. The average height of the gland is $130\mu \pm 7\mu$ and the width $51\mu \pm 3\mu$. The salt granules are often grouped together into small clusters. A typical calcium gland is shown in Fig. 7.

The nature of the secretion of the calcium glands was determined histochemically.

1. Spherical granules, solitary or arranged in clusters, were found only in glands from material treated with a non-acid fixative, e.g. ethanol or neutral formalin.

2. Treatment of such sections with 1% hydrochloric acid resulted in loss of these granules, whilst hydrogen peroxide caused effervescence. From these results it can be inferred that the glands contain a water-insoluble carbonate salt.

3. Silver substitution by the Von Kossa (1901) method, (cited in Pearse, 1960), gave a black deposit within the glands, indicating the presence of carbonates and/or phosphates. Black melanin granules in the connective tissue outside the glands made it impossible to decide if salts were present there also.

4. Treatment of alcohol-fixed, frozen sections with nuclear fast red (McGee-Russell, 1955) which forms a dye-lake with calcium salts, was followed by a positive reaction. Red, birefringent precipitates of the calcium lake were produced both inside the glands and, to a
lesser extent, in the adjacent blood spaces. The staining time was short (30 seconds) and there was little diffuse coloration of the surrounding tissue. With paraffin-embedded material the results were less satisfactory; glands known to contain mucopolysaccharides stained heavily also, though treatment of control sections with 1% hydrochloric acid showed that the coloration here was not due to calcium salts.

5. Treatment of alcohol-fixed, frozen sections with alizarin red S gave poorer results. The spherical granules in some of the glands stained orange, the expected colour response for calcium salts, while granules in similar glands did not stain at all. The alizarin method is known to be extremely pH sensitive (Dahl, 1952) which may explain the inconsistency of the response.

6. The reactions above indicate that insoluble salts of calcium, carbonate and/or phosphate, are present in the glands and in the adjacent blood spaces. It is also possible that soluble calcium salts are present. The ammonium oxalate method of Rabl (1923) for demonstrating such salts was tried but with little success. There are no methods of established reliability for demonstrating the presence of such salts in tissue sections (Pearse, 1960).

7. The use of acid fixation, e.g. Bouin, or treatment of prepared sections with 1% hydrochloric acid resulted in the removal of calcium salts from glands and blood spaces. The glands still contained a secretion, however, which reacted in a way similar to that of the protein glands, (pp. 28, 32 ). The matrix of the calcium glands is thus proteinaceous.
Fig. 7. Alcohol-fixed frozen section of mantle edge showing deposits of calcium salts.

Stain: Nuclear fast red.

b.s., blood spaces containing calcium salts; c.g., calcium gland; c.t.c., connective tissue cell containing calcium salts; d.e., dorsal epithelium.
2. Connective tissue.

Underlying the dorsal epithelium is a layer of connective tissue whose thickness varies with the size of the animal and the state of contraction of the muscle cells. This connective tissue can be subdivided, (see p.21 ), into a dorsal pigmented region, a median highly vascular region, and a ventral region containing a mat of muscle fibres.

A. Dorsal pigmented region.

Scattered in the connective tissue immediately beneath the dorsal epithelium are numerous black pigment granules. After fixation, these granules are not confined to chromatophores but lie freely in the connective tissue. They are most densely aggregated just below the epithelium and form a thin layer around even the deepest-lying gland cells, often obscuring the basement membrane. The connective tissue below the level penetrated by the gland cells is devoid of pigment granules, (Fig. 2b).

The granules are insoluble both in water and in organic solvents. They dissolve in alkalis and are precipitated from solution by dilute acids. They are decolorised by hydrogen peroxide and reduce ammoniacal solutions of silver. Alkaline extractions of the pigment show no fluorescence in ultra-violet light. The above features are all characteristic of melanin, (Fox, 1953).

Examination of acidified methanol extracts of mantle edge tissue by ultra-violet light revealed no red fluorescence, and hence no uroporphyrins are present.

B. Median vascular region.

(i) Blood spaces.

Beneath the pigmented region of the mantle edge is a zone of highly vascular connective tissue. The blood spaces which ramify through this tissue give it a spongy appearance. Most of these
spaces are bordered by an extremely thin cellular layer, in which
tangentially flattened nuclei can occasionally be seen, (Fig. 8).
The lining layer is often indistinct. In some regions it is impos­
sible to discover by optical microscopy if the blood spaces do have
a lining layer of cells; they appear not to have, but it may be
that the cytoplasm of the cells is here too thin to be revealed by
light microscopy. The cytoplasm of those lining cells which are
clearly visible is basophilic. Muscle fibres are found close to
the walls of the blood spaces.

(ii) Blood.

The blood of Arion hortensis is composed of a non-coagulating
transparent plasma, of a pale straw colour though acquiring a bluish
tint after standing, suspended in which are blood cells. When
smears of blood taken from the heart, fixed in cold neutral formalin
and stained with either Delafield's haematoxylin and eosin or with
Ehrlich's thionine are examined, three types of blood cell can be
recognised, (Fig. 9).

Type I. cell.

These blood cells are fairly small and irregular in shape. Their
average dimensions after fixation in neutral formalin are $5 \mu \pm 0.4 \mu$
by $6 \mu \pm 1.5 \mu$. The cytoplasm is granular and frequently contains
darkly staining inclusions; it is distinctly eosinophilic, staining
deeply with eosin. The nucleus is polymorphic. The cell membrane
often has a crumpled appearance.

Type II cell.

These cells are larger, with a smooth, occasionally indistinct,
cell membrane; their average dimensions are $12 \mu \pm 1.2 \mu$ by $6 \mu \pm 0.4 \mu$.
They have clear, basophilic cytoplasm and an ovoid nucleus of length
$5 \mu \pm 0.5 \mu$. 
Fig. 8. **Sectioned blood space showing cellular lining.**

b.s., blood space;
n.l.c., nucleus of lining cell;
pr., protein gland, (sectioned transversely).
Fig. 9. Blood cells.

Fixative: Neutral formalin.
Stain: Delafield's haematoxylin and eosin.

b.c. I, II, and III., blood cells of types I, II, and III.
Type III cell.

These cells are relatively large, of average dimensions $12 \pm 0.1 \mu$ by $14 \pm 0.5 \mu$. The cell membrane is wrinkled after fixation and the cytoplasm is granular and acidophilic, staining lightly with eosin. Inclusions are sometimes present. The nucleus is spherical, of diameter $9 \pm 0.4 \mu$ and sharply defined.

(iii) Connective tissue ground substance.

The ground substance, which forms the bulk of the connective tissue, has two components; one amorphous and one fibrillar (Meyer, 1959). The amorphous component is a gel which disappears after digestion with hyaluronidase solution, applied as described in Pearse (1960). This gel gives a negative response to treatment with 2,2'-dihydroxy-6,6'-dinaphthyl-disulphide, (D.D.D.); shows pronounced basophilia; and stains lightly with Steedman's alcian blue. Considered together, these features indicate that it is a non-sulphated mucopolysaccharide, probably hyaluronic acid (Casselman, 1962).

For more precise identification the gel would first have to be extracted from the tissue. This was not attempted since other substances might also be extracted thus making any subsequent 'identification' valueless.

Embedded in the amorphous component of the ground substance are collagen fibres. These stain black after silver impregnation and yellow with Van Gieson's stain. They are all extremely thin.

(iv) Connective tissue cells.

Scattered through the connective tissue ground substance are a variety of cells, many of which are similar to those of the blood. All three types of blood cell, (p.37) occur in the connective tissue proper and it must be assumed that they can pass out from the blood spaces. A similar movement of blood cells into the connective tissue has been described by Haughton (1934) in Helix aspera,
Connective tissue cells.

c.t.(a), (b) and (c), connective tissue cells (a), (b), and (c);
n., nerve;
s.c.n., sheath cell nucleus.
The following cells are also present.

(a) Cells containing calcium granules are often seen near the calcium glands. Such cells are usually ovoid in shape (Fig. 10a, 10b) though occasionally cells with projections are seen (Fig. 7). The average dimensions of the cell are $16 \mu \pm 0.5 \mu$ by $12 \mu \pm 0.4 \mu$. Similar cells have also been seen in the digestive gland.

(b) Cells with darkly staining nuclei and reticular cytoplasm are also present (Fig. 10). The cytoplasm shows distinct basophilia and the cell membrane is wrinkled after fixation. The average dimensions of the cell are $13 \mu \pm 0.5 \mu$ by $15 \mu \pm 0.6 \mu$, while the diameter of the spherical nucleus is $5 \mu \pm 0.3 \mu$.

(c) A third type of connective tissue cell has faintly acidophilic cytoplasm of a reticular nature. The nucleus is spherical and its contents dense. The dimensions of the cell are $16 \mu \pm 1 \mu$ by $12 \mu \pm 0.6 \mu$. The diameter of the nucleus is $5 \mu \pm 0.4 \mu$, (Fig. 10a, 10b).

(v) **Muscle fibres.**

Smooth muscle fibres lie in the connective tissue between the blood spaces. They are very numerous; in an $8 \mu$ transverse section of the mantle edge an average of 20 fibres or parts of fibres can be seen in an area $25 \mu$ square.

In transverse, sagittal, and frontal sections a lattice-like arrangement can be seen. The muscle fibres forming this arrangement can be divided up into 6 groups, here arbitrarily named groups 1 to 6, and the fibres in any one group all lie roughly parallel to one another.

**Group 1.** These fibres lie at an oblique angle to the dorsal and ventral epithelia and in a vertical plane. They are sectioned longitudinally when the mantle edge is cut transversely.

**Group 2.** These lie at an angle to the group 1 fibres and are also sectioned longitudinally when the mantle edge is cut transversely.
Group 3. These lie at right angles to those of groups 1 and 2. They, too, lie in a vertical plane and at an oblique angle to the epithelia. They are sectioned longitudinally when the mantle edge is cut sagitally.

Group 4. These lie at an angle to those of group 3 and are also sectioned longitudinally when the mantle edge is cut sagittally. In most specimens they lie parallel to the ventral epithelium.

Group 5. These lie at an oblique angle to the dorsal epithelium but in a horizontal plane. They are sectioned longitudinally when the mantle edge is cut frontally.

Group 6. These lie at an angle to those of group 5 and are also sectioned longitudinally when the mantle edge is cut frontally.

The six groups of muscle fibres described above are illustrated in Figs. 11a, b, and c, and also in Fig. 12.

The dorsal epithelium of the mantle edge is curved and the muscle fibre arrangement found within the mantle tissue is such that when the fibres contract they exert a pull in a direction approximately normal to the tangent at the point of intersection of the epithelium and the fibre. This arrangement is mechanically efficient and the complexity of the muscle arrangement is probably correlated with the curvature of the epithelium.

The structure of the individual muscle fibres was investigated by both optical and electron microscopy in the course of this work. The fibres are definitely smooth in nature. No bands can be seen. Their diameter ranges from 1μ to 4μ in Bouin-fixed material. The total length of the fibres is not known for they have so far only been studied in sectioned material. Portions of fibres of length 175μ have, however, been noted. The microscopical appearance of the fibres is dependent on the method of fixation used. After the use of alcoholic fixatives followed by Azan staining the fibres take up the stain uniformly and no differentiation into central and peri-
Fig. 11 (a) Transverse section of mantle edge showing fibres of muscle lattice (i) microscopically, (ii) diagrammatically.

(i)

(ii)

d.e.m., dorsal epithelium of head;
d.e.m., dorsal epithelium of mantle;
v.e.m., ventral epithelium of mantle.
Fig. 11 (b) Sagittal section of mantle edge showing fibres of muscle lattice (i) microscopically, (ii) diagrammatically.

(i)

(ii)

d.e.m., dorsal epithelium of head; d.e.m., dorsal epithelium of mantle edge; m.c., mantle cavity; v.e.m., ventral epithelium of mantle edge; v.m., ventral mat of muscle fibres.
Fig. 11 (c) Frontal section of mantle edge showing fibres of muscle lattice (i) microscopically, (ii) diagrammatically.

(i)

(ii)

c.g., cutaneous glands; d.e.m., dorsal epithelium of mantle edge; m.c., mantle cavity.
Fig. II. Anterior region of slug with portion of mantle edge removed to show relative positions of fibre groups of muscle lattice.
Pheral zones can be seen. After Bouin fixation and Azan staining the effect is similar, but a distinct non-cellular polysaccharide sheath is seen around the fibres. Similar sheaths have been reported in many cells and Bennett (1963) has proposed the term "glycocalyx" for them. Using Susa fixation, again followed by Azan staining, the fibres show differentiation into a clear central core and a peripheral region which stains readily. The nucleus of the fibre is found in the central cytoplasmic core (Fig. 13).

Electron microscopy shows that there is a distinct, though thin, layer of clear cytoplasm around the extreme periphery of the muscle fibres. This region contains numerous mitochondria. The cytolemma, homologous to the sarcolemma of striated muscle, shows numerous prolongations, and the cytolemmas of adjacent cells interdigitate (Fig. 14). The amorphous appearance of the cytoplasm in alcohol and Bouin-fixed material is an artefact, for electron microscopy reveals that the muscle fibres are differentiated into myofilaments. These organelles are longitudinally disposed and lie oblique to the main fibre axis though roughly parallel to one another (Fig. 15). From the relatively low power electron microscopy used here it is impossible to discover if the myofilaments are all alike or if they are differentiated into two types, as has been shown by Hanson and Lowy (1957, 1961) in the oyster, and by Hanson (1957) in the muscle fibres of the squid and of *Helix pomatia*.

Muscle fibres are also found associated with the cutaneous glands. They are so arranged that they form a mesh-work around some of the glands. A similar arrangement has been noted in *Helix aspersa*, (Campion, 1957). The disposition of the muscle fibres is such that contraction would result in pressure on the gland followed by exudation of its contents.

(vi) **Nerves.**

The connective tissue of the mantle edge also contains branches
Fig. 13. Portion of mantle edge showing muscle fibres in transverse and longitudinal section.

Fixative: Susa.
Stain: Azan.

c.c., cytoplasmic core of fibre; m.f., muscle fibre in transverse section; m.f', muscle fibre in longitudinal section; n., nucleus of fibre; p.s., peripheral sheath of fibre.
Fig. 14. Electron micrograph of junction between adjacent muscle fibres.


- c.c., layer of clear cytoplasm immediately beneath the cytolemma;
- m., mitochondria; p.c., prolongation of cytolemma;
- p.s., peripheral sheath of muscle fibre containing myofilaments.
Fig. 15. Electron micrograph showing myofilaments lying oblique to the longitudinal axis of the muscle fibre.

Fixative: Osmium tetroxide.
Stain: Phosphotungstic acid.

my., myofilaments;
p.s., peripheral sheath of muscle fibre sectioned longitudinally.
of the pallial nerves. Attempts to trace the fine nerve endings by silver impregnation proved unsuccessful. The branches of the nerves are covered by sheath cells (Fig. 10a, 10b).

C. Ventral muscular region.

Immediately above the ventral epithelium the connective tissue contains a mat of muscle fibres. These fibres are arranged either at right angles to or parallel to the longitudinal axis of the body. The mat is best developed at the junction of the mantle edge with the roof of the mantle cavity and least developed at the free margin of the mantle edge, where it consists of a layer only two or three fibres in depth (Fig. 16). Where the ventral epithelium is highly glandular the muscle mat is interrupted and the fibres form a thin network around the glands. The structure of the fibres forming the ventral muscle mat appears to be similar to that of the other muscle fibres (p. 48).

3. Ventral epithelium.

This, like the dorsal epithelium, consists of non-glandular and glandular cells.

A. Non-glandular cells.

These are usually cubical in form and of $11\mu \pm 1\mu$ in height. The cells nearest the free margin of the mantle edge are somewhat taller than those forming the rest of the epithelial sheet. Each cell has an ovoid nucleus, whose longitudinal axis lies at right angles to the free surface of the epithelium. The length of the nucleus is $4\mu \pm 0.6\mu$. The cytoplasm of the cells is slightly granular. Those cells lying near the pneumostome are ciliated.

The cells lie on a thin basement membrane which is not masked by pigment as in the case of the dorsal epithelium. After Azan
Fixative: Bouin.

Stain: Heidenhain's iron haematoxylin and orange G.

b.s., blood spaces; m.g. IV, mucous gland type IV; v.e., ventral epithelium; v.m., ventral muscle mat.
staining the basement membrane appears as a thin blue line; after P.A./S. treatment it has a faint pink colour.

B. Glands.

The glands associated with the ventral epithelium are all unicellular and extend into the sub-epithelial connective tissue. They are of two types:

(i) mucous glands;
(ii) calcium glands.

No protein glands are present though these are common on the dorsal surface.

(i) Mucous glands.

The most common are of a type not found on the dorsal surface. To avoid confusion these will be designated 'Type IV' glands.

Type IV. Large numbers of these glands are frequently found clustered together, and where contact occurs the cell bodies are polygonal. The bodies of isolated cells are spherical. The glands extend deeply into the connective tissue, forming a thick, compact mass. All open separately to the exterior, and consequently the deepest-lying glands have the longest necks. The average diameter of the glands is 14 \( \mu \) ± 0.7 \( \mu \), and the length of the central, ovoid nucleus is 5 \( \mu \) ± 0.4 \( \mu \). The glands extending furthest into the connective tissue are as much as 75 \( \mu \) long.

The cytoplasm of the glands appears granular after Bouin, Susa, and Zenker fixation, and is therefore quite distinct from the reticular cytoplasm of the mucous glands previously described (pp.23-28). After formalin fixation, however, numerous spindle-shaped units are visible within the glands. The gland secretion reacts histochemically in a manner similar to that described for the dorsal mucous glands, (p. 26), and is therefore acid mucopolysaccharide in character. It gives a faintly positive response to P.A./S. treatment, does not
take up Sudan black B, is not affected by amylase digestion, and
gives strong, (γ), metachromasia with toluidine blue and thionine.
The significance of these reactions is explained elsewhere (p. 26).
In two respects the Type IV glands stain differently from the other
mucous glands: they stain pink with Mallory's triple stain whilst
the other mucous glands stain blue; and they give a stronger meta-
chromasia with thionine than do the others. Type IV cells are
illustrated in Fig. 17.

Near the junction of the ventral with the dorsal epithelium
mucous glands of Types I, II, and III, are found. Calcium glands are
also occasionally present.
Fig. 17. Transverse section of mantle edge showing Type IV mucous glands.

Fixative: Susa.
Stain: Delafield's haematoxylin, eosin, and alcian blue.

b.s., blood space; m.g.IV, mucous gland type IV; showing central nucleus and granular secretion; n.m.g., neck of mucous gland; v.e., non-glandular cells of ventral epithelium.
DISCUSSION.

The structure of the mantle edge of *Arion hortensis* is similar, in its main features, to that of homologous regions of other gastropods. Any significant differences can be correlated with the mode of life of the animal.

*A. hortensis* is a terrestrial mollusc, but one restricted to regions of relatively high humidity. It is found on the surface of the soil, usually hidden beneath vegetation and debris. It is inactive during the day and emerges to feed at dusk when the temperature falls. It cannot tolerate desiccation.

As in all gastropods, the surface epithelium is only one cell thick, and lubrication is essential if damage by friction is to be reduced. There is no keratinisation nor cuticle production, and consequently no waterproofing of the skin. This in itself restricts the species to moist environments. Added to this is the absence of a shell, a structure which normally gives protection and reduces desiccation. In the slug one would expect the body wall to be modified to compensate for loss of the shell.

Mucous, calcium and protein glands, all large though unicellular, extend deeply into the sub-epithelial connective tissue. In this way the local concentration of the glands, and the amount of secretion produced, is increased. In aquatic gastropods, e.g. *Gibbula cineraria* and *Diodora apertura*, deeply-placed cutaneous glands are found in the foot only, (Fretter and Graham, 1962). In terrestrial gastropods with shells deeply-placed glands are present in the mantle skirt as well as in the sole of the foot, (Campion, 1957). In *A. hortensis* they are distributed over the whole of the body surface. It is possible that the distribution and concentration of cutaneous glands is correlated with their function and the demand for secretory products. Campion, (1957, '61), found that in *Helix* the mucous glands are responsible for lubrication, the calcium glands for
adhesion, and the protein glands, enhanced by the calcium secretion, for defence. In a gastropod without a shell, lubrication and defensive secretion will be greatly in demand, and this may help explain the high concentration of glands.

Four types of mucous gland are present in the mantle edge of *A. hortensis*. Of these Type IV is restricted to the ventral epithelium. The gland designated as Type I appears, from its form and staining reactions, to be homologous to the Type A gland found by Campion (1957, '61) in *Helix*, and to the "Faketdrusen" of Herfs (1922). It is possible that the Type I and Type II glands of *Arion* are phases in the secretion of a single type. Evidence from comparative cell dimensions and histochemistry (pp. 23-28) supports this view. The Type III glands may represent an early stage in the development of the mucous glands. The Type IV gland is probably homologous with the Type B gland described by Campion in *Helix*, since their morphology and histochemistry are similar. It is also interesting to find that, apart from differences in size, the morphology and staining reactions of the protein glands of *Helix* and *Arion* are similar.

Specific staining shows that calcium salts are present in quantity in the mantle and associated tissues of gastropods. The actual position of these salts has been a matter of dispute for some time. Manigault (1939) described calcium cells in the epithelium of *Helix pomatia* but Heaysman (1951) did not think this interpretation was correct. She held that the "calcium cells" of Manigault were actually mucous cells. Her work on *Helix pomatia*, *Paludina vivipara*, and *Nucella lapillus* brought her to the conclusion that in these animals calcium salts were present in the blood spaces only and not in cellular glands. This interpretation was challenged by McGee-Russell (1954) and Campion (1957, '61), who both claimed that calcium glands exist in the mantle tissue. In *A. hortensis* crystals of calcium salts are present in quantity in definite glands and to a lesser extent in the blood spaces around those glands. Cells,
sometimes with pseudopodia-like projections, and containing calcium salts, are also found in the connective tissue. The methods involved in the preservation and staining of calcium salts are not conducive to good fixation and staining of the surrounding tissue; distortion and loss of cellular detail are inevitable. It is possible that these factors are responsible for the differences in interpretation noted above.

A further problem is the origin of the cutaneous glands. The theory that they might originate in the connective tissue was first proposed by Leydig (1876). He referred to the calcium and protein glands specifically. Prenant (1924), Barr (1928), and Campion (1957) also supported this theory but extended it to cover all the cutaneous glands. A second theory, that some or all the gland cells might have an epithelial origin, also had its supporters. Leydig (1876) thought that mucous glands might be epithelial but the chief exponent of this concept was Roth (1929). In *A. hortensis* the mucous glands are certainly epithelial in origin. Developmental stages of these glands, the Type III glands, are often found intercalated between the non-glandular epithelial cells; only later do they extend into the connective tissue. Furthermore, the thin basement membrane underlying the non-glandular cells is continuous around the deeply-placed mucous glands. Had these glands been of connective tissue origin and grown up towards the epithelium it would be difficult to account for this finding. Experimental evidence supporting the epithelial origin of the mucous glands is to be found elsewhere (pp. 119, 242), as is evidence indicating that the calcium glands are probably of connective tissue origin (pp. 123, 242). Nothing is known of the origin of the protein glands.

It is surprising to find that while in *Arion ater* large quantities of free uroporphyrin are present in the body wall (Kennedy, 1959), none is to be found in the mantle edge of *A. hortensis*. Kennedy suggested that in *A. ater* there is a directly proportional relationship between the amount of free uroporphyrin, a photosensitive
pigment, and the degree of masking by melanin. In *A. hortensis* melanin is present in some quantity but it is not here masking uroporphyrin.

The connective tissue of the mantle edge is highly vascular in all pulmonates. Through it ramify blood spaces or sinuses which give the tissue a spongy appearance. According to Fretter and Graham (1962) these spaces are, in prosobranchs, lined by an endothelium, although the larger venous sinuses occasionally have no such lining. In *A. hortensis* it is usual to find that the smaller blood spaces are bounded by a distinct endothelium, while it is doubtful if the larger are so lined. The endothelial cells are very flattened and the nucleus peripherally placed,—features which may well be fixation artefacts as was recognised by Barfurth, (1885), and later by Cuénot (1892).

The blood cells of molluscs have been described in many different terms. They have been called "leucocytes" (Kollmann, 1908; Goodrich, 1919); "mucus cells" (Cretin, 1923); "amoebocytes" (Wagge, 1951); and "blood cells" (Fretter and Graham, 1962). The term 'blood cell' is used in this account because of its lack of ambiguity; any cell found in the blood plasma is termed a blood cell. The presence of three distinct types of cell in the blood of *A. hortensis* is of interest, since George and Ferguson (1950) also report the presence of three cell-types in the blood of the stenoglossans they examined. In *A. hortensis*, as in the stenoglossans, two of the cells have granular cytoplasm, while the third has clear, basophilic cytoplasm. Cuénot's view (1892) that only one type of cell is present in the blood of gastropods, is now known to be incorrect, as is Kollmann's opinion that few gastropods have "leucocytes" with granular cytoplasm. There is no self-evident reason why all gastropods should have the same types of blood cells, however, and to generalise on this would be rash. The presence of cell similar to the blood cells in the connective tissue of *A. hortensis* indicates that here, as in *Helix aspersa*, (Haughton, 1934), diapedesis can occur.
The muscle fibres of the mantle edge of A. hortensis are in some respects similar to the smooth muscle fibres of the squid, as described by Hanson and Lowy (1957) and by Lowy and Hanson (1962). In both cases the fibres have a central core of cytoplasm, containing a nucleus, with the myofilaments arranged around this core at an oblique angle to the longitudinal axis of the fibre. In the fibres of A. hortensis, however, there is a peripheral sheath of cytoplasm containing mitochondria beneath the cytolemma. In the smooth muscle fibres of the squid the mitochondria are restricted to the fibre core. In A. hortensis, where two muscle fibres touch their cytolemmas interdigitate. The interdigitating processes are long and fine and ensure intimate contact between the cells. There is thus a possibility that the fibres, which are arranged in a lattice-work in the connective tissue of the mantle edge, can act as an anatomical entity in a co-ordinated way, rather than as individual muscle fibres. It is not possible to say with certainty whether or not true intercellular bridges link adjacent muscle fibres in A. hortensis. Such bridges have been found in vertebrate smooth muscle, (Bergmann, 1956; Mark, 1956), though other authors, (Caesar, Edwards, and Ruska, 1957), question their presence.

The arrangement of the muscle fibres of the mantle edge, though at first sight somewhat complex, is of a basically simple pattern. There is a ventral muscle mat, consisting of fibres arranged at right angles to each other, with a three-dimensional lattice of muscle fibres above it, forming a net-work in the connective tissue. The lattice is composed of 6 different groups of fibres, and it is through the meshes of the lattice that the blood spaces ramify. It is shown elsewhere (pp. 81-87, 184) that the size of the blood spaces is determined by the state of contraction of the lattice and mat fibres. A muscle fibre arrangement of this type has not, as far as is known, been previously reported in the body wall of pulmonates.
IV. STRUCTURAL AND METABOLIC CHANGES IN THE MANTLE EDGE DURING WOUND HEALING.

INTRODUCTION.

The ability of organ regeneration is said to decline as structural complexity increases (Pribram, 1909). It is perhaps because of this 'law' that regeneration studies in invertebrates have been concentrated on members of the lower phyla. There is little evidence, however, that the ability of tissue regeneration follows the same trend. Studitskii (1954, cited in Vorontsova and Liosner, 1960) recently advanced a totally different view. He believes that as animal structure becomes more complex, the level of regenerative ability not only does not diminish but may even increase. Vorontsova and Liosner think this interpretation to be of great theoretical importance. It is obviously of considerable interest to see if tissue regeneration such as wound healing can be readily effected in a complex invertebrate such as Arion hortensis.

The molluscs were among the first animals in which regeneration was studied. As early as 1768, Spallanzani discovered that garden snails could regenerate the anterior part of the head. Interest in this phenomenon waned and at the end of the nineteenth century research on molluscan shell regeneration was commenced. Since then most of the work on molluscan regeneration has been on repair of the shell, (de Villepoix, 1892; Manigault, 1939; Wagge, 1951; et al.). Regeneration of other molluscan organs has also been studied though to a lesser degree. Techow (1910) studied regeneration of the mantle, foot, and tentacles after wounding by excision; Hanco (1913) and Lange (1920) investigated healing of the cephalopod arm; regeneration of the foot in gastropods was studied by Abeloos (1941) and Pande (1953) et al.; and regeneration of the gonad by Laviollette (1950). Interest in physiological regeneration, such as radula replacement, (Runham, 1962), has increased of late.
What is known, from previous work, of the stages in molluscan wound healing is listed below.


2. Blood cells accumulate at the wound site, provisionally closing the wound, (Techow, 1910; Hanko, 1913; Cucagna and Nusbaum, 1915; Lange, 1920; Vorontsova and Liosner, 1960).

3. Soon after wounding the epithelial cells surrounding the wound move over its surface and become very attenuated, (Pande, 1958; Vorontsova and Liosner, 1960). The epithelial cells may divide, (Techow, 1910).

4. After covering the wound the epithelial cells regain their original cuboid shape, (Pande, 1958). If the wound is of the excised type, the epithelium covers the agglutinated blood cells rapidly, (Lange, 1920); if, however, the wound was produced by cautery, a scab of burnt tissue lies external to the blood cells, and this is shed before epithelial migration commences, (Pande, 1958). Retardation of epithelial movement after cautery is also found in mammals.

5. The demolition phase of wound healing follows provisional closure of the wound. Damaged cells and foreign particles are phagocytosed by amoebocytic blood cells, (de Villepoix, 1892; Pande, 1958; et al.).

6. The next stage is the development of a blastema, a mass of undifferentiated cells which form replacement tissue and complete wound healing. There is some doubt as to the origin of molluscan blastemata. Techow (1910) thought that the blastema was composed of immigrant cells; Hanko, (1913), thought it was indigenous, that is, composed of local cells; Lange (1920) described a bipartite blastema, partly immigrant and partly indigenous. Kriukova (1935, cited in
Vorontsova and Liosner, 1969) provided evidence that muscle cells regenerate from cells produced by de-differentiation at the wound site, while Pande (1958) claimed that muscle cells regenerate from de-differentiated local cells which immigrate into the wound from the surrounding uninjured tissue. Further work is obviously needed on this point.

7. The blastema gradually increases in size. Workers on molluscan regeneration seem wary of indicating definitely how blastema growth is effected. Cell division must occur, but where and of which cells is a matter of some doubt. Lange (1920), working on cephalopod regeneration, claimed that myoblasts divide mitotically within the blastema but did not report mitoses within regenerating nerve tissue. Kriukova (1935) also reported mitoses in molluscan myoblasts, while Pande (1958) was unable to identify mitotic figures in either myoblasts or blastema epithelium and claimed, furthermore, that the amoebocytes which accumulate at the wound divide amitotically. It has been suggested that in insects epithelial mitoses are rare early in healing, though they become more frequent later, (Wigglesworth, 1937). Similar findings have been reported for the epithelial cells of vertebrates, (Abercrombie and Harkness, 1951).

8. Blastema growth is followed by differentiation. Where the blastema is produced by immigrant cells it is usual to find that these cells are pluripotent, e.g. in the Turbellaria and Annelida, (Needham, 1952). Whether or not the blastema cells of molluscs are also pluripotent is not yet known. It seems likely that while some blastema cells are immigrant others, e.g. the myoblasts, are produced by de-differentiation of local cells. The evidence available, (Kriukova, 1935; Pande, 1958; et al.), suggests that myoblasts are capable only of re-differentiation into muscle cells.
Little information is available on the metabolic changes which take place during molluscan regeneration, though this aspect of the regenerative process has been studied extensively in mammals, especially during the past two decades. The following review is restricted to the enzymes acid and alkaline phosphatase and the metabolites glycogen, ribonucleic acid (R.N.A.), and the SH and SS-containing compounds.

The acid phosphatases are thought to play a role in the demolition phase of regeneration. There is an increase, in vertebrates, in local acidity during this phase (Needham, 1952) and this is accompanied by an increase in the level of acid phosphatase activity, (Bodian and Mellors, 1945). Acid phosphatase is generally associated with cell degeneration, (Yao, 1950), and its function is, presumably, to liberate energy by the breaking of energy-rich phosphate bonds, such as those available in adenosine triphosphate (A.T.P.).

It has been suggested that alkaline phosphatase is associated, in shell-bearing molluscs, with growth and regeneration of the shell, (Wagge, 1951; Bevelander, 1952), possibly because of its role in calcium metabolism, where it controls the deposition of calcium as a phosphate. A possible relationship between phosphatase activity and protein synthesis has been brought to light by the work of Fell and Danielli (1943) and Danielli, Fell, and Kodicek (1945), using vertebrate material. Needham (1952) claimed that the enzyme is most active in the differentiation phase of regeneration, as shown for ontogenesis by Brachet (1950). Recently Carranza, Fermina, and Cabrini (1963) showed that in mammals, though connective tissue repair is associated with high alkaline phosphatase activity, epithelial proliferation is not. The main function of the enzyme is in most cases not the deposition of inorganic phosphate but the liberation of energy by the breaking of high-energy phosphate bonds. It is thus of interest that A.T.P., which contains such bonds, does in fact promote differentiation, (Jaeger and Barth, 1948).
Danielli (1954) and Bradfield (1951) have also pointed out that there is a correlation between the distributions of alkaline phosphatase and fibrous proteins.

Weisz (1948), working on regeneration in the protozoan Stentor, observed that carbohydrate reserves were used up during the healing process, and that regeneration could not occur in total absence of carbohydrate. Pande's work (1958) on glycogen distribution during molluscan wound repair, showed that glycogen accumulates in the blastema early in the process and is gradually used as a source of energy by the developing cells in the wound region. During glycolysis glycogen combines with inorganic phosphate to give glucose mono-phosphate by a reversible process (Soskin and Levine, 1949). The latter is degraded stepwise into other phosphorylated compounds which are used either to provide energy or for synthesis of cell components. The phosphatases are actively concerned in glycogen degradation.

It is well known that R.N.A. participates in protein synthesis, a process essential to growth and consequently to regeneration and wound healing. Long ago it was suggested that R.N.A. was synthesized during regeneration (Ide-Rozas, 1937; Weitzmann, 1937; Kedrowski 1941; et al.). More recently Pande (1958) showed that in Helix aspersa the amount of R.N.A. within regenerating tissues is at first low, then gradually increases during blastema growth, but decreases during differentiation. Williamson and Guschlbauer (1961, '63) showed by autoradiography that R.N.A. is formed rapidly during the early stages of mammalian wound healing.

SH and SS groups are found in a variety of enzymes and also in the fibrous protein of muscle, etc., all of which are formed during regeneration. The importance of sulphydryl groups in regeneration has been realised for some time. They are, for instance, more common in the regeneration blastemata of the oligochaete Stylaria than in other tissues of the body, (Chu, 1948). They are also characteristic...
of those planarian tissues which have high regenerative powers, (Goldsmith, 1934). They promote the action of cathepsins in the demolition phase of vertebrate regeneration, (Hammett and Levine, 1940), and also promote mitosis, (Riley, 1940, et al.). Disulphide groups seem to promote differentiation, (Hammett and Smith, 1931). Work on the relative importance of the sulphydryl and disulphide groups was reviewed by Williamson, (1956, '57).

In the present investigation into wound healing in *Arion hortensis* special attention was paid to the following aspects of the process:
1. The method of wound closure.
5. The onset of differentiation.

The distribution of the following substances was determined histochemically:
1. Acid phosphatase.
2. Alkaline phosphatase.
3. Glycogen.
4. Ribonucleic acid.
5. Sulphydryl and disulphide containing compounds.

An attempt was made to associate changes in structure with changes in metabolism.
METHODS USED.

The slugs used in this investigation were collected at least one week before wounding and kept in the laboratory in the manner described on p. 9.

Each experiment involved 30 animals, which were taken from the stock jars immediately before use. The slugs were narcotised in groups of 3 at intervals of 2 minutes, during which period specimens already narcotised were operated on. 450 animals were used in the course of this work.

1. Method of narcosis.

The three animals in each group were placed in a covered crystallising dish. A piece of folded, moistened filter paper sprinkled with a few crystals of menthol was placed in the dish to one side of the slugs. Once movement had ceased the animals were operated on.

2. Design of operating forceps.

A cylindrical portion of mantle edge tissue was excised from each specimen. The excisions were made with sterile operating forceps, specially designed so that the wounds were uniform in size and shape (Fig. 18 a). The cutting edge was the hardened silver-steel rim of a steel cylinder of length 4 mm. and diameter 2.5 mm., screwed near the end of one blade of a pair of stainless steel watchmaker's forceps. The original pointed tips of the forcep blades had been cut off so that they ended in a straight, blunt edge. The rim of the steel cylinder met the inner face of the opposing forcep blade when the forceps were closed.

3. Wounding technique.

The forceps were held with the blades horizontal. The lower
blade was inserted between the mantle edge and the dorsal integument of the head in the mid-line. The forceps were gently eased forwards till the tip of the lower blade touched the junction of the head integument with the mantle edge (Fig. 18 b). They were then closed and a cylindrical portion of the mantle edge excised. The forceps were removed by gently pulling them forwards. The narrow strip of tissue anterior to the cut made by the steel cylinder always tore as the forceps were removed, but apart from this the wound was cleanly made, (Fig. 19 a). During the operation the slug was held in a loop of Kleenex tissue.

4. Post-operative procedures.

After wounding, the three animals in each group were placed in a covered crystallising dish, lined with moist filter paper, and containing food. They were kept at a constant temperature of 15°C and under constant conditions of illumination throughout the experiment.

5. Histological techniques.

After fixation, usually in Bouin, the anterior region of the body was removed, dehydrated, and embedded in paraffin wax, m.p. 54°C, to which a trace of ceresin had been added. Sections were cut at 5μ or 8μ and then stained. The chief stains used were:

(i) Heidenhain's Azan;
(ii) Delafield's haematoxylin, with eosin and Steedman's alcian blue
(iii) Heidenhain's iron haematoxylin, counterstained with orange G.

The study of nervous tissue presented some difficulty. There are no wholly specific methods for staining nerve fibres and cells so it was necessary to compare the results of a number of different methods:

(i) Combined fixation and staining with 1% thionine in 10% formalin, (Gurr, 1956).
**Fig. 18 (a) Structure of operating forceps.**

**Fig. 18 (b) Method of wounding.**

f.b., forcep-blade inserted between mantle edge and head; m.e., mantle edge; p., pneumostome; s., screw attaching steel cylinder to forceps; s.c., steel cylinder with cutting rim; t., tip of forceps with point removed; w.f., watchmakers' forceps.
(ii) Treatment of freshly dissected blastemata with acidified methylene blue, (Alexandrowicz, 1932).

(iii) Treatment of freshly dissected blastemata with reduced methylene blue, (Batham, Pantin, and Robson, 1960).

After treatment (i) the material was embedded, sectioned, and mounted in D.P.X. After treatments (ii) and (iii) the blastemata were washed in distilled water, fixed in 8% ammonium molybdate at 0°C, (Pantin, 1948), dehydrated, and mounted whole in Canada balsam.

Other stains were occasionally used where this was thought desirable.

6. Histochemical techniques.

The methods of fixation and staining used were dictated by the composition of the substance under investigation. For each experimental series, 3 animals were killed at intervals of 1, 2, 4, 7, 14, 21, 28, and 35 days after wounding.

(a) Acid phosphatase. The material was fixed in acetone at 4°C for 3 hours and then dehydrated and embedded in paraffin wax in the usual manner, allowing one hour for infiltration. Sections were cut at 8μ and then treated according to the modified lead nitrate method for acid phosphatase, (Pearse, 1960, after Takeuchi and Tanoue). Two hours were allowed for incubation. For control slides, the enzyme substrate, sodium β-glycerophosphate, was omitted from the incubation medium. Care was taken to keep the pH of all incubation media constant at 5. The sites of acid phosphatase are marked by a dark brown precipitate with the above method.

(b) Alkaline phosphatase. The material was fixed in 95% ethyl alcohol at 4°C for 24 hours according to Gomori's method, (1946, cited in Pearse, 1960). It was dehydrated, embedded in paraffin wax, allowing one hour for infiltration, and sectioned at 8μ.

For one series of experiments the sections were treated according to Fredricsson's modification of the Gomori method for alkaline phosphatase, (Pearse, 1960).
For a further experimental series the material was fixed and embedded in the same way, but the paraffin sections were then treated according to Pearse's (1960) modified azo-dye method. Using this technique the sites of enzyme activity appear reddish-brown. The azo-dye used in this work was 5-chloro-o-toluidine.

Control sections were incubated in a medium similar to that with which the experimental sections were treated, except that the enzyme substrate, (sodium-α-glycerophosphate in the first series of experiments and sodium α-naphthyl phosphate in subsequent work), was omitted.

All incubation media were maintained at pH 9.

(c) Glycogen. The material was fixed in cold absolute alcohol for 24 hours and then sectioned at 8 μ in the usual way. Control sections were incubated in a 1% solution of diastase in distilled water at 37°C for 2 hours to remove the glycogen. The remainder of the sections were incubated in pure distilled water under similar conditions. The material was then stained with Best’s carmine according to the method of Gurr (1953), using Ehrlich’s haematoxylin as a counterstain.

In trial experiments the Periodic acid / Schiff, (P.A./S.), reaction was used, but it was found that the molluscan integument contained so much mucopolysaccharide, another P.A./S. positive substance, that the glycogen could not be accurately located.

The mode of action of the carmine solution depends on physical and stereochemical factors not yet fully understood and the use of an empirical method such as that of Best is not easily reconcilable with the histochemical point of view. It does, however, give clearly defined results and its application presents few technical difficulties.

(d) Ribonucleic acid. (R.N.A.). The material was immersed in Carnoy fixative for 3 hours, dehydrated, embedded in paraffin wax, and sectioned at 8 μ. Control sections were incubated in a solution
of 0.03 gs. crystalline ribonuclease in 60 ml. distilled water for 1 hour at 37°C (Casselman, 1962). To ensure that the ribonuclease solution was free from active protease it was heated to 80°C for 10 minutes before use, (Sanders, 1946). The remainder of the sections were incubated in pure distilled water under similar conditions. After incubation the sections were stained in a 1% solution of toluidine blue, and counterstained with 2% orange G in 90% alcohol for 30 seconds. They were dehydrated rapidly with tertiary butyl alcohol (Stowell, 1946), cleared in xylol and mounted in D.P.X. The ribonucleic acid content of the regenerating tissue was estimated by visual comparison of the basophilia of control slides treated with crystalline ribonuclease with that of others which had not been so treated.

In a further experiment similarly incubated material was treated according to the methyl-green/pyronin method of Brachet (1942) but the R.N.A. did not stain as expected. The reason for this failure is not known.

(e) SH and SS groups. The material was fixed in cold, neutral formalin for 3 hours, dehydrated, embedded in paraffin wax, (allowing 1 hour for infiltration), and sectioned at 8μ. In one series of experiments sections were treated according to the ferric ferri cyanide method of Chéremont and Fréderic (1943) and the nuclei counterstained with carmalum. In a further experimental series sections were treated according to the dihydroxy-dinaphthyl-di-sulphide (D.D.D.), method of Barrnett and Seligman (1952, '54, cited in Pearse, 1960).

For both series control sections were incubated in a solution of 0.1 M n-ethyl maleimide in a 0.1 M phosphate buffer at pH 7.4 for 4 hours at room temperature (Pearse, 1960). n-ethyl maleimide is an effective block for SH groups, entirely preventing them from reaction in subsequent tests.
OBSERVATIONS.

STRUCTURAL CHANGES.

A. Macroscopic observations.

The changes in the external form of the wound are most marked during the early stages of the healing process.

1. Immediately after wounding.

As soon as the operating forceps are withdrawn the wound appears as shown in Fig. 19 a. The surface of the wound is grey in colour, due to the scattering of pigment granules through the connective tissue when the excision was made. The head integument, where normally covered by the mantle edge, is non-pigmented, and this lighter zone is visible through the perforation made on wounding. The injured surface is covered by a mixture of blood and mucus.

2. 10 minutes after wounding.

The margin of the wound, originally circular, now forms a horizontal crescent, (Fig. 19 b). The lateral edges of the wound have been withdrawn, presumably by muscular contraction, and the general appearance suggests that a crescentic perforation had initially been made. The cut surface, originally vertical, now faces downwards and is therefore hidden from view.

3. 3 days after wounding.

The cut surface is again vertical in position. If it is examined closely, a thin band of greyish-white tissue, readily distinguishable from the heavily-pigmented tissue of the adjacent uninjured regions, can be seen. This is the newly-formed blastema, which will ultimately form the regenerate. There is no pronounced scab formation. The appearance of the wound at this stage is shown in Fig. 19 c.
4. 7 days after wounding.

The blastema has increased in size and is now more readily seen. It extends forwards, anterior to the excision line, which is marked by the junction of black, uninjured mantle tissue with greyish-white blastema, (Fig. 19 d). The crescentic wound margin is now somewhat shallower than at earlier stages.

5. 10 days after wounding.

The wound has become increasingly shallow, partly as a result of blastema growth and partly because the lateral wound edges have retracted, (Fig. 19 e).

The later stages of healing are concerned mainly with differentiation of the blastema. These changes cannot be followed macroscopically. At 3 weeks, differentiation is near completion; the regenerate, which the blastema has now become, has gradually darkened due to pigment deposition, but is still lighter than the uninjured mantle tissue. At 2 months, the only indication of wounding is the slighter colour of the regenerate as compared with the rest of the mantle edge, (Fig. 19 f). After 4 months the regenerate is indistinguishable externally from the original mantle edge.
Macroscopic appearance of mantle edge after wounding.

(a) On wounding.

(b) After 10 minutes.

(c) After 3 days.

(d) After 7 days.

(e) After 10 days.

(f) After 2 months.

b., blastema; j., junction between mantle edge and head; m.e., mantle edge; n-p.z.h., non-pigmented zone of head integument; p.z.h., pigmented zone of head integument; p., pneumostome; r., regenerate; w.e.(1) wound edge initially circular; w.e.(2) wound edge now crescentric and facing downwards.
B. Microscopic observations.

1. 1 hour after wounding.

The wound margin is slightly ragged. A thin layer of blood and mucus covers the injured surface.

The dorsal epithelium was torn during wounding and a considerable area of connective tissue exposed. The non-glandular cells were stripped off cleanly, but the glands, firmly embedded in the connective tissue, remain in position although damaged. The non-glandular cells adjacent to the exposed connective tissue have flattened and, as experimental work described elsewhere (p.202) has shown, have already begun to move over the wound surface. Some piling up of the epithelial cells has occurred at the free edge of the migrating epithelial sheet which is here and there several cells deep. The longitudinal axes of the oval nuclei, normally arranged at right angles to the basement membrane, now lie parallel to it. No mitotic figures can be seen. The migrating cells of the dorsal epithelium are separated from the basement membrane by a narrow space (Fig. 20).

The non-glandular cells of the ventral epithelium, in regions adjoining the wound surface, are also attenuated. Their nuclei are now some 19 μ apart, as compared with 7μ in regions distant from the wound. The cells near the wound surface are not, however, separated from the basement membrane by a well-defined space such as was found below the dorsal epithelium.

In transverse sections of the mantle edge, the blood spaces appear smaller immediately adjacent to the wound than in either the injured tissue of the wound region itself or in the more distant uninjured tissue (Fig. 21 and Appendix 1). The areas of the sectioned blood spaces were estimated with the aid of an ocular graticule under an oil immersion objective.

There is no apparent increase in the number of blood cells in the
Fig. 20. *Migrating epithelium separated from basement membrane.* (1 hour after wounding).

b.m., basement membrane; b.s., blood space; d.e., dorsal epithelium (multi-layered in places); e.n., epithelial cell nucleus; s., space separating epithelial cells from basement membrane.
Fig. 21a. Frequency histograms showing change in area of blood spaces during early stages of healing.

I unit of area represents 625 μ².
Fig. 21 (b). Change in area of blood spaces of regenerate with age.
wound region, but a few are found in the mucus which coats the damaged ends of the blood spaces.

The connective tissue also shows little change. There is no increase in the number and distribution of the connective tissue cells and the hyaluronic acid component of the ground substance stains normally. The only noticeable change is that the collagen fibres, which in uninjured tissue are usually straight, are now wavy.

Many muscle cells were severed on wounding, and such cells are now twisted in form. This distortion leads to loss of their lattice like arrangement, though in adjacent undamaged regions it persists.

The cells or fibres of the muscle lattice lie between the blood spaces and it is possible that they control the size of the latter. To test this an attempt was made to measure the degree of contraction of the muscle fibres, and any changes in their positions relative to one another, with a view to correlating these findings with the size of the blood spaces.

Degree of contraction was estimated by measuring the diameter of the muscle fibres, since decrease in length must be accompanied by increase in diameter if it is assumed that the volume remains constant. Length could not be measured directly, for the whole of a muscle fibre is seldom visible in one thin section. The measurements made were restricted to fibres of groups 1 and 2 (see Figs. 11 and 12) and are shown in Fig. 22a and Appendix 2a. The acute angles formed when fibres of the two groups cross were also measured using a graticule marked in degrees and these measurements are shown in Fig. 22b and Appendix 2b. Changes in the diameter of fibres from the ventral muscle mat were also noted, (Fig. 23 and Appendix 3). Three sets of measurements were taken:

(a) from tissue injured on wounding;
(b) from uninjured tissue adjacent to the wound;
(c) from uninjured tissue distant from the wound.
Variation of lattice fibre diameter, (groups I and 2), from uninjured tissue adjacent to wound, with time after wounding.

(I unit of diameter = 1. 25μ)

Fig. 22a Variation of lattice fibre diameter, (groups I and 2), from uninjured tissue adjacent to wound, with time after wounding.
Fig. 22b  Variation in acute angles between lattice fibres of groups I and 2, from uninjured tissue adjacent to wound, with time after wounding.
In uninjured tissue adjacent to wound.

I hour after injury.  
After 1 day.  
After 2 days.  
After 3 days.

(Diameter, measured in units of 1.25 μ).

In tissue from uninjured animals.

Fig. 23a Frequency histograms showing changes in diameter of fibres of ventral muscle mat during early stages of healing.
Changes in diameter of ventral mat muscle fibres in uninjured tissue adjacent to the wound.
If Figs. 21, 22, and 23 are compared, it will be seen that though there is some contraction of muscle fibres in the injured tissue zone, there is no marked decrease in the size of the blood spaces in this region. In the uninjured tissue adjacent to the wound, however, there has been a marked contraction of both lattice and ventral mat fibres, together with a decrease in the size of the blood spaces. Here too the acute angles between crossing fibres of groups 1 and 2 have decreased, i.e., the meshes of the muscle lattice have closed up. In the distant uninjured tissue neither the size of the blood spaces, nor the degree of contraction of the muscle fibres, show any change.

It would seem that the size of the blood spaces and the degree of contraction of the muscle fibres are related. Normally when the muscle fibres contract the meshes of the lattice close up, decreasing in size and changing in shape, as is illustrated by the decrease in the acute angles between fibres of groups 1 and 2. The blood spaces lie in the meshes of the lattice and since they also decrease in size it seems likely that they are controlled by the fibres of the lattice. Should the attachment of the muscle fibres be damaged and the lattice arrangement interrupted, as is found in the injured tissue of the wound region, the fibres could contract and yet not influence the size of the blood spaces, as does in fact occur.

The evidence presented above indicates that the local blood supply of the mantle edge is controlled by the size of the lattice meshes, which are themselves controlled by the muscle fibres of the lattice and mat.

It should be noted that, in the case of the lattice, only fibres of groups 1 and 2 have so far been considered. Antagonistic fibres which relax when these contract must also be present, and are probably represented by certain of the other fibre groups. As far as the ventral mat is concerned, only those fibres lying at
right angles to the longitudinal axis of the body have been considered. Other fibres lying parallel to this axis are also present and these may be antagonistic.

Several of the fine branches of the pallial nerves were severed on wounding. It is not possible to trace the nerves to the wound surface; they appear to end approximately 8 μ from it. It is not known if this is a fixation artefact or if the nerves retracted after cutting.

2. 1 day after wounding.

The wound margin is now less ragged. The wound surface has been covered partly by epithelium and partly by agglutinated blood cells. A thin scab of dead tissue is found in some specimens, external to the cells sealing the wound. Only traces of the initial mucous covering remain.

The epithelial cells have extended over the whole of the dorsal and part of the vertical surfaces of the wound. Traces of mucus are visible above and between the cells. There is still some piling up of the cells dorsally, but this is less common than at 1 hour after injury. The epithelial cells again adhere firmly to the basement membrane, except at the extreme edges of the sheet, and are extremely attenuated, especially those of the ventral epithelium. No mitotic figures can be seen at this stage, (Fig. 24).

The blood spaces in the uninjured tissue adjacent to the wound are greatly constricted, (Fig. 21 and Appendix 1). Those spaces in the injured tissue which did not constrict initially are now filled with blood cells. Their boundaries are therefore indistinct and their size cannot be measured with any accuracy. The blood cells contain pigment granules taken up from the wound region. Those of the blood cells which are at the wound surface and are as yet uncovered by epithelial cells have agglutinated and seal the
Fig. 24. T.S. wound showing attenuated ventral epithelium. (1 day after wounding).

Fig. 25. T.S. wound tissue (1 day after wounding).

b.s., blood space; d.e., dorsal epithelium; g.s., ground substance; h.i., head integument; p.c., phagocytic cells; s.p.g., scattered pigment granules; v.e., ventral epithelium.
wound, preventing blood loss. Initially bleeding was arrested by the immediate secretion of mucus; then by sinus constriction caused by muscular contraction, which reinforced the action of the mucus; and finally the wound was closed by the epithelial sheet and by agglutinated blood cells.

There has been a pronounced change in the connective tissue of the wound region. The mucopolysaccharide ground substance now stains more densely here than in the surrounding uninjured tissue. This change in staining is not, however, uniform throughout the wound. A ventral band of tissue, approximately 60\(\mu\) thick, stains deeply, but above this is a lighter staining zone. There is no clear difference in cell-distribution in the two zones, but the lower zone contains more damaged muscle fibres and is less vascular than the upper. Collagen fibres are scattered through the wound connective tissue. They are thin, being only just visible by optical microscopy, and are wavy in outline. The increase in stain-affinity of the ground substance may indicate an increase in the amount of hyaluronic acid present. If so, then for this to occur so early in the healing process is of some significance, for it is known that hyaluronic acid has physical properties which make it capable of acting as a tensile matrix increasing wound strength (Watts, Baddeley, and Wellings, 1964).

There has been a marked increase in the number of cells present in the connective tissue of the wound region. The zone of tissue extending some 100\(\mu\) from the excision line contains much cellular debris: damaged cells, displaced pigment granules, and degenerating free nuclei. Into this zone the phagocytic cells have migrated. Their cytoplasm contains ingested pigment granules and if carmine is introduced into the wound region it is readily taken up by these cells. Mitotic division of the nuclei of phagocytic cells is rarely seen. Such cells are shown in Fig. 25.
The uninjured muscle fibres adjacent to the wound region are still contracted and the size of the blood spaces in that region is quite small. The damaged muscle fibres are beginning to degenerate, and their fibrillar appearance is no longer visible by optical microscopy, while their nuclei are more readily visible. Meanwhile, other muscle fibres are de-differentiating to form myoblasts, cells which will contribute to the blastema, from which the regenerate will later develop. Degenerating muscle fibres can be recognised by their pycnotic nuclei and by the fact that they become greatly swollen. This swelling is probably an endosmotic effect. The muscle fibres normally contain glycogen; if there were an increase in glycolysis resulting in production of excess glucose the resulting increase in osmotic pressure within the fibres would cause uptake of water with consequent swelling. Glycogen can no longer be detected in degenerating muscle fibres, a fact which supports this possible explanation. In de-differentiating fibres the nuclei are not pycnotic and there is no such swelling.

There has been an interesting change in the appearance of the sheath cells of the pallial nerves in the wound region and in the tissue adjacent to it. They appear to be moving along the nerves to the injured region. Since the nerves are finely divided they cannot be examined in the living condition and evidence for this movement must be arrived at indirectly, by the examination of fixed material. Nerves which had been sectioned longitudinally were examined and the distances between the nuclei of the sheath cells measured. It was found that generally the distances separating the nuclei decreased as the wound was approached. This suggests that the sheath cells are moving towards the cut end of the nerve and that the rate of movement decreases as the cut end is approached. Occasionally recently divided nuclei, separated by a space of only $\frac{1}{\mu}$ or $2\mu$ were found. The onset of cell division marks the beginning of the repair phase of healing, and it is of interest to find that the sheath
cells, i.e. the cells closest to the nerves, begin to divide at a time when the surrounding tissue is still in the demolition phase. There is no evidence that the nerve fibres have begun to regenerate.

3. 2 days after wounding.

The wound surface is completely sealed by epithelial cells and agglutinated blood cells. Slight traces of a scab of dead tissue remains in a few specimens, though this has usually disappeared by 2 days. Demolition of damaged tissue is well in progress. Blastema cells are beginning to accumulate at the wound surface.

Movement of the epithelial cells has apparently now ceased, and though the ventral cells are still thin and attenuated, those of the dorsal epithelium are now squamous to cubical in form. Any increase in the height of the cells is, however, only slight. No dividing cells can be seen. The remains of damaged gland cells are still present in the connective tissue; these slowly degenerate and their contents are removed by phagocytes.

The blood spaces in the uninjured tissue adjacent to the wound region are now more distended (Fig. 21 and Appendix 1), and the amount of blood reaching the wound is thus increased. Small though numerous blood spaces are to be found amongst the damaged tissue which thus has a rich vascular supply. The dilation of the blood spaces increases the amount of fluid in the wound region and causes a slight swelling, visible externally in the living animal. The increased blood supply results in more phagocytes being brought to the wound, whilst the rate of demolition and waste removal also increases.

Some phagocytic cells are found in the fine endings of the blood spaces, while others are found in the connective tissue ground substance. Most of the pigment, which was scattered through the connective tissue by the act of wounding, has been taken up by phagocytes. Cells containing debris can be seen in the connective
tissue at some distance from the wound. It would be of interest if some of these cells could be labelled and their movements traced.

There has been a further increase in the stain-affinity of the connective tissue ground substance in the wound region. As was found after 1 day of healing, there is still a ventral band of tissue which stains more deeply than the rest of the demolition zone, (Fig. 26). Collagen fibres are present, scattered apparently at random through the amorphous component of the ground substance.

Blastema cells have begun to accumulate postero-ventral to the demolition zone with its characteristic phagocytes, (Fig. 26). The origin of these cells is not known with certainty. The cells which migrate to the wound, (that migration does occur is shown elsewhere, p. 241), are probably of 4 types:

(a) epithelial cells;
(b) non-phagocytic blood cells;
(c) non-phagocytic connective tissue cells;
(d) nerve sheath cells.

None of the above cells contain debris, hence the suggestion that they are probably non-phagocytic. Their appearance coincides with increased vascularity of the wound region, and it is therefore probable that some have been brought by the blood and are in fact blood cells. It has already been noted, (p. 90) that there is an increase in the number of sheath cells at the cut ends of the nerves, and a movement of such cells towards the wound. This, though not proving that the sheath cells contribute to the blastema, indicates that they may do so. There are as yet no myoblasts within the blastema tissue. Mitotic figures are rarely seen, and then only after special treatment, (p. 222).

There has been a marked relaxation of the muscle fibres at and around the wound region, (Fig. 22, 23 and Appendices 2 and 3). Degeneration of damaged fibres and de-differentiation of other local fibres are still in progress.

There has been no further change in the progress of nerve regeneration.
**Fig. 26.** T.S. mantle edge showing early blastema. (2 days after wounding).

**Fig. 27.** T.S. wound region showing dorsal phagocytic cells. (3 days after wounding).

b.s., blood space; c.f., collagen fibres; d.c., degenerating cell; d.g.s., dorsal ground substance, stains lightly; m.b., migratory cells of blastema; p.c., phagocytic cells; p.g., pigment granules; v.e., ventral epithelium; v.g.s., ventral ground substance, stains deeply.
4. 3 days after wounding.

The wound surface is covered partly by epithelial cells and partly by agglutinated blood cells. The demolition phase is still in progress and much debris remains. The number of blastema cells is slowly increasing. The scab, i.e. those traces of dead tissue external to the cells sealing the wound, has in most cases been lost.

Although the epithelial cells do not yet cover the wound surface completely, migration has already apparently ceased and there is no longer a well-defined space between the cells and the underlying tissue. It seems that final epithelial coverage is achieved by cell-attenuation and growth, rather than by active migration. The cells on the dorsal aspect of the wound surface are squamous to cubical, whilst those on the ventral surface are still very flattened. No dividing cells can be seen. Traces of degenerating glands can be seen in the connective tissue underlying the epithelium.

The wound region is now well supplied with blood, for though the blood spaces within it are small they are very numerous. The blood spaces of the wound region are circular or oval in transverse section, while those of uninjured tissue are much larger and irregular in shape. They are bounded by a thin, well-defined membrane, though whether or not this is cellular is not yet known. The blood spaces of the healthy tissue immediately adjacent to the wound are somewhat distended.

Yet more debris has been removed from the wound area. Phagocytes containing this debris can be seen in the blood spaces at some distance from the wound, indicating that they are at least in part responsible for its removal. Some debris still lies free in the wound connective tissue but most of it has either been removed by the phagocytes or has at least been taken up by them. Degeneration of damaged glands and muscle fibres is now well advanced, but since they are not attacked by phagocytes it must be assumed that their
gradual breakdown is due to cytolysis.

The ground substance of the wound region now stains evenly, though more deeply than that of normal uninjured tissue. The collagen fibres present are still very slender, and are extremely numerous. Scattered through the ground substance are pigment granules, phagocytes, and traces of degenerating cells, while blastema cells are found in the ventral regions (Fig. 27).

The number of blastema cells within the wound region has increased, and myoblasts, as well as the migratory cells mentioned earlier, are now present (p. 92). The myoblasts have been produced by the de-differentiation of local muscle cells. During de-differentiation the myofibrils gradually disintegrate, but there is no swelling of the cells or fibres, such as is found during degeneration (p. 90). By the end of the third day after wounding the myofibrils can no longer be distinguished by optical microscopy, and the fibres have lost their earlier bright eosinophilia. The muscle fibres have become myoblasts. They are roughly spindle shaped and mononucleate, with the nucleus elongated along the longitudinal axis of the cell and clearly visible, (in differentiated muscle fibres the nucleus is obscured by the myofibrils). The myoblasts of Arion are structurally reminiscent of myoblasts formed by de-differentiation of striated muscle fibres in amphibian limb regeneration, (Hay, 1961).

The nerve sheaths now extend beyond the cut ends of the nerve fibres and penetrate the blastema as thin, hollow tubes. The sheath cell nuclei are closest together near the blastema-end of the sheaths, indicating either:

(a) that the cells towards the blastema end of the sheath have divided,
or (b) that sheath cells have been migrating towards the blastema and accumulating there.

It is not known whether or not sheath cells, having reached the blastema, leave the tubes and contribute to it; their function
may be simply to link the severed nerve fibres with the developing blastema.

5. 4 days after wounding.

The demolition and repair phases of healing still overlap, though there is evidence that the latter is taking over from the former. Fewer phagocytes are present than at earlier stages in the healing process, while the number of blastema cells is gradually increasing.

The epithelial cells are now moving forwards centripetally over the surface of the agglutinated blood cells. The former have elongated nuclei, all orientated in the same direction, and are separated by distinct cell membranes. They can readily be distinguished from the blood cells, which have smaller, deeply staining nuclei with no common orientation, and indistinct cell membranes (Fig. 28).

The wound region is richly supplied with blood and still contains many phagocytes. The blood spaces within the wound region are small, with a smooth, regular outline which seems quite characteristic. In the uninjured tissue beyond the wound, the blood spaces are distended.

The ground substance of the wound region stains uniformly throughout, but more deeply than that of adjacent uninjured connective tissue. Collagen fibres are plentiful, but are too slender and difficult to see to make any quantitative study of their distribution by visual means practicable. Much of the debris has been removed from the wound, but some large pigment granules still remain, scattered at random through the connective tissue. Numerous small pigment granules are also present and are frequently found lying on the surface of the myoblasts, though they never actually penetrate these cells.
Fig. 28. Surface view of part of wound showing epithelial and blood cells. (4 days after wounding).

Fig. 29. T.S. ventral region of wound. (4 days after wounding).

d.d.z., dorsal demolition zone; e.c., epithelial cell; m., myoblast; (with superficial pigment granules); n.e.c., nucleus of epithelial cell; n.b.c., nucleus of blood cell; p.c., phagocytic cell containing pigment granules; p.m., plasma membrane; v.b., ventral blastema; v.e., ventral epithelium.
Fig. 30. T.S. wound showing nerve-sheath tube extending towards blastema. (4 days after wounding).

Fig. 31. Mitotic figures in epithelial cells. (5 days after wounding).

b.s., blood space; c.s.t., cavity of sheath tube; c.t.c., connective tissue cells; d.e.c.(s.), dorsal epithelium, (sectioned); e.c.a., epithelial cell at anaphase; e.c.t., epithelial cell at telophase; l.e.c.(s.v.), lateral epithelial cells in surface view; m., myoblasts; n.s.t., nerve sheath tube; s.c.n., sheath cell nucleus; v.e., ventral epithelium; v.e.c.(s), ventral epithelium (sectioned).
At this stage the blastema is ventral in position, though its epithelial layer also extends over much of the dorsal demolition zone, covering many of the agglutinated blood cells. Blastema formation is now well advanced. Three cell types can be distinguished within the blastema: flattened superficial epithelial cells; spindle-shaped myoblasts; and roughly isodiametric undifferentiated cells of uncertain origin, (see p.78). The latter are probably blood and connective tissue cells, though it is possible that cells derived from nerve sheath cells may also be present. The myoblasts are ventral in position and lie horizontally, with their longitudinal axes at right angles to the longitudinal axis of the body (Fig. 29).

There is no evidence of nerve fibre regeneration, and the severed ends of the fibres remain connected to the growing blastema by hollow tubes consisting of sheath cells (Fig. 30). There is indirect evidence that the sheath cells are dividing, in that their nuclei are frequently arranged in pairs, as though they had just divided. No mitotic figures have, however, been seen.

6. 5 days after wounding.

The demolition phase of healing is now being succeeded by the repair phase. The number of blastema cells in the wound region has increased and the number of phagocytic cells has decreased.

The epithelial cells continue to spread over the surface of the agglutinated blood cells, few of which still remain uncovered. Mitotic division of the epithelial cell nuclei has commenced (Fig. 31).

The wound region is still well supplied with blood, and it is now noticeable that the blood spaces are larger in the dorsal region where phagocytosis is still in progress, than in the ventral where the bulk of the blastema cells accumulate. In the latter
Fig. 32. T.S. wound showing blastema. 
(5 days after wounding).

b., blastema; c., collagen; c.s.t., cavity of nerve sheath tube; d.d.z., dorsal demolition zone; d.r.c.t., dorsal region containing many connective tissue cells; m., myoblasts; s.c., sheath cell; v.e., ventral epithelium; v.r.m., ventral region of blastema containing myoblasts.
region the spaces are small in section but very numerous.

The number of phagocytic cells within the wound region has decreased, and most of the debris has now been removed. In some specimens a few degenerating muscle fibres can still be seen but this is atypical.

The ground substance of the connective tissue in the wound region stains uniformly, though more deeply than that of surrounding uninjured tissue. There has been a marked increase in the number of collagen fibres present, especially interspersed amongst the myoblasts.

The blood cells, connective tissue cells, epithelial cells and fibroblasts, which up to this stage formed the bulk of the blastema, have now been joined by a large number of myoblasts (Fig. 32). Those myoblasts near the ventral epithelium are arranged parallel to it, with their longitudinal axes at right angles to the longitudinal axis of the body. They are not distributed uniformly throughout the blastema, but are closely packed ventrally for a depth of 30μ to 35μ. Above this region the myoblasts are loosely packed, lack common orientation, and are interspersed amongst the other blastema cells. Some mitotic figures can be seen.

The severed nerve fibres show no sign of regeneration and are still linked to the blastema by apparently empty sheath tubes. The sheath tubes now penetrate the blastema. Structurally undifferentiated cells are frequently found on the outer surface of the tubes at this stage, and it is possible that they may be migrating along the nerves towards the blastema, though it is impossible to obtain direct evidence either for or against this view from examination of fixed material. Such cells are rarely found in this position in uninjured tissue. It is also of interest that the growth of the sheath tubes into the blastema
is accompanied by an increase in mitosis in that tissue.

7. 6 days after wounding.

The demolition phase of healing is near completion and the repair phase well advanced.

The epithelial sheet is still interrupted by small localised pockets of agglutinated blood cells. The epithelial cells are now growing and dividing and mitotic figures are quite common. The cells are frequently covered by a thin sheet of mucus, which must have been secreted by adjacent gland cells from the uninjured tissue for there has been as yet no gland development in the blastema.

The wound region remains highly vascularised. There has been a noticeable increase in the size of the blood spaces around the margins of the wound region (Fig. 33) though within the blastema the spaces are still small and regular in shape. The number of phagocytic blood cells within the wound region has decreased still further. Those that remain are heavily laden with debris and may contain granules of pigment collected from the wound.

The connective tissue ground substance within the wound region remains more deeply staining than that of the uninjured tissue. The number of collagen fibres appears to be still increasing, though as mentioned earlier the fibres are too small and slender to be counted accurately. The fibres are most plentiful amongst and immediately above the myoblasts. Some displaced pigment granules remain scattered through the connective tissue ground substance though most of the wound debris has been removed. The division of the connective tissue into two zones, a ventral zone comprised mainly of blastema cells and a dorsal demolition zone, is much less marked than in earlier stages. The blastema is more extensive and the demolition zone reduced, while the junction
Fig. 33. T.S. showing blood spaces in the wound region. (6 days after wounding).

b.s.b., blood spaces of blastema; b.s.d.z., blood spaces of demolition zone; b.s.u.t., blood spaces of uninjured tissue; d.e., dorsal epithelium; v.e., ventral epithelium.
between the two regions is rather ill-defined.

There has been a change in the orientation of the myoblasts. Though the ventral myoblasts still all lie parallel to the ventral epithelial sheet, some now have their longitudinal axis parallel to the longitudinal axis of the body, while others lie at right angles to it. Yet other myoblasts lie in positions intermediate between these two extremes. In Fig. 34 those myoblast nuclei which appear circular in section are from cells lying parallel to the longitudinal axis of the body; those nuclei which are oval in section are from cells lying at right angles to the longitudinal axis of the body. Mitotic figures are occasionally seen, and there is a lack of uniformity in cell size, a further indication that the myoblasts are dividing. Many of the myoblasts have a superficial covering of pigment granules.

There has been no further change in the progress of nerve regeneration. Those sheath tubes which have penetrated the blastema remain empty of nerve fibres.

8. 7 days after wounding.

The repair phase of healing is progressing rapidly. Demolition and removal of damaged cells is complete.

The wound surface is now completely covered by epithelial cells. The sequence of extension, growth, and division of the cells is constantly repeated, as the epithelial sheet increases in area with the increased volume of the rest of the blastema. The sequence is not synchronous throughout the epithelial sheet and all three phases can be seen in any specimen sectioned at this stage. A number of epithelial mitoses are illustrated in Fig. 35. The ventral epithelial cells are squamous, while the remainder are cubical in form. No gland cells have differentiated and the epithelial cells are all structurally similar.
Fig. 34. T.S. ventral region of blastema showing change in myoblast orientation. (6 days after wounding).

l.s.m.n., longitudinally sectioned myoblast nucleus;
O.s.m.n., obliquely sectioned myoblast nucleus;
t.s.m.n., transversely sectioned myoblast nucleus;
v.e., ventral epithelium.
Fig. 35. Mitotic figures in blastema cells. (7 days after wounding).

b.c.(s)., blastema cells in section; d.e.(s)., dorsal epithelium in section; d.n.c.c., dividing nucleus of connective tissue cell; d.n.e.c., dividing nuclei of epithelial cells; l.e.(s)., lateral epithelium in surface view.
Fig. 36. T.S. through wound tissue.
(7 days after wounding).

c., collagen; c.t.c., connective tissue cell; m., myoblast;
p.c., phagocytic cells; v.e., ventral epithelium.
There has been no change in the appearance of the blood spaces. Fewer phagocytic blood cells are found within the wound region, though numbers of such cells have accumulated at the boundary of the wound tissue and the adjacent uninjured tissue. Such cells are readily identified by their granular, darkly-staining contents.

The amorphous component of the ground substance is masked either by the closely packed collagen fibres or by blastema cells, the number of the latter having greatly increased.

Division of the blastema cells is at a maximum by the end of the seventh day after wounding (see p. 226). Highly specific fixation and staining methods must be used if mitotic figures are to be revealed (p. 227). The appearance of the blastema at this stage is shown in Figs. 35 and 36, in the former of which dividing nuclei can be seen.

The ventral myoblasts are now arranged either parallel to the longitudinal axis of the body or at right angles to it, both groups of cells lying parallel to the ventral epithelial sheet. In the dorsal regions of the blastema the myoblasts are arranged obliquely, at an angle to the epithelium. The ventral myoblasts eventually form the ventral muscle mat while the dorsal cells, when they have attained their final orientation, form the six fibre groups of the muscle lattice.

There has been no further progress in nerve regeneration.

9. 8 days after wounding.

The blastema is still growing rapidly and differentiation has commenced.

The epithelial cells are generally squamous to cubical in form though in some regions attenuated cells are still found.
Classic mitotic figures are occasionally seen, and the nuclei are frequently grouped in pairs, indicating that they have recently divided.

There has been no further change in the microscopic appearance of the blood spaces. The number of phagocytic blood cells within the wound region has decreased yet further.

There has been a marked increase in the number of connective tissue fibres within the wound region, especially in the vicinity of the myoblasts. This increase in the collagen content of the wound tissue presumably increases its tensile strength. Herrick (1945) and Herrick and Brown (1952) found this to be so in the fowl. According to Harkness (1961) the collagenous framework of the organism forms a structure limiting its size. Such a framework is relatively inextensible and of high tensile strength. Should the size of the framework increase, then the limiting factor will be removed and growth could presumably occur. It is thus interesting to find that in Arion collagen production is high at a time when blastema growth is occurring.

There are now fewer mitotic figures in the blastema cells than at 7 days after wounding. This observation is supported by the results of experimental work described elsewhere (p. 233). Differentiation of the blastema cells has progressed sufficiently for epithelial cells, fibroblasts, differentiating muscle fibres and certain connective tissue cells to be distinguishable. Fibroblasts are most common in these regions containing much collagen. They are elongated cells with large nuclei and are apparently scattered at random through the ground substance. The myoblasts, which are now differentiating into muscle fibres, are already more eosinophilic than the fibroblasts and are arranged in a more orderly manner (see p. 108). The epithelial cells may be recognised from their superficial position. Perhaps the most enigmatic of all the constituents of the blastema are the connective
The darkness of the section in the ventro-lateral regions is caused by increased eosinophilia of the newly differentiated muscle cells and the high collagen content of the area.

d.e., dorsal epithelium; d.m., differentiated muscle cells; u.m., undifferentiated myoblasts; u.t., uninjured tissue; v.e., ventral epithelium.
Fig. 38. **T.S. blastema showing re-formation of muscle lattice.**

- u.m.: undifferentiated myoblasts
- u.t.: uninjured tissue of mantle edge
- v.e.b.: ventral epithelium of blastema
- n.s.t.: nerve sheath tube
- d.e.h.: dorsal epithelium of head
- G.1. m.f.: Group 1 muscle fibre
- G.2. m.f.: Group 2 muscle fibre
- c.t.c.: connective tissue cell
- c.: collagen

**Legend:**
- c., collagen; c.t.c., connective tissue cell; G.1. m.f., Group 1 muscle fibre; G.2. m.f., Group 2 muscle fibre; d.e.h., dorsal epithelium of head; n.s.t., nerve sheath tube; u.m., undifferentiated myoblasts; u.t., uninjured tissue of mantle edge; v.e.b., ventral epithelium of blastema.
Sketch of anterior region of slug to show position of sections.

Section I

Section 2

Section 3

Base of tentacles.

Posterior limit of blastema.

Fig. 39 Diagrams showing progress of differentiation into the blastema.

Ant., anterior; Post., posterior; D., dorsal; V., ventral.

Undifferentiated tissue.
tissue cells other than the fibroblasts; many are probably amoebocytic blood cells which have become lodged in the wound tissue. Their origin and subsequent fate are not, however, known with certainty.

The blastema cells in which differentiation is most marked are the myoblasts, and the first visible stage in this differentiation is an increase in their eosinophilia. At this stage other myoblasts are still increasing in size. Differentiation of myoblasts into muscle fibres begins postero-ventrally, adjacent to the muscle of the uninjured mantle edge bordering the wound region, and extends obliquely upwards into the rest of the blastema tissue (Figs. 38, and 39). The differentiating muscle fibres take up their final orientation so that they form a continuation either of the uninjured muscle lattice or of the ventral muscle mat (Fig. 39).

There has been no noticable progress in nerve regeneration.

From the 8 day stage, which marks the onset of differentiation, the healing process is one of spatial organisation, growth, and further differentiation. The changes which take place in later 24 hour periods are no longer sufficiently extensive to warrant detailed description and the time intervals between the stages described are therefore longer.

10. 12 days after wounding.

The wound tissue is now completely free from debris. The blastema is well formed and differentiation has extended a considerable way into it.

Mitotic figures are still occasionally seen in the epithelial tissue. The dorsal region of the epithelial sheet is indented in most specimens, indicating that the underlying musculature of
the regenerate or differentiated blastema is in some regions contractile (Fig. 40).

The blood spaces are no longer rounded but are irregular in section, though they are still small. This change in shape is probably caused by the surrounding, newly-differentiated muscle fibres becoming contractile. The blastema and the adjacent uninjured tissue are both highly vascular. No blood cells containing debris can be found in the wound region, though cells basically similar in appearance but not containing debris are present in some number.

The distribution of collagen fibres is not uniform; collagen content is greatest where differentiation into muscle is most advanced, i.e. adjacent to the uninjured tissue.

The blastema cells are now dividing at a diminished rate (see p. 233). They are widely spaced in differentiated regions but closely packed where the tissue is still undifferentiated. Myoblast differentiation is well advanced and in only a small zone distal to the junction with uninjured tissue can undifferentiated myoblasts still be found. The ventral muscle mat has been re-formed and it is thicker than that of undamaged tissue. The newly formed muscle fibres dorsal to the mat are not yet arranged into a symmetrical lattice pattern throughout the tissue. Spacing and orientation, like differentiation, commence adjacent to the uninjured tissue and progress forward into the rest of the blastema.

The nerve sheaths which have penetrated the differentiating blastema are still devoid of nerve fibres. As the blastema cells divide, replacing the tissue excised, the sheath cells also divide and the sheath tubes extend to keep pace with this growth. In whole mounts of blastemata the tubes formed by the sheath cells cannot be distinguished from intact nerves; it is only when sections
Fig. 42. T.S. blastema showing indented dorsal epithelium.
(12 days after wounding).

d.e.b., dorsal epithelium of blastema;
u.d.e., uninjured dorsal epithelium;
u.t., uninjured tissue.
are examined that the sheath tubes which penetrate the blastema can be seen to be empty (Fig. 41).

11. 14 days after wounding.

Differentiation of the blastema is now well advanced, and the muscle fibres are functional in many regions. Normal orientation of the muscle lattice is still restricted to a zone bordering on the uninjured tissue.

The dorsal epithelium is often indented, and the cells lining the indentations are squamous, while the surface cells are low-columnar. In some specimens newly-differentiated mucous glands can be seen. These glands, which are few in number, arise from the epithelium and are structurally similar to the Type III glands described elsewhere (p. 28). A number of these glands are shown in Fig. 42.

There has been no differentiation of glands associated with the ventral epithelium at this stage, and a further difference between dorsal and ventral epithelia is that the latter is never indented. This is probably because the fibres of the muscle lattice lying in the vertical plane, and which are attached directly to the dorsal epithelium, are not connected with the ventral epithelium directly, but with the ventral muscle mat above it (Fig. 11c). Thus when the fibres of the lattice contract the dorsal epithelium indents, while the ventral epithelium remains relatively smooth.

There has been no further change in the appearance of the blood spaces.

Collagen fibres are now uniformly distributed in the regenerate, and it is of interest to note that at this stage myoblast differentiation has also extended throughout the whole of the regenerate.
Fig. 41. T.S. showing empty nerve sheath tube in blastema tissue. (12 days after wounding).

b.s., blood space; c.t.c., connective tissue cell;
d., dorsal; n.s.t., nerve sheath tube;
s.c.n., sheath cell nucleus;
v., ventral.
Fig. 42. T.S. of regenerate showing newly formed mucous glands.
(14 days after wounding).

d.e., dorsal epithelium;
T.III. m.g., Type III mucous glands.
It is not yet known how collagen fibres are produced in Arion and it would be of interest to follow the process by electron microscopy, using the technique of Bradbury and Meek (1958) in their studies of fibrogenesis in Hirudo medicinalis.

Although myoblast differentiation is complete, many cells still appear to be undifferentiated on examination. These cells, however, closely resemble the blood cells (described on p. 37). Empty nerve sheaths are present within the blastema. It is impossible to state definitely whether or not regeneration of the severed nerve fibres has commenced.

Since most of the cells of the blastema are now differentiated it will henceforth be termed the regenerate.

12. 21 days after wounding.

Protein and mucous glands, associated with the dorsal epithelium, have now appeared. The mucous glands correspond structurally to the Type III glands of uninjured tissue. This lends weight to the opinion stated elsewhere (p. 57) that Type III glands are developmental stages of the other dorsal mucous glands. The newly formed protein glands are essentially similar to those of uninjured tissue, though they may be somewhat smaller (Fig. 43). No ventral glands have yet appeared.

A mat of thin collagen fibres has been laid down immediately below the epithelial sheet. It is possible that these fibres form part of the basement membrane. Where it underlies the dorsal epithelium the collagen mat is quite thick (3 to 5 μ), though under the ventral epithelium it is much thinner. The thinness of the basement membrane in uninjured tissue may be caused by stretching when the epithelial cells differentiate into glands and grow downwards into the connective tissue.

The non-glandular cells of the dorsal epithelium are now low-
Fig. 43. T.S. of regenerate. (21 days after wounding).

d.e.h., dorsal epithelium of head; d.e.r., dorsal epithelium of regenerate; m.g.T.I, T.II, T.III, mucous glands of regenerate, Types I, II, and III respectively; m.f., muscle fibres of regenerate; prg., protein gland of regenerate; u.t., uninjured tissue; v.e.r., ventral epithelium of regenerate.
columnar and those of the ventral epithelium squamous to cubical, as in uninjured tissue.

The regenerate is highly vascularised, though the blood spaces are still smaller in section than those of uninjured tissue. Blood cells are plentiful, but restricted to the spaces or to the connective tissue close by.

More collagen is present within the regenerate than in corresponding regions of uninjured tissue.

The myoblasts have now all differentiated into muscle fibres and where the muscle mat and lattice have been re-formed (i.e. proximal to the uninjured tissue), these fibres are now functional. A portion of the regenerated muscle mat and lattice is illustrated in Fig. 44.

The nerve sheath tubes which have penetrated the regenerate still do not contain nerve fibres. The sheaths, which can be distinguished from the blood spaces by their well-defined cellular walls and regular outline (Fig. 41), can be seen lying close to the epithelium, as well as in the central connective tissue zone. It is noticeable that epithelial differentiation is most advanced in the neighbourhood of these sheaths.

13. 28 days after wounding.

Differentiation is now near completion in most specimens. The regenerate can be distinguished from the rest of the mantle edge tissue by the following features:

(a) it is supplied by a large number of small blood spaces, while in uninjured tissue fewer but larger blood spaces are present;
(b) the glandular epithelial cells do not penetrate as deeply into the connective tissue as do those of the uninjured
Fig. 44. T.S. regenerate showing portion of regenerated muscle mat and lattice. (21 days after wounding).

b.s., blood space; D., dorsal; G.1.m.f., G.2.m.f., muscle fibres of Groups 1 and 2 respectively; V., ventral; v.e., ventral epithelium; v.m.m., ventral muscle mat;
(c) the ground substance of the regenerate stains more deeply than that of uninjured tissue; 
(d) there is less pigment in the regenerate than in uninjured tissue.

The non-glandular epithelial cells are now similar to those of uninjured tissue in all respects. Glands are no longer restricted to the dorsal surface, for a few widely scattered glands have appeared in the ventral regions of the regenerate. Downward growth of those mucous and protein glands associated with the dorsal epithelium has caused considerable stretching of the basement membrane which is now thin and inconspicuous.

In contrast to the mucous and protein glands, it seems probable that calcium glands originate from connective tissue cells. Small cells (diameter 10 \( \mu \)) containing spherules of calcium salts are to be found within the connective tissue of the regenerate at a depth of over 100 \( \mu \). These cells are not connected to the dorsal epithelium (Fig. 45). The cells develop a dorsal-pointing extension, the neck. It is interesting that calcium glands are not surrounded by a basement membrane, as are the other glands, but since the basement membrane is extremely thin and difficult to see it may have been overlooked.

The orientation of the muscle fibres is now similar to that of uninjured tissue throughout the whole of the regenerate. The fibres are, however, shorter and thinner than those of uninjured tissue.

Nerve fibres now extend into the nerve sheaths which penetrated the regenerate early in the healing process. Only the most distal parts of the regenerate do not contain these fibres.
Fig. 45. T.S. regenerate showing developing calcium glands. (28 days after wounding).

b.s., blood space; c.g., calcium gland; D., dorsal; n.c.g., nucleus of calcium gland; ne.c.g., neck of calcium gland; V., ventral.
14. **35 days after wounding.**

Differentiation is complete. The regenerate can now be distinguished from uninjured tissue only by its higher cellular content and lesser depth.

The non-glandular epithelial cells are similar to those of uninjured tissue. In vertical section the cells appear to be similar to each other in size while in surface view they form a pavement of straight-edged hexagonal units of different sizes. The units are not isodiametric but have their longest edges parallel to the free anterior border of the regenerate.

The full complement of glands is now present: opening onto the dorsal surface of the regenerate are mucous glands of types I, II, and III, protein glands, and calcium glands, while opening onto the ventral surface are mucous glands of types I, II, III, and IV, and calcium glands. None of the above glands can be distinguished from their counterparts in uninjured tissue.

The blood spaces are slightly smaller, more numerous and more regular in outline than those of uninjured tissue, though these differences are less noticeable at earlier stages in wound healing. Blood spaces are no longer more numerous in the regenerate than elsewhere.

There has been a gradual increase in pigmentation of the regenerate. The pigment appears to be confined within the long, fine, branching extensions of the chromatophores, though this is difficult to demonstrate convincingly. The wound collagen is now less obvious and much has presumably been resorbed. The amorphous, muco-polysaccharide component of the ground substance also takes up stain less readily than it did earlier in the healing process.

Muscle fibre size and orientation is now similar in all
respects to that of uninjured tissue. The lattice has regenerated completely so that it now forms a functional entity with that of the rest of the mantle edge. The ventral muscle mat of the regenerate is now the same thickness as that of uninjured tissue.

Short lengths of empty nerve sheath can occasionally be seen in the more distal regions of the regenerate, but there is evidence that the nerve fibres have extended well into the newly formed tissue. The regenerating nerve fibres do not completely fill the sheaths, as in uninjured tissue, but are separated from the sheath cells by a clearly defined space.

15. 42 days after wounding.

The excised portion of the mantle edge has now been replaced and can only be distinguished from uninjured tissue by the following characteristics:

(a) regenerated tissue is slightly more collagenous;
(b) scattered pigment granules are occasionally found in the ventral connective tissue of the regenerate; they are never found in this position in uninjured material;

There has been no further change in the epithelium, which cannot be distinguished from that of uninjured tissue.

There is no longer any significant difference in blood space size in the regenerate and in uninjured tissue.

Regeneration of the muscle mat and lattice is complete.

Nerve sheaths devoid of nerve fibres can no longer be found, but it cannot be deduced from this that nerve regeneration is therefore complete. The outgrowth of the fibres to the muscles and glands can only be traced adequately by electron microscopy, and this is beyond the scope of the present investigation.

Healing and regeneration of the mantle edge is thus complete.

The observations described above are summarised in Table 3.
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METABOLIC CHANGES.

A. Distribution of acid phosphatase.

1. In uninjured tissue.

There is relatively little acid phosphatase activity in the tissues of the uninjured mantle edge. The epithelial cells do not react to the modified lead nitrate test for the enzyme, (p. 71). Large black precipitates are produced in the dorsal connective tissue in the vicinity of the mucous glands after treatment, while finer deposits are to be found in the ventral connective tissue (Fig. 46 experimental). The distribution of the precipitate has been shown (Pearse, 1960), to be influenced by physico-chemical conditions as well as by enzyme distribution. It has been shown, (p. 27), that the mucous glands of the mantle edge produce acid mucopolysaccharides. Combination of lead ions with acid radicals which were available here before the action of the enzyme might explain the large deposits of lead salt in the connective tissue close to the glands. A further source of acid radicals might well be the ground substance itself, which has been shown to be composed in part of a non-sulphated mucopolysaccharide, probably hyaluronic acid, (p. 40). Control sections incubated in a medium from which the enzyme substrate, sodium β-glycerophosphate, had been omitted, also showed deposits of lead salts around the glands, though there were few fine deposits in the ventral connective tissue (Fig. 46 control). This indicates that the large deposits around the glands are not formed as a result of enzyme activity, though the finer ventral deposits may be.

2. In regenerating tissue.

1 day after wounding.

Neither the epithelial cells nor the blood cells sealing the
Fig. 46. Distribution of acid phosphatase in uninjured tissue.

EXPERIMENTAL SECTION (incubated with substrate).

CONTROL SECTION (incubated without substrate).

c.t.d., fine deposits in connective tissue; d.e.m., dorsal epithelium of mantle edge; m.g.d., heavy deposits around mucous glands.
130

wound contain detectable concentrations of acid phosphatase. The ground substance of the demolition zone contains numerous small granules of lead salt after treatment, indicating the presence of acid phosphatase. In certain regions the granules are localised and occupy areas the size and shape of the smaller blood cells. This may indicate that they are lying on the site of a blood cell though this has not been proved conclusively, (Fig. 47). Thus the blood cells of the demolition zone may contain acid phosphatase and the ground substance certainly does. A false, strongly positive response is sometimes obtained from the connective tissue along the excision line. A similar response is found in control sections, and is thought to be caused by the combination of lead ions with free acid radicals present in the mucus which was pushed into the tissue during the operation.

2 days after wounding.

Neither the epithelial cells nor the blood cells sealing the wound contain detectable acid phosphatase. There has been an increase in the positive response of the tissue of the demolition zone to tests for the enzyme. The wound tissue is now at the stage equivalent to the 'acid' phase of repair found in mammalian wound healing( Arey, 1936; Okunev, 1928), and in repair of the crustacean limb (Okunev, 1929). It is reasonable to expect to find the acid phosphatases active at such a stage. The sites of enzyme activity are not restricted to the cells; the ground substance of the wound connective tissue also shows acid phosphatase activity, (Fig. 48).

4 days after wounding.

The acid phosphatase activity of the demolition zone is high. This activity appears to be greatest in the dorsal regions of the zone, though this may be an artefact caused by the proximity of acid radicals in the secretion of the dorsal glands. There is now
Fig. 47. Acid phosphatase activity in demolition zone. (1 day after wounding).

Fig. 48. Increased acid phosphatase activity in demolition zone. (2 days after wounding).

**EXPERIMENTAL SECTION.**

b.d., acid phosphatase activity in structures thought to be blood cells; b.s., blood spaces; g.s., ground substance.
a noticeable difference in the location of acid phosphatase sites at the free edge of the wound and at the junction between wound tissue and uninjured tissue. Near the free edge of the wound the sites of activity are scattered, and positive responses are obtained from the ground substance as well as from the cells of the demolition zone. In control sections there is some deposition of lead salt at the free edge of the wound, but far less than in experimental sections (Fig. 49), indicating that the positive response obtained here is partly caused by enzyme activity and partly by the presence of other substances.

In that region of the wound bordering the uninjured tissue, acid phosphatase activity is more localised, being restricted to what are probably the sites of blood cells. The epithelium continues to give a negative response to tests for the enzyme.

7 days after wounding.

The demolition tissue continues to show high acid phosphatase activity, especially over the sites of degenerating muscle fibres and blood cells (Fig. 50). The blastema tissue, which is now well-formed, shows only low phosphatase activity.

14 days after wounding.

There has been a significant decrease in the acid phosphatase activity of the wound region. The regenerating muscle fibres of the ventral mat show some slight activity, but far less than that seen in degenerating fibres earlier in the process. A positive response is not obtained from control sections, indicating that acid phosphatase is present in the new muscle fibres, though in small quantity (Fig. 51).

21 days after wounding.

Differentiation is now near completion. The newly formed tissue
Fig. 49. Sagittal section of mantle edge showing distribution of acid phosphatase. (4 days after wounding).

D.

V.

EXPERIMENTAL SECTION (High power).

D.

V.

CONTROL SECTION (Low power).

ANT., anterior.; b.w., body wall; d.r.h.a., dorsal region of experimental section with high enzyme activity; d.r.l.a., dorsal region of control with less enzyme activity; e., negative epithelium; g.s., ground substance with scattered sites of enzyme activity; POST., posterior.; V., ventral.
**Fig. 50.** T.S. demolition zone showing acid phosphatase activity. (7 days after wounding).

**EXPERIMENTAL SECTION.**

D., dorsal;
d.d.z., dorsal demolition zone with high acid phosphatase activity;
V., ventral;
v.b., ventral blastema with low acid phosphatase activity.
Fig. 51. T.S. blastema to show distribution of acid phosphatase.
(14 days after wounding).

EXPERIMENTAL SECTION.

CONTROL SECTION.

m., myoblasts of control section showing no enzyme activity;
p.c., phagocytic cells with high enzyme activity;
r.m., regenerating muscle fibres with slight enzyme activity;
v.e., ventral epithelium of experimental and control sections both show no enzyme activity.
shows little acid phosphatase activity and this in those regions
where undifferentiated cells still remain. The ventral muscle
fibres also show traces of precipitate after treatment according
to the lead nitrate method. These deposits are absent from the con-
trol sections and are therefore due to enzyme activity. They are
fine and well scattered along the length of the muscle fibre
surfaces (Fig. 52).

28 days after wounding.

The regenerated tissue is now, in all major respects, similar to
that of the uninjured mantle edge. The acid phosphatase content
resembles that of normal uninjured tissue. Changes, caused by
wounding, in the content and location of the enzyme are now at an
end.

The observations described above are summarised in Table 4.
**Fig. 52.** T.S. regenerate showing acid phosphatase activity.
(21 days after wounding).

**V. EXPERIMENTAL SECTION.**

**CONTROL SECTION.**

b.s., blood space; D., dorsal; d.e.h., dorsal epithelium of head;
m., myoblast reacts positively; mu't', mu.t., differentiated
muscle tissue reacts negatively in both experimental and control
sections; V., ventral; v.e., v'e', ventral epithelia of experi-
mental and control sections both showing no enzyme activity;
**TABLE 4.**

**DISTRIBUTION OF ACT MUSCLEASE DURING HEALING**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Before Injury</th>
<th>1 day</th>
<th>2 days</th>
<th>4 days</th>
<th>7 days</th>
<th>14 days</th>
<th>21 days</th>
<th>28 days</th>
<th>35 days</th>
</tr>
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<tbody>
<tr>
<td>Normal epithelium</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Ventral epithelium</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood cells</td>
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<td>+</td>
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<td>-</td>
<td>+</td>
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<tr>
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<td>+</td>
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<tr>
<td>Damaged cells</td>
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<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Myoblasts (or muscle fibres)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Other blastema cells</td>
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<td></td>
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<tr>
<td>Ground substance</td>
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<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>
B. Distribution of alkaline phosphatase.

1. In uninjured tissue.

The dorsal and ventral epithelial cells of the uninjured mantle edge show no sign of alkaline phosphatase activity after treatment according to the Fredricson modification of the Gomori technique. Small deposits of cobalt sulphide indicate presence of traces of the enzyme in the ground substance near the ventral muscle mat. The muscle fibres give a negative response to the test. An apparently positive response is given near the mucous glands but controls show that this is caused by melanin deposits and not by alkaline phosphatase. Some of the blood cells give a well-defined positive response to the tests applied, (Fig. 53).

2. In regenerating tissue.

1 day after wounding.

The migrating epithelial sheet and the agglutinated blood cells sealing the wound contain no detectable quantities of the enzyme. The damaged tissue and the majority of the phagocytes within it also give a negative response to tests. Some few phagocytes do contain detectable amounts of alkaline phosphatase. At the junction of injured with uninjured tissue, the cytoplasm of the cells lining the blood spaces shows clear black precipitates of cobalt sulphide, indicating presence of the enzyme. Sites of phosphatase activity are also apparently located in the connective tissue ground substance. This may be false localisation caused by diffusion of either the enzyme or the products of enzyme hydrolysis during the incubation period. Lison (1960) drew attention to the fact that phosphatases are present as soluble (lyo-) and fixed (desmo-) enzymes. Diffusion of the former occurs readily in his opinion. Fig. 54 shows the wound tissue at this stage.
**Fig. 53.** T.S. mantle edge showing alkaline phosphatase distribution in uninjured tissue. (Modified Gomori technique).

**D.**

**EXPERIMENTAL SECTION**

D., dorsal; d.e., d'.e', dorsal epithelia of experimental and control sections showing no enzyme activity; g.s., ground substance near ventral muscle mat showing slight activity; m.c., m'.c', muscle cells of experimental and control sections showing no enzyme activity; v., ventral.

**V.**

**CONTROL SECTION**
Fig. 54. T.S. wound region showing alkaline phosphatase distribution. (1 day after wounding). (Modified Gomori technique).

**EXPERIMENTAL SECTION**

- **b.s., b'.s',** blood space with positive border in experimental section and negative border in control section, respectively; **d., dorsal; g.s., g'.s',** ground substance positive in experimental section and negative in control section, respectively; **p.c., p'.c',** phagocytic cells positive in both sections, though fewer positive cells are found in the control; **v., ventral.**

**CONTROL SECTION**
2 days after wounding.

The epithelial cells and agglutinated blood cells sealing the wound usually contain no detectable alkaline phosphatase. The degenerating muscle fibres also show no phosphatase activity and relatively few of the phagocytic blood cells now present in the wound region react positively to tests for the enzyme. The ground substance in the demolition zone shows scattered sites of enzyme activity. The latter may be caused by the release of the enzyme from cells injured during wounding or it may be false localisation of the enzymes as a result of diffusion during incubation. The cytoplasm lining the blood spaces in the adjacent uninjured tissue shows evidence of high alkaline phosphatase activity.

4 days after wounding.

Alkaline phosphatase activity is now higher in the wound region than in the adjacent uninjured tissue. The epithelium shows slight enzyme activity with the modified Gomori method and the response is definitely positive with the azo technique. The phagocytic cells of the demolition zone react positively to tests but it is known, (p. 97), that they contain melanin granules and the apparently positive response may in many cases be simply due to the presence of black pigment. Control sections, however, show less black deposits than do the experimental sections, indicating that the reaction is partly caused by the enzyme. The degenerating muscle fibres, accumulating blastema cells, and the agglutinated blood cells at the wound surface, all give a negative response to tests, (Fig, 55).

7 days after wounding.

There has been a marked increase in alkaline phosphatase activity within the wound region. After treatment according to the coupling azo dye method, using 5-chloro-o-toluidine, the phagocytic cells stain a very dark brown. The dark coloration is caused partly by
Fig. 55. T.S. wound region showing alkaline phosphatase distribution at 4 days after wounding. (Azo-dye method).

- **e.c.**, epithelial cells of experimental section showing positive response to test; **e'c'**, epithelial cells of control section showing negative response; **m.g.**, mucous gland in uninjured tissue; **u.t.**, uninjured tissue; **w.r.**, wound region.
the particles of debris they contain, and partly by enzyme activity. The epithelial cell nuclei show less heavy coloration but give a clear, positive response to tests for alkaline phosphatase. The myoblasts, now accumulating ventrally in the wound region, still give a negative response, while the other cells of the blastema are slightly positive. The latter response is strongest in those parts of the blastema from which most of the wound debris has been cleared. There is a definite indication that the alkaline phosphatase content of the wound region increases as demolition is succeeded by repair. This stage is illustrated in Fig. 56.

14 days after wounding.

The alkaline phosphatase activity of the wound region has increased still further. The regenerating muscle fibres stain far more deeply with the azo dye than do the muscle fibres of uninjured tissue, indicating that they have a higher alkaline phosphatase content. Both the nuclei and cytoplasm of the epithelial cells give a well-defined positive response to tests for the enzyme. Those phagocytes remaining within the wound region, and the structurally undifferentiated cells comprising much of the blastema at this stage, react positively to both the coupling azo dye method and the Gomori technique, (Fig. 57).

The blastema tissue is now at the growth and differentiation stage and the alkaline phosphatase content is still increasing. It has been shown (Bradfield, 1951) that this enzyme is very active in fibrous protein synthesis, and also (Fell and Danielli, 1944) that it plays an important part in the differentiation of collagen. It is of interest to recall that at this stage in the development of the blastema, muscle differentiation, involving the production of fibrous protein, and collagen formation, (p. 116), are both in progress.
EXPERIMENTAL SECTION.

**Fig. 56.** T.S. demolition zone showing alkaline phosphatase distribution (7 days after wounding). (Azo-dye method).

**Fig. 57.** T.S. blastema showing alkaline phosphatase distribution (14 days after wounding). (Azo-dye method).

e.b.c., epithelial and other blastema cells positive to test for enzyme; e.c., epithelial cells positive; m.f., muscle fibres of uninjured tissue negative; p.c., phagocytic cells positive; r.m.f., regenerating muscle fibres positive.
21 days after wounding.

The epithelial tissue shows a complete absence of alkaline phosphatase activity. Where both differentiation and orientation of the newly formed muscle fibres has occurred, the alkaline phosphatase content of the muscle fibres is much decreased. Only scattered deposits of cobalt sulphide are found after treatment according to the modified Gomori method, (Fig. 58a). Where there has been differentiation only, the deposits are greater, indicating a higher enzyme concentration, (Fig, 58b). The cytoplasm of the cells lining the blood spaces of the regenerate also react positively to tests for the enzyme.

28 days after wounding.

The epithelium, now fully differentiated, shows no alkaline phosphatase activity. The differentiated and orientated muscle fibres are usually unreactive also. Some of the blood cells, those thought to be phagocytic, give a positive reaction to tests for the enzyme. Such positively reacting cells are not restricted to the regenerate but are found in other regions of the body also, e.g. the foot. Their cytoplasm, after use of the modified Gomori technique, is pale grey in colour, and their nuclei almost black. The connective tissue surrounding the newly-formed glands gives an apparently positive response, but controls show that this is caused by the presence of melanin and not by alkaline phosphatase activity.

35 days after wounding.

The distribution of alkaline phosphatase resembles that found in uninjured tissue. Healing is now complete in most specimens and the tissue of the regenerate is metabolically as well as structurally back to normal.

The observations described above are summarised in Table 5.
Fig. 58. Distribution of alkaline phosphatase. (21 days after wounding). (Modified Gomori technique).

(a) T.S. of region of regenerate where myoblasts are differentiated and finally orientated.

(b) T.S. regenerate where myoblasts are differentiated only.

d.e.c., dorsal epithelial cells of regenerate react negatively to test; d.e.h., dorsal epithelium of head; d.m.f., differentiated muscle fibres react positively; d.o.m.f., muscle fibres which are differentiated and orientated show scattered sites of enzyme activity; v.e.c., ventral epithelial cells of regenerate negative to test.
### TABLE 5.

**DISTRIBUTION OF AKALINE PHOSPHATASE DURING HEALING**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Before Injury</th>
<th>1 day</th>
<th>2 days</th>
<th>4 days</th>
<th>7 days</th>
<th>14 days</th>
<th>21 days</th>
<th>28 days</th>
<th>35 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal epithelium</td>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
<td>Ventral epithelium</td>
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<td>Blood cells</td>
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<tr>
<td>Damaged cells</td>
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<tr>
<td>Myoblasts (or muscle fibres)</td>
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<tr>
<td>Other blastema cells</td>
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<td>+</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood space lining</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
C. Distribution of glycogen.

1. In uninjured tissue.

The epithelial cells contain glycogen, as do many of the connective tissue cells, especially those resembling blood cells. Free glycogen granules are also found in the connective tissue ground substance. Blood cells laden with glycogen are occasionally found in the blood spaces of the mantle edge and the muscle fibres may also contain it. Glycogen has not been detected in the glands. (Fig. 59).

2. In regenerating tissue.

1 day after wounding.

Many 'type c' connective tissue cells (see p. 42), laden with glycogen, have arrived at the wound, where demolition of damaged tissue is in progress. Similar cells are found in the uninjured tissue adjacent to the wound region. The cytoplasm of the cells is so laden with glycogen that the whole cell (excluding the nucleus) reacts positively to Best's carmine (Fig. 60). Positive cells are not found in the blood spaces.

The uninjured epithelium adjacent to the wound no longer contains detectable amounts of glycogen, and it is likely that the carbohydrate has been transferred to local connective tissue cells ready for transportation to the cells of the demolition zone, though this has not been proved. Those epithelial cells which have moved out over the injured tissue surface still contain traces of glycogen.

There are no free glycogen granules in the connective tissue ground substance.

2 days after wounding.

The epithelial cells and the agglutinated blood cells sealing
Fig. 59. T.S. mantle edge showing distribution of glycogen in undamaged tissue. (Stained with Best's carmine & haemalum)

Experimental section.

Control section (treated with diastase).

c.t.c. and b.c., connective tissue and blood cells, glycogen-positive in experimental section; negative in control section;
d.e., dorsal epithelium, glycogen-positive in experimental section, negative in control section;
v.e., ventral epithelium, glycogen-positive in experimental section, negative in control section.
Fig. 60. T.S. wound region showing distribution of glycogen. (1 day after wounding). (Stained with Best's carmine and haemalum).

c.t.c., connective tissue cells containing glycogen;
d.z., demolition zone immediately behind line of excision;
d.e.h., dorsal epithelium of head;
e., glycogen-free epithelium;
u.t., uninjured tissue adjacent to wound, containing no glycogen.
the wound react negatively to tests for glycogen. The adjacent uninjured epithelium is also negative in response. Connective tissue cells containing glycogen are present in the wound and have presumably migrated to it from the surrounding connective tissue. Small glycogen granules are present in the blastema cells and also in the tissue of the demolition zone. That part of the wound bordering the uninjured tissue contains larger granules of glycogen. The presence of small granules instead of the large ones normally found in the mantle edge tissue marks a stage in the breakdown process which ultimately results in the glycogen being made available as an energy source. The distribution of glycogen at this time is illustrated in Fig. 61.

4 days after wounding.

Though the epithelial cells covering uninjured tissue around the wound contain no detectable glycogen, those cells now stretching over the blood cells to complete wound closure give a slight positive response with Best's carmine. This response is not well marked and was noted in only one third of the specimens examined. Connective tissue cells containing glycogen are still moving into the blastema, but there are fewer positive cells within it than previously. The blastema contains many small glycogen granules, while there is little glycogen in the adjacent uninjured tissue (Fig. 62). These two facts seem to indicate that the glycogen is being converted to some other compound, such as glucose, for use as an energy source for demolition and growth activities now in progress (p. 96).

7 days after wounding.

The epithelial cells of the blastema give a diffuse, positive response to Best's carmine, indicating the presence of glycogen, and verified by the absence of the response from control sections. The demolition tissue also contains glycogen, mainly in the connective tissue cells though some free granules occur in the ground substance
Fig. 61.  T.S. of wound region showing distribution of glycogen.
(2 days after wounding).  (Stained with Best's carmine and haemalum).

bl.c., blastema cells containing small glycogen granules;
e.c.b.c., glycogen-free epithelial and blood cells sealing the wound;
t.b.d., tissue bordering demolition zone with cells containing large granules of glycogen;
u.e., uninjured epithelia with cells containing no detectable glycogen.
Fig. 62. T.S. wound region showing distribution of glycogen.
(4 days after wounding). (Stained with Best's carmine and haemalum).

c.t. and b.c., glycogen-positive connective tissue and blood cells of wound region;
d.e., and v.e., dorsal and ventral epithelia respectively.
g.g., small glycogen granules in connective tissue;
u.t., uninjured tissue containing little glycogen.
The blastema, now well established, contains little glycogen; more is to be found in the adjacent uninjured tissue (Fig. 63).

10 days after wounding.

The epithelial cells of the regenerate react positively to tests for glycogen. This reaction is most marked on the ventral surface, where the epithelium is still squamous in form. The red coloration given with Best's carmine is not restricted to granules but dispersed throughout the cytoplasm. The demolition zone no longer contains detectable glycogen, nor do the connective tissue cells of the blastema, though those of the uninjured tissue nearby give a slight positive response to tests. It seems probable that the connective tissue cells are moving to the blastema and there releasing their glycogen. If the glycogen were then converted to some other compound, the negative response obtained within the blastema would be explained. At this stage in the healing process energy is required for cell division, differentiation, and protein synthesis. Glycogen carried to the wound region probably provides the source of this energy.

The differentiating myoblasts contain no detectable glycogen; they are actively engaged in the production of fibrillar protein (p. 178) and all the glycogen they obtain is used up in this process. The distribution of glycogen within the tissues at this stage is illustrated in Fig. 64.

14 days after wounding.

The newly formed muscle fibres are now functional but do not yet contain glycogen. In specimens where the epithelium has become columnar it, also, does not contain glycogen; in those where it is still squamous to cubical a diffuse, positive reaction is obtained. During the change from squamous to columnar increase in volume occurs.
Fig. 62. T.S. of wound region showing distribution of glycogen.
(7 days after wounding). (Stained with Best's carmine and haemalum).

b., blastema, containing less glycogen than surrounding tissue;
c.t.c., connective tissue cells adjacent to blastema containing glycogen;
d.z., demolition zone;
v.e.b., ventral epithelium of blastema.
Fig. 64. T.S. ventral region of wound showing distribution of glycogen. (10 days after wounding). (Stained with Best's carmine and haemalum)

b., blastema cells, other than epithelial, containing no detectable glycogen; (in some sections traces were found);
c.t.c., connective tissue cells adjacent to wound containing glycogen;
v.e.b., ventral epithelium of blastema giving a diffuse, positive response for glycogen.
The cells are not vacuolated and it is therefore unlikely that this increase is due solely to water uptake by endosmosis. Protein synthesis resulting in growth must be at least partly responsible. For this energy and raw materials are required. Stores of glycogen are present in the connective tissue of the mantle edge and foot. It is probable that this glycogen is transferred to the epithelium from local sites by connective tissue cells and from distant regions by blood cells. This would explain the presence of cells reacting positively to tests for glycogen in the connective tissue of uninjured regions of the mantle edge, of cells giving a negative response but otherwise similar in appearance in the blastema, and of a positive squamous epithelium at the 10 day stage. Once glycogen is present in the epithelium glycolysis can occur, with the release of energy which could be used in protein synthesis and growth. This would explain why the growing epithelial cells become glycogen-negative, (Fig. 65).

21 days after wounding.

Small, widely scattered glycogen granules can be seen in the ground substance near the few undifferentiated cells remaining in the regenerate. There is also some glycogen in the uninjured tissue nearby. The amount of glycogen in the wound region has been steadily decreasing since the fifth day after wounding and is now near complete depletion.

29 days after wounding.

The regenerate is now completely devoid of glycogen, though the uninjured tissue nearby contains some glycogen reserves. It might be expected that complete depletion would coincide with the peak of protein synthesis and growth; it would seem, however, that it succeeds these processes, (see Tables 6 and 7).

33 days after wounding.

Glycogen reserves are now being built up in the regenerate.
Fig. 65. T.S. wound region showing distribution of glycogen.
(14 days after wounding). (Stained with Best's carmine and haemalum)

b., blastema containing little glycogen;
d.e.b., dorsal epithelium of blastema, now columnar, and containing no glycogen;
g.u., glycogen present in uninjured tissue adjacent to blastema;
v.e., ventral epithelium of blastema.
Granules are present in the connective tissue and the epithelium also reacts positively to Best's carmine. Glycogen distribution is now essentially like that in normal mantle edge tissue.

The observations described above are summarised in Table 6.
### Table 6.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Before Injury</th>
<th>Time after Injury</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 days</td>
<td>4 days</td>
</tr>
<tr>
<td>Normal epithelium</td>
<td>++</td>
<td>+</td>
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<tr>
<td>Neural epithelium</td>
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<td>+</td>
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<td>Blood cells</td>
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<td>++</td>
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<td>Connective tissue cells</td>
<td>++</td>
<td>+++</td>
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<td>++</td>
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<tr>
<td>Myoblasts (or muscle fibres)</td>
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<td>+</td>
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<tr>
<td>Other blastema cells</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Ground substance</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>
D. Distribution of ribonucleic acid.

1. In uninjured tissue.

In the uninjured mantle edge the chief locations of basophilic substances are the nuclei and mucous glands. After treatment of control sections with ribonuclease, (see p. 73), and subsequent staining with toluidine blue, it can be shown that only the nuclei contain detectable quantities of ribonucleic acid (R.N.A.). The mucous glands continue to stain and the response here is thus not caused by R.N.A. The epithelial nuclei stain more intensely with toluidine blue than do the nuclei of other cells. R.N.A. was not detected in the cytoplasm of any cells, (Fig. 66).

2. In regenerating tissue.

1 day after wounding.

The demolition tissue of the wound contains phagocytic cells with faintly basophilic cytoplasm and strongly basophilic nuclei. Control sections indicate that the basophilia is caused by R.N.A. The epithelial cell nuclei also contain detectable quantities of R.N.A. though none is found in their cytoplasm.

2 days after wounding.

The cytoplasm of the phagocytes in the wound region is faintly R.N.A positive and their nuclei more strongly so. The damaged mucous glands are strongly basophilic, due to their mucopolysaccharide contents, and this masks any basophilia which might be caused by R.N.A; the glands are still basophilic after treatment with ribonuclease.

4 days after wounding.

The nuclei of some of the epithelial cells which at this stage help seal the wound show a more pronounced basophilia than at earlier
Fig. 66. T.S. mantle edge (at junction with dorsal body wall) showing distribution of R.N.A. in uninjured tissue.

**EXPERIMENTAL SECTION**

**CONTROL SECTION** (treated with ribonuclease prior to staining).

d.e.n., nuclei of dorsal epithelial cells showing basophilia caused by R.N.A.; d'.e'.n', dorsal epithelial nuclei on control slide showing no marked basophilia; m., mucus; m.g., m'.g', basophilic mucous glands in both experimental and control sections, indicating that the basophilia is not caused by R.N.A.
stages (Fig. 67). Since they show no basophilia when treated with ribonuclease prior to staining the basophilia is caused by R.N.A. The nuclear membrane and nucleoli are particularly basophilic and the nuclear sap and cytoplasm faintly so. This would seem to indicate that movement of R.N.A. from the nucleolus, across the nuclear membrane, and into the cytoplasm is occurring. Such movement is a necessary preliminary to protein formation. The agglutinated blood cells which also help to seal the wound show a slightly increased basophilia but this is not as pronounced as that of the epithelial cells. The nuclei of the blastema cells give a faintly positive response; at this stage they are simply accumulating at the wound and it is unlikely that cell growth has yet commenced. The phagocytes no longer have basophilic nuclei and are consequently difficult to distinguish in sections treated with toluidine blue.

7 days after wounding.

The epithelial cells covering the wound now all have basophilic nuclei. As at 4 days, this basophilia, which is due to the presence of R.N.A., is most pronounced in the nuclear membrane and nucleolus. The cytoplasm of the epithelial cells is only weakly basophilic (Fig. 68a). Phagocytic cells are still present in the wound debris (p.109), but since they are no longer basophilic they are difficult to detect in toluidine blue stained sections. The blastema cells show a definite increase in the amount of R.N.A. they contain. The cytoplasm, nucleolus, and nuclear membrane of each cell shows a clear increase in basophilia which cannot be demonstrated after treatment with ribonuclease and is thus caused by R.N.A. This increase coincides with the peak of cell division in the cells of the blastema (p.233). The blastema cells at this stage are illustrated in Fig. 68b).

14 days after wounding.

The epithelium covering the wound is intensely basophilic. The
Fig. 67. Oblique section through wound region showing distribution of R.N.A. (4 days after wounding).

b.e.n., high basophilia (caused by R.N.A.) found in certain epithelial cell nuclei;
d.e., obliquely sectioned dorsal epithelium, with many cells seen in surface view;
d.z., tissue of demolition zone;
Fig. 68. T.S.'s of wound region showing distribution of R.N.A. (7 days after wounding).

(a) **Dorsal region showing epithelial cells.**

(b) **Ventral region showing blastema.**

b.c., blastema cells, (connective tissue and blood cell types), with basophilic nuclei; d.e.c., dorsal epithelial cells, all with basophilic nuclei; d.z., demolition zone; m., basophilic myoblasts; m.g., ventral uninjured basophilic mucous glands (here the basophilia is caused by acid mucopolysaccharides and is present in control sections).
cytoplasm of its cells gives an intense, positive response for R.N.A., though this is less marked than that given by the nucleus. In the case of the epithelium covering adjacent uninjured tissue, the positive response for R.N.A. is restricted to the nucleus. The ventral epithelium of the regenerate is more basophilic than the dorsal epithelium. Glandular and non-glandular cells are becoming differentiated in the dorsal epithelium, whilst the ventral epithelium is as yet structurally undifferentiated. It is possible that the R.N.A. content of the cells decreases as differentiation increases. The nuclei of the few phagocytes remaining in the wound also contain detectable quantities of R.N.A. The undifferentiated myoblasts at the edge of the wound furthest from the uninjured tissue (i.e. at the distal tip of the regenerate) are highly basophilic (Fig. 69a). Both nucleus and cytoplasm contain detectable concentrations of R.N.A. The proximal myoblasts have now differentiated into muscle fibres containing myofibrils (p. 119) and are less basophilic (Fig. 69b). Their cytoplasm gives a slight response to tests for R.N.A. while the nuclei are clearly positive. Again it seems likely that the R.N.A. content decreases when differentiation occurs.

21 days after wounding.

The nuclei of the epithelial cells give a strong, positive response for R.N.A. but the response is much weaker in the cytoplasm. The undifferentiated blastema cells still have a high R.N.A. content, both in the nucleus and in the cytoplasm. In differentiated cells, only the nuclei remain R.N.A. positive. Undifferentiated cells are illustrated in Fig. 70.

28 days after wounding.

The wound is now almost completely healed and often difficult to locate (p. 121). Differentiation has been accompanied by a
**Fig. 69.** T.S.'s of regenerate showing change in basophilia with differentiation. (14 days after wounding).

(a) T.S. through zone containing undifferentiated myoblasts.

(b) T.S. through zone containing newly differentiated muscle fibres.

d.m.f., differentiated muscle fibres only slightly basophilic; u.m., undifferentiated myoblasts showing intense basophilia; v.e., ventral epithelium with high basophilia.
EXPERIMENTAL SECTION.
(Toluidine blue counterstained with orange G).

d., debris, taken up by phagocytes showing little basophilia;
d.e.h., dorsal epithelium of head;
u.b.c., few remaining undifferentiated blastema cells still noticeably basophilic;
v.e.m., ventral epithelium of mantle edge showing a strong positive response for R.N.A., in the nuclei and a weaker response in the cytoplasm.
decrease in R.N.A. Dorsal and ventral epithelial cells stain quite clearly with toluidine blue but the stain is taken up mainly by the nuclei. Control sections indicate that this basophilia is caused by R.N.A. The newly formed epithelial glands are also basophilic but in this case the staining is caused by their acid mucopolysaccharide contents in many instances. Any R.N.A. they may contain is of course masked by this response. The differentiated cells of the regenerate contain detectable amounts of nuclear R.N.A. but none within their cytoplasm; nuclear R.N.A. is restricted to the nucleolus, and the nuclear membrane is no longer basophilic.

35 days after wounding.

The distribution of R.N.A. within the regenerate is similar to that in corresponding regions of uninjured tissue. Healing is now complete.

The observations described above are summarised in Table 7.
### TABLE 7

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Before Injury</th>
<th>1 day</th>
<th>2 days</th>
<th>4 days</th>
<th>7 days</th>
<th>14 days</th>
<th>21 days</th>
<th>28 days</th>
<th>35 days</th>
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</thead>
<tbody>
<tr>
<td>Epithelial</td>
<td>++</td>
<td>*</td>
<td>*</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+?</td>
<td>+?</td>
</tr>
<tr>
<td></td>
<td>N.M.</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>C.</td>
<td></td>
<td></td>
<td>*</td>
<td>*</td>
<td>+?</td>
<td>+?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood cells</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N.M.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C.</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Connective tissue cells</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
<td></td>
<td>N.M.</td>
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<tr>
<td></td>
<td>C.</td>
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<td></td>
</tr>
<tr>
<td>Damaged cells</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>N.M.</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>C.</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial (or muscle fibres)</td>
<td>++</td>
<td>*</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>N.M.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C.</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other blast-type cells</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N.M.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C.</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

C., cytoplasm; N., nucleus; N.M., nuclear membrane.
E. Distribution of SH and SS groups.

1. In uninjured tissue.

The chief sites of compounds containing sulphydryl (SH) and disulphide (SS) groups are the protein glands. Dorsal and ventral epithelial cells, blood cells, muscle fibres and nerve cells give a negative response to the dihydroxy-dinaphthyl-disulphide (D.D.D.) and ferric ferricyanide tests for SH and SS groups. Since it is most unlikely that these cells contain no SH or SS groups it would appear that the tests used are not of high sensitivity. Fig. 71 shows the appearance of the tissue after the ferric ferricyanide test.

2. In regenerating tissue.

1 day after wounding.

There is a diffuse positive response to the ferric ferricyanide test in the damaged cells at the edge of the wound. Migrating epithelial cells, phagocytes and the blood cells sealing the wound are all SH and SS negative while traces of glandular secretion overlying the wound are rich in SH and SS groups (Fig. 72).

2 days after wounding.

The cytoplasm around the pigment granules scattered by the act of wounding gives a faint positive response to tests, while the rest of the cytoplasmic debris is negative in response. The undamaged tissue adjacent to the wound region contains some detectable SH and SS groups, and a band of positively reacting tissue, some 100 μ in width, separates the negative wound region from the negative tissue forming the rest of the uninjured mantle edge. The cytolysing damaged cells, phagocytes, migrating epithelium and the blood cells sealing the wound do not contain detectable
Fig. 71. T.S. mantle edge showing distribution of substances containing SH- and SS- groups.

(Ferric ferricyanide method).

b.s., blood spaces;
d.e.h., dorsal epithelium of head;
d.e.m., dorsal epithelium of mantle edge;
pr., protein glands, giving a clear positive response to test;
v.e.m., ventral epithelium of mantle edge.
Fig. 72. T.S. ventral region of wound region showing distribution of SH- and SS- groups. (1 day after wounding).

(Ferric ferricyanide method).

b.s., blood spaces;
d.c., damaged cells at edge of wound giving diffuse positive response to test;
m., traces of mucus on surface of wound also give SH- and SS-positive response;
v.e., ventral epithelium.
concentrations of SH or SS groups.

4 days after wounding.

The blastema cells now accumulating at the wound are SH and SS negative, as are the phagocytes. Where the secretion of damaged, degenerating protein and calcium glands has entered the blood sinuses, a positive response is given to the ferric ferricyanide test (Fig. 73). The blood cells, however, remain SH and SS negative. The zone of diffuse SH and SS positive tissue lying next to the wound region is less well marked than at 2 days after wounding.

7 days after wounding.

The epithelial cells at the wound surface still do not contain detectable quantities of SH and SS groups. In most specimens cell demolition is complete and the connective tissue ground substance in the demolition zone (marked by displaced pigment granules) gives a faintly positive response to the ferric ferricyanide test. This may be due to the release of SH and SS containing material from the protein and calcium glands. Occasionally blood cells containing detectable quantities of SH and SS group-containing compounds can be found. Cells corresponding to the Type I and Type II blood cells (p. 37) also give a positive response. The myoblasts and ventral epithelial cells still do not contain detectable quantities of SH and SS groups. Evidence has been given elsewhere (p. 106) that these cells are undergoing division.

14 days after wounding.

There has been an interesting increase in the amount of SH and SS positive material present in the wound area. The epithelial cells remain negative, but the myoblasts, blood cells, connective tissue cells and nerve sheath cells all give a positive response to ferric ferricyanide tests (Fig. 74). At this stage in the healing process growth and differentiation are proceeding concur-
Fig. 73. Sagittal section of wound margin showing distribution of SH- and SS- groups. (4 days after wounding).

(Ferric ferricyanide method).

b.s., blood spaces;
d., debris; (displaced pigment granules responsible for dark colour);
m.e.c., migrating epithelial cells, SH- and SS- negative;
pr., protein glands of uninjured tissue, faintly SH- and SS- positive.
b., myoblasts, connective tissue cells, and blood cells of blastema showing positive response for SH- and SS- groups; d.e., dorsal epithelium; v.e., ventral epithelium.
rently. Many myoblasts have now differentiated into functional muscle fibres (p. 116) and the increase in SH and SS groups found here probably reflects their production of new fibrous protein. Cell division, followed by growth and involving protein synthesis, could account for the increase in SH and SS positivity found in other blastema cells at this stage. More SH and SS positive blood cells are present in the spaces or sinuses than was noted at 7 days. The connective tissue ground substance in those regions where displaced pigment granules still remain also gives a positive response due to SH and SS groups.

21 days after wounding.

The few structurally undifferentiated cells which remain within the regenerate are all positive to tests for SH and SS groups as are those which are newly differentiated and including the epithelial cells. In all preceding stages the epithelium was negative. The connective tissue ground substance, blood cells, and connective tissue cells, with the exception of types (a) and (c), react positively. The appearance of the regenerate at this stage is shown in Fig. 75a. If this is compared with Fig. 75b, which shows a region of uninjured tissue taken from the same section, it can be seen that the response inside the regenerate is stronger than that outside it and it is therefore likely that more SH and SS group-containing compounds are present in the regenerate than in the uninjured tissue. At 21 days the concentration of SH and SS groups within the regenerate reaches its highest level.

28 days after wounding.

Cellular differentiation is near completion. Both dorsal and ventral epithelia have lost their brief SH and SS positivity (found at 21 days). The connective tissue ground substance remains faintly positive at the sites of displaced pigment granules but elsewhere
**Fig. 75.** Distribution of SH- and SS- groups at 21 days after wounding. (Ferric ferricyanide method).

(a) **T.S. regenerate.**

(b) **T.S. adjacent uninjured tissue.**

c.t., m.f., v.e., connective tissue, muscle fibres, and ventral epithelium of uninjured tissue showing weak, positive response to test; r., regenerate showing strong positive response to test; v.e.r., ventral epithelium of regenerate SH- and SS- positive, (only a few cells of regenerate epithelium are to be seen in lower figure).
the disposition of positively reacting material resembles that of uninjured tissue. The appearance of the regenerate at this stage is shown in Fig. 76.

The period of intensive accumulation of SH and SS positive material is thus at an end.

35 days after wounding.

The ground substance around any displaced pigment granules remains slightly SH and SS positive. In all other respects the reactions of the tissues of the regenerate to D.D.D. and ferric ferricyanide treatments are similar to those of uninjured tissue.

The observations described above are summarised in Table 8.
Fig. 76. T.S. regenerate to show distribution of SH- and SS-groups. (28 days after wounding).

(Ferric ferricyanide method).

d.e., v.e., dorsal and ventral epithelia respectively, both no longer SH- and SS- positive;
g.p.g., ground substance near pigment granules giving a faint, positive response to test.
Table 6.

DEVELOPMENT OF SH AND BH CUSHION DURING HEALING

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Before Injury</th>
<th>Time after injury</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 day</td>
<td>2 days</td>
</tr>
<tr>
<td>Dorsal epithelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventral epithelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood cells</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Connective tissue cells</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Damaged cells</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Myoblasts (or muscle fibres)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Other blastema cells</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Ground substance</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nerve sheaths</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Protein glands</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Calcium glands</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

TABLE 8.
SUMMARY AND DISCUSSION.

Excision was used in preference to cautery for the following reasons:

(a) Cautery injures the tissue for a considerable distance beyond the apparent wound margin, causing protein denaturation and enzyme destruction (Pande, 1958). With excision there are no such side effects and the extent of the wound is clearly defined.

(b) It is easier to inflict uniform wounds, provided that a suitably designed operating instrument is used.

(c) It is quickly performed.

(d) Excision methods have been used successfully on molluscs by other authors (Techow, 1910; Lange, 1920).

Healing occurs rapidly, and where the method and degree of excision described here are used, the excised tissue is eventually replaced.

1. Provisional wound closure.

The first stage in the healing of any wound is the closure of the injured surface. Blood-loss must be arrested and the entry of micro-organisms prevented. Within minutes of injury the epithelial cells are released from the basement membrane and begin to extend and migrate over the surface of the wound (p. 77). Blood cells agglutinate (p. 87), and epithelial and blood cells jointly effect a provisional wound closure. The process is not completed by the end of the first hour after injury, though by this time blood-loss appears to have ceased. Other factors must therefore be involved in arresting blood flow.

Mucus, secreted by the mucous glands, penetrates a little way into the cut ends of the blood spaces and may partially block them, so preventing blood-loss, (p. 31). The most important factor is,
however, the constriction of blood spaces by the muscle fibres. It has been shown (p. 36) that local muscle fibres contract when the mantle edge is injured. Muscular contraction also pulls the edges of the wound together and this helps in provisional wound closure. Furthermore, when the muscle fibres contract, the meshes of the muscle lattice are partially closed (p. 36), thus constric­
ting the blood spaces which ramify through them and presumably reducing the flow of blood to the wound. Constriction is only temporary, for after 2 days of healing when provisional wound closure is complete, the blood spaces around the wound regain their normal size (p. 71).

Wound closure by epithelial stretching and migration is appar­
ently universal for minor wounds (Needham, 1952). After injury, the blood cells of Arion hortensis agglutinate and so help to seal the wound. The ability of molluscan blood cells to agglutinate after wounding has been observed by several authors (Lange, 1920; George and Ferguson, 1950; et al.).

Constriction of blood spaces after wounding, though not previously reported for molluscs, is by no means unusual. In vertebrates (Needham, 1952) bleeding is reduced by constriction of local capil­
laries and arterioles. In A. hortensis the muscular contraction resulting in constriction of the blood spaces also helps in pro­
visional wound closure by decreasing the area of the exposed wound surface.

At this stage the migrating epithelial cells and agglutinated blood cells contain no detectable amounts of SH and SS groups, and show no acid or alkaline phosphatase activity. The epithelial cells contain traces of glycogen, though less than in normal uninjured tissue. The glycogen is presumably being utilised as a source of energy for the stretching and migration of the cells.
2. Demolition of damaged tissue.

Before an injury is repaired the damaged tissue must be removed from the wound area. The demolition phase of healing begins during the first 24 hours after injury and continues until all debris has been removed. It commences with the immigration of phagocytic cells into the wound tissue. Opinion is unanimous that there are two types of phagocyte: one a blood cell, the other a tissue cell (Needham, 1952). In Arion these cells phagocytose tissue debris and foreign particles, gradually removing them from the wound. Damaged cells are cytolyzed.

Neither damaged cells nor phagocytes of the demolition zone contain detectable quantities of SH and SS groups, though the ground substance does, presumably because of the release of SH and SS group-containing compounds from the protein and calcium glands. The tissue of the demolition zone does not contain detectable alkaline phosphatase, though acid phosphatase is certainly present especially in the phagocytic cells and the ground substance. Degenerating muscle fibres also show high acid phosphatase activity. This is not unusual for acid phosphatases are known to be associated with cell degeneration, (Yao, 1950). They have also been shown to become active in the late regression phase of nerve regeneration (Bodian and Mellors, 1944, '45). Bodian, however, later associated increased acid phosphatase activity with the re-synthesis of Nissl bodies rather than their destruction (Bodian, 1947). Yao, working on Drosophila melanogaster, found evidence of acid phosphatase activity in histolyzing tissue fragments. There would seem to be a definite relationship between phosphatase activity and cellular degeneration, and the experimental results obtained for Arion add weight to this opinion.

It is noteworthy that in the demolition or regression phase of healing in amphibians (Okunev, 1928), crustaceans (Okunev, 1929),
and mammals (Arey, 1936), there is increased local acidity in the wound area. Although the pH of wound tissue has not been investigated for Arion, it is interesting to find that at this stage it is the acid phosphatases which are active, indicating that the wound environment is acidic. It has been suggested (Arey, 1936), that the difference in pH between a wound and its surroundings sets up a potential difference and an action current. It is known that weak galvanic currents induce electrolysis (Doljanski, et al, 1944), and therefore probably proteolysis, an essential part of tissue demolition.

Another feature of interest connected with the demolition phase is the dilation of the blood spaces which follows provisional wound closure. This increases the amount of blood carried to the wound and causes slight swelling (p. 91). Increase in the volume of blood flowing through the wound is accompanied by increase in the number of phagocytic blood cells carried to it, and the rate of demolition and removal of waste thus increases. It has been suggested that in vertebrates oedema may also dilute any poisons in the wound and may facilitate the movement of leucocytes and other cells in the tissue spaces (Needham, 1952). It may have the same effect in Arion also. In vertebrates inflammation appears within a few minutes of injury and lasts for up to 5 days. In Arion the blood supply does not begin to increase until the second day after injury. This delay is understandable in view of the way in which wound closure is effected. Since the wound is not closed rapidly by a blood clot as in vertebrates, blood vessel constriction at the wound site must continue until wound closure by cell agglutination has been achieved. More rapid increase in the blood supply would be harmful, probably promoting further blood loss rather than healing.
3. Final wound closure.

The wound is finally closed by the epithelial cells which gradually cover the agglutinated blood cells, but pass underneath any dead superficial tissue. Wound closure is complete after 5 days, but the epithelial cells continue to grow and divide, keeping pace with the growth of the rest of the blastema. The superficial blood cells do not dry and harden to form a scab, as found in vertebrates, but are constantly lubricated by mucus.

At the time of final wound closure the epithelial cells contain no detectable SH and SS groups, though they do give a positive response for alkaline phosphatase. Their R.N.A. content gradually increases, especially in the nuclear membrane and nucleolus, from the fourth day onwards, up to the time at which the glandular and non-glandular cells are distinguishable. During this stage the cells effect final wound closure and growth and division occur. The increase in R.N.A. content indicates a stage in protein production associated with growth.

When the epithelial cells are actively migrating over the wound surface the glycogen content of the tissue falls, presumably because it (the glycogen) is being oxidised as a source of energy for migration. After final wound closure, while the epithelium is still squamous, the glycogen content rises, only to fall once more when the epithelium begins to grow, changing from squamous to low columnar. Presumably the glycogen has now been converted to glucose (Soskin and Levine, 1949), and is being used as an energy source for protein synthesis (p. 158).

4. Formation and growth of the blastema.

In Arion the blastema is formed by de-differentiation of local cells, migration of more distant cells and cell division.

The only cells which certainly undergo de-differentiation are the muscle fibres. Local, undamaged fibres in the vicinity of
the wound lose their fibrillar structure and de-differentiate forming myoblasts which later divide and re-differentiate, replacing the excised muscle tissue. Fibre de-differentiation becomes noticeable during the third day after wounding. The cells gradually lose their eosinophilia and as the myofibrils disappear the nucleus, usually obscured by them, becomes clearly visible (p. 95). The process is basically similar to that reported by Hay (1961) during de-differentiation of muscle fibres in amphibians.

In some animals, e.g. amphibians (Thornton, 1949) de-differentiation begins when demolition ends and continues after epithelial closure. In Arion de-differentiation and demolition overlap to some extent. Both processes involve proteolysis so it is hardly surprising that they can both proceed at the same time and in the same chemical environment. It is of interest that atrophy, another form of tissue regression, also involves proteolysis (Belfer, Koran, Eder and Bradley, 1943). Proteolytic enzymes are concentrated in the epithelium of young regenerates (Orechowitsch and Bromley, 1934) and these may be responsible for de-differentiation.

The factors responsible for the commencement and cessation of de-differentiation in Arion are not known. It has been shown that in amphibians a 'wound factor' exists whose main function is probably the initiation of de-differentiation. Thornton (1949) has shown that beryllium salts which inhibit the wound factor also inhibit de-differentiation. The chemical nature of the wound factor is not known, and its presence is not universal. In the limbs of Asellus there is no evidence of a wound factor (Needham, 1947, '49). Whether one exists in Arion is a problem beyond the scope of this thesis.

The cells which migrate to and form part of the blastema are of the following types: epithelial, blood, connective tissue, and nerve sheath cells. Whether they de-differentiate on reaching the
wound site has not been investigated, nor has the possibility of metaplasia. Algire (1959) suggested that one of the ways in which mammalian connective tissue cells regenerate is from "specialised wandering cells" in the blood stream by metaplasia. It would be of interest to investigate this possibility in Arion, for it has already been noted that cells similar to the blood cells are present in the connective tissue (p. 40). Until there is a reliable method for long-term labelling of de-differentiated cells, however, it will be difficult to prove conclusively if a cell is metaplastic.

Migration of cells is extremely important in blastema production amongst the lower invertebrates. In coelenterates interstitial cells migrate to the wound (Messing, 1903; Kanajew, 1926; et al); in annelids migratory neoblasts are thought to be responsible for blastema formation (Faulkner, 1932; Stone, 1932; Turner, 1934; et al.); in crustaceans migratory cells are thought to be responsible for forming the internal tissues of regenerating limbs (Charniaux-Legrand, 1951). In 1951 Stephan-Dubois surveyed the histological potentialities of migratory undifferentiated cells in the regeneration of coelenterates, planarians, and oligochaetes. Little work has been done on this aspect of regeneration in molluscs. Techow (1910) thought that the molluscan blastema was immigrant; Hanko (1913) that it was indigenous; while Lange (1920) claimed that in cephalopods it was bipartite consisting of:

(a) agglutinated blood cells which gave rise to new connective tissue,

and

(b) local de-differentiated cells which formed the rest of the regenerate.

It is clear from the results of the current investigation that in Arion the myoblasts are local in origin, while epithelial, blood, connective tissue, and nerve sheath cells migrate to the wound. The latter claimed is also supported by results discussed later (pp. 246, 248).
The nature of the immigrant cells of *Arion* blastemata is somewhat uncertain. As mentioned above, blood and connective tissue cells contribute to the blastema. The uncertainty lies in whether or not the so-called connective tissue cells are in fact blood cells. Since the blood system is of the 'open' type and the walls of the blood spaces are extremely thin it is possible that there is an interchange of cells between blood and connective tissue. It has already been noted (p. 40) that three of the connective tissue cell types are similar in appearance to the blood cells and (p. 97) that many of the blastema cells are also similar to the blood cells. In considering glycogen content, it was recorded that type 'c' cells (p. 42) containing glycogen entered the blastemata (p. 47). These cells are unlike any found in the blood and are truly connective tissue cells. The fate of these cells, after losing their glycogen, is not known. They may or may not remain within the blastema. It was recently suggested (Wollfe, 1961) that migratory cells in invertebrates are attracted to the wound tissue by diffusible wound substances. This may explain the movement of connective tissue cells to the wound region.

Nerve sheath cells also migrate to the wound. Hollow nerve sheath tubes, which in uninjured tissue would contain an intact nerve, grow into the blastema and extend to keep pace with its forward growth. This extension is a result of both cell migration and cell division (p. 95). Into each tube nerve fibres later regenerate. It is not known if the sheath cells leave the ends of the tubes and form part of the blastemata. It is of interest that the Schwann cells of mammals migrate down the nerves after injury and it has been suggested that there is also some outwandering, in material cultured 'in vitro' at least (Abercrombie and Johnson, 1942; Abercrombie, Johnson and Thomas, 1949). The Schwann cells of mammals though not strictly comparable in position to the sheath cells of *Arion* show parallel behaviour in this respect. Recently Nathaniel
and Pease (1963) suggested that the Schwann cells formed collagen and basement membranes during regeneration.

The importance of the migration of epithelial cells is investigated more fully in a later section (p. 194).

At first considerable difficulties were encountered in demonstrating cell division in Arion blastemata. It is well known that dividing cells are difficult to locate in somatic tissue (Montagna, 1952) but in general workers on vertebrate regeneration report no difficulty in locating such cells. It was eventually found that by fixation in Carnoy for 1 hour at 4°C mitotic figures could be located, and examples of these are illustrated in Figs. 31, 35. Once this was discovered further work (pp. 232, 246) showed that cell division was of considerable importance in formation and growth of the regenerate.

The first cells to divide are the nerve sheath cells (p. 90). There is indirect evidence of division in these cells 24 hours after wounding. This is interesting in light of findings of Abercrombie and Johnson (1946) and of Thomas (1948) who showed that some product of the degenerating nerve stimulated proliferation of Schwann cells and local blood vessels, although Karczmar (1946) had shown that similar products induced de-differentiation in other tissues. Needham (1952) suggested that proliferation of Schwann cells was necessary for the regeneration of nerve, which "must in turn anticipate the regeneration of other tissues in order to control their regeneration". Thus it is reasonable to suppose that Schwann cells would multiply at a time when other tissues are de-differentiating. The proliferation of nerve sheath cells in Arion at a time when other tissues are de-differentiating is a situation similar to that concerning the Schwann cells, and it would be of considerable interest to see if here, too, some product of degenerating nerve is responsible for the concurrent processes of de-differentiation and proliferation.
The other cell types of the blastema divide much later. Myo­
blast division commences on the fifth day after wounding (p.101),
and division of the blastema cells is at a maximum on the seventh
day after injury (pp.233,246), when they show intense basophilia
due to their R.N.A. content. The epithelial cells seem to divide
as required to keep pace with the increase in size of the regenerate.
and mitotic figures are quite common as late as the twelfth day
after wounding.

5. Differentiation.

The tissue in which differentiation has been studied most fully
in the course of this work is the muscle tissue. Structural differ­
entiation has reached a level at which it is visible by optical
microscopy by the eighth day after wounding (p.113), though changes
in spatial orientation of the myoblasts commence at the fifth day
(p.101). Differentiation, both spatial and structural, begins in
those regions adjacent to the uninjured tissue and progresses out­
wards into the rest of the blastema (Fig. 39). It would appear
that the uninjured tissue organises development of the regenerating
muscle in such a way that by the end of the process the excised
portions of the ventral muscle mat and lattice have been replaced.
The regenerated muscle fibres are functional by the twelfth day
after injury.

Collagen fibres have been shown by Watts (1961) to be developed
from the mucopolysaccharide ground substance and it is noticable
that in Arion the ground substance takes up stain most deeply
before and during collagen fibre production (pp.93,102). It is a
marked feature that collagen fibres, which increase the tensile
strength of the regenerate, are most abundant in those places where
the muscle fibres are differentiated and functional and are thus
capable of exerting a pull on the wound tissue.

Structural differentiation of the cells of the blastema is
accompanied by changes in their metabolism. As differentiation proceeds any positive SH and SS response the cells may have had is lost (p. 178). The alkaline phosphatase activity of the myoblasts is greatest when the myofibrils are being formed, possibly because the energy-rich phosphate bonds broken by enzyme activity provide energy for protein synthesis. The cytoplasmic R.N.A, content appears to decrease as differentiation increases. The glycogen store is exhausted at differentiation, presumably because it is being used as a source of energy for the concurrent processes of growth and differentiation.
V. ANALYSIS OF BLASTEMA FORMATION.

A. MOVEMENT OF EPITHELIAL CELLS.

INTRODUCTION.

When tissue is excised from the mantle edge of *Arion hortensis* (p. 77) the epithelium of the dorsal surface is torn so exposing some of the connective tissue behind the excision line. The wound is sealed initially by blood cells and later by epithelial cells (pp. 87, 104) the latter forming the outermost layer of the blastema. The aim of this investigation was to determine:

(a) how soon after wounding migration of the epithelial cells commenced;
(b) the rate of epithelial cell migration;
(c) the stage at which migration ceased;
(d) the effect of multiple wounding on the direction of epithelial cell movement.

Peters (1885) was one of the earliest authors to report the rapid migration of epithelial cells over a fresh wound surface. It was recognised that epithelial movement resulted in wound closure and the method by which this movement was achieved became a matter of some controversy. Fraissé (1885) thought that wound covering resulted from epithelial proliferation, but his opinion was not supported by experimental evidence. Barfurth (1891) noted the absence of proliferation and showed that covering was caused by the amoeboid movement of cells from the edges of the wound.

If closure is effected by movement of epithelial cells from the wound edge and not by their proliferation, as is now generally supposed, there remains the problem of how this movement is achieved. Two explanations are possible:

(a) cells are drawn passively over the wound;
(b) cells move actively, either as a cohesive sheet or as individual cells.
Rand (1905) claimed that in the case of the earthworm epithelium wound closure was due to the active movement of individual cells. Oppel (1913) and Osowski (1914), while agreeing that closure was caused by the active movement of epithelial cells, believed that this was a "Massenbewegung" or mass movement, and not necessarily amoeboïd in nature. It had earlier been claimed (Born, 1897) that epithelial flattening, resulting in the coverage of a greater surface area, might be responsible for wound closure. Holmes (1914) working with tadpole tails 'in vitro' claimed that here the coverage of the injured surface was due to passive mass movement of the epithelium. Only the cells at the very edge of the wound advanced, according to this view, pulling the rest of the epithelial sheet behind them. Arey (1936) believed that, in mammalian wound healing at least, wound closure was caused by the active movement of epithelial cells, and that after wound closure cell proliferation followed, terminating in the reconstitution of the normal epidermis. Lash (1953) studying wound closure in the urodele skin, also supported active cell migration, but did not investigate the possibility of cell proliferation after closure. He concluded that though the epithelial cells appear to move 'en masse' they still behaved as "individual locomotor units". He also found that the breaking of the attachment of the epithelial cells with the substratum (the basement membrane) enabled the cells to move freely over the wound surface. Weiss and Ferris (1954) had shown earlier by electron microscopy that in the larval amphibian skin the epidermis was attached to the basement membrane by osmiophilic adhesive discs. When the skin was injured the discs lost their adhesive power and the cells were able to move.

In the investigations described above, the authors had assumed that the epithelial cells which closed the wound remained there as healthy, permanent tissue. There is still some doubt on this point. In studies on mammalian wound healing Billingham and Medawar (1955) stated that the migratory epithelium was a temporary repair
tissue, disappearing when its work was completed. Other authors (Carrel and Hartmann, 1916; Abercrombie and James, 1957) thought it was a permanent tissue, while Joseph and Townsend (1961) working with the mobile skin of the rabbit, found that the migratory epithelium covering some 10% of the denuded area was permanent, eventually differentiating into new epidermis containing hairs.

Few authors, with the exception of Eycleshymer (1907), Herrick (1932) and Lash (1955) have followed the movement of individual marked cells during wound closure, and their work has been restricted to the vertebrates. In the present investigation a modification of the methods used by Lash (1955) on urodele skin has been used in the study of epithelial movement in Arion. This involved the insertion of carmine granules into individual cells, using the granules as markers to trace the movement of these cells.
METHODS USED.

The animals were lightly narcotised with diethyl ether vapour. They were put in a covered petri dish into which a ball of cotton wool, soaked in diethyl ether and supported on a solid watch glass, had earlier been placed. The vapour caused an initial extrusion of mucus followed by writhing movements. When movement ceased the animals were taken from the dish and operated on.

The epithelial cells were removed from an area 1 mm. square, on the dorsal surface of the mantle edge, by scraping with a fine glass knife. This method of wound was chosen rather than that used in previous experiments (p. 63) because the movement of cells could be traced more easily over a dorsal, horizontal, injured surface than over a vertical one. It was found, on subsequent microscopic examination, that the thin basement membrane and underlying pigment granules were also removed and the connective tissue exposed.

Using a binocular microscope, granules of carmine were placed on the surface of the epithelium around the margin of the wound. With the aid of fine needles, mounted on a simple micromanipulator attached to the mechanical stage of a second microscope, the granules were thrust into the epithelium, where they remained readily visible.

Two types of needle were tried out in the preliminary stages of this work:-

(a) Needles made from stainless steel or tungsten wire of s.w.g. 47 and etched down to a fine point according to the method of Lash (1955). Lash's method was modified in that the wire was placed in the glass rod at an angle of 45° to the longitudinal axis of the rod. This was found desirable since the micromanipulator used only allowed limited freedom of movement.
(b) Glass needles made according to the method in Rugh (1948), but also with the point at an angle of 45° to the rod.

The wire needles, though they had the advantage of having very fine points, tended to bend under the slight pressure applied when thrusting the carmine granules into the epithelium. Glass needles, though of wider tip diameter and more brittle, were found to bend less than the wire needles and were therefore used in most of the experiments.

The experimental animals were placed in covered petri dishes lined with moist cotton wool. They were examined every 15 minutes under a monocular microscope. Displacement of the marked epithelial cells was measured with a micrometer graticule. The wound margin, which is readily seen because pigment granules were excised with the epithelium making the wound lighter in colour than the surrounding, black, uninjured tissue, was used as a reference line. The term "wound margin" here refers to the junction between black and lighter areas.

When movement of the marked cells had ceased, the specimens were fixed in Bouin, sectioned at 8 μ and stained with Ehrlich's haematoxylin. Only measurements for cells into which carmine granules had been correctly inserted were accepted as valid.

In the course of these experiments the movement of 41 successfully marked cells was traced.
RESULTS.

1. Direction of epithelial cell movement after single wounding.

After an initial period of inactivity, correlated with the distance of the marked cell from the wound margin, movement commenced. In 22 out of 23 marked cells movement was towards the wound margin. In only one case was there a temporary reversal of the direction of movement and this only for a 15 minute period. The migration of marked cells towards a typical single wound is shown in Fig. 77. Here the movement of four cells has been plotted and it can be seen that all move towards the wound margin, that the two cells initially nearest to the wound travel some distance over the wound surface before movement ceases, and that the cells furthest from the wound are the last to begin to move.

2. Direction of epithelial cell movement after double wounding.

In the double wounding experiments, two wounds were made one behind the other on the dorsal surface, and in all cases the anterior wound was made first. A distance of one to two mms. was left between the wounds, which were both of the same size, 1 mm. square. The posterior wound was made as soon as the anterior wound had been completed.

Of the 18 successfully marked cells studied, all moved towards the nearest wound margin. In one case the movement of a cell equidistant between the two wounds was traced and it was found that the cell moved towards the anterior wound. There was an initial period of inactivity prior to the commencement of epithelial movement. The movement of marked cells after a typical experiment in double wounding is shown in Fig. 78. In this experiment 4 cells had been marked.
Fig. 77 Movement of epithelial cells after single wounding.
Fig. 78 Movement of epithelial cells positioned between two wounds.
3. Delay in mobilisation of cells.

As mentioned earlier, there is an initial delay in the mobilisation of the epithelial cells. The length of this delay is correlated with the original distance of the marked cell from the wound margin. Fig. 79 illustrates this, showing the delay of mobilisation with distance from the wound margin for cells from both single and double wounded animals. The best-fit line has been drawn and the equation for this line and the data for the diagram are to be found in Appendix 4. The correlation coefficient, \( r \), was calculated, and the probability found to be less than 0.001 indicating that the correlation is highly significant.

The length of delay is similar after both single and double wounding, as Fig. 79 shows. 23 of the cells traced were from single and 18 from double-wounded animals. The delay ranged from 15 to 135 minutes, and in general the cells nearest to the wound margin were the first to be mobilised.

4. Rate of movement of cells.

The mean rate of movement for 38 marked cells initially at distances of between 0\( \mu \) and 900\( \mu \) from the wound margin was calculated as 75\( \mu \) per hour. This rate is much lower than that reported by Lash for urodele skin (119\( \mu \) per hour).

The rate of movement of marked cells is not affected by their distance from the wound margin. This is illustrated in Fig. 80 where the rates for the 38 cells have been plotted against their original distance from the wound edge, and the best-fit line drawn. The data for this diagram and the equation of the line are given in Appendix 5.

It can be seen from Fig. 80 that there is less variation in the rate of movement of epithelial cells from double-wounded than from single wounded animals. It was therefore decided to
Fig. 79 Variation in delay of epithelial cell movement with distance of cell from nearest wound edge.

- Single wounding.
- Double wounding.
Fig. 80  
Variation of rate of movement of epithelial cells with original distance from wound edge.

- Single wounding.
- Double wounding.
test statistically the possibility that there might be a significant difference between the mean rate of movement after single and that after double wounding. The mean rate after single wounding is \( 73 \mu \) per hour and that after double wounding \( 76 \mu \) per hour. Student's t-statistic was calculated as \(-0.341\), but since this figure lies inside the limits for \( t \) at the 5\% level of significance (± 2.01) the difference between the two means is not significant.

5. Time of cessation of epithelial cell movement.

There is no significant correlation between the time at which a cell stops moving towards the wound margin and its original distance from it. This is illustrated in Fig. 8 which shows the variation in the time of cessation of 23 cells, 12 from double wounded and 11 from single-wounded animals. The cessation times of the cells have been plotted against their original distance from the wounds, and the best-fit line drawn. The data on which the figure is based and the equation of the line are given in Appendix 6.

The possibility that there might be a significant difference between the mean time of cessation after single and that after double wounding was tested statistically. The mean cessation time after single wounding is 166 minutes and that after double wounding 179 minutes. Student's t-statistic was calculated as \(+4.112\), and since this figure lies outside the limits at the 5\% level of significance chosen for the test (± 2.08), the difference between the two means is probably significant.


Within the first hour following injury the undamaged epithelial cells adjacent to the wound become detached from the thin basement membrane. At one hour after injury, in material fixed in Bouin and sectioned, the cells are separated from the basement membrane by a distance of the order of \( 0.5 \mu \), (p. 73). Subsequent events
Fig. 81 **Variation of time of cessation of epithelial cell movement with original distance from nearest wound edge.**

- o Single wounding.
- o Double wounding.
show that the cells are now free to move. The cells flatten, and since they are firmly attached to the basement membrane distal to the injury and free from the membrane proximal to it they begin to move over the injured surface. The cells do not, however, move as a completely cohesive sheet; cells sometimes slip out of the sheet, lie dorsal to it, and cause piling up, (see Fig. 20). This may indicate that epithelial cell movement is more than just a passive flattening of the cells, or a simple mass movement; individual cell movement also plays a part.

Movement of the epithelial cells over the wound surface ceases before wound closure is complete. It is not, therefore, caused by contact with homologous cells, as found by Chiakulas (1952) in his work on tissue specificity in urodele wound healing. The reason for cessation of movement in Arion is not known with certainty, but a possible explanation is given on p. 212.

Temporary closure of the wound surface is achieved in part by epithelial cells but also by agglutinated blood cells (see p. 87). Although the latter cells are much in evidence at 24 hours after wounding they are not very numerous after 2 hours, by which time the movement of the epithelial cells has usually ceased. Final closure of the injured surface is effected by growth and division of the epithelial cells. The evidence for this has already been given (p. 104).
SUMMARY AND DISCUSSION.

The results indicate that the epithelial migration following wounding is an organised process. The initial effects of injury on the epithelium can be summarised as follows:

(a) The cells immediately surrounding the wound become separated from the underlying tissue.

(b) After a short period of inactivity the cells nearest the wound margin flatten and move over the injured surface.

(c) This mobilisation and flattening of the cells extends to the more distant uninjured tissue. There is a definite correlation between the time of mobilisation and the distance of the cell from the wound margin.

(d) The rate of movement of the individual cells shows some variation, though this is not correlated either with the original distance of the cell from the wound or with whether one or two wounds had been made.

(e) While epithelial movement appears to be primarily due to cell flattening, there is evidence that movement of individual cells, as distinct from movement of the cell sheet as a whole, is also possible.

(f) The time at which individual cell movement ceases shows some variation though this cannot be correlated with the original distance of the cells from the wound margin. It is possible that cell movement ceases earlier when only one wound has been made.

(g) Cessation of epithelial cell movement is not caused by contact with homologous cells.

These effects will now be considered more fully.

That there is some change in the relationship between the
epithelial cells surrounding the wound and the basement membrane in response to the stimulus of injury is unquestionable. From histological evidence it would seem that the cells separate from the membrane. It is possible, however, that the attachment between the cells and the membrane is simply weakened and that contact with fixative causes complete detachment of the cells. The effect of weakening or breaking the bonds between cells and membrane is that the cells are now free to spread over the surface of the wound coagulum. A similar pattern of events has been reported by Lash (1955) in the urodele skin. It is possible that the gradual acquisition of movement by the epithelial cells is the direct result of loss of adhesion between cells and basement membrane.

The variation in the rate of movement of individual marked cells is probably a result of their capacity for individual movement. It has been noted already that though as is usually found for epithelia (Curtis, 1962) the mass movement of the cells as a sheet is of primary importance, individual cells frequently slip out of the sheet and migrate above it, resulting in the piling up of cells. Those cells whose rates of movement are furthest from the mean are probably those which have slipped out of the sheet while those nearer to the mean are presumably still part of it. This must mean that contact inhibition is incomplete, for it has been suggested (Abercrombie, 1957) that such inhibition probably prevents the overlapping of epithelial cells and so co-ordinates their movements. Curtis (1962) stated that epithelia in embryogenesis, wound healing and tissue culture "move together as apparently coherent sheets of cells. The cells neither climb over one another nor lag behind the rest when the sheet moves". This is not fully confirmed by the results obtained for Arion. It should however, be emphasised that relatively few cells leave the epithelial sheet and that by the time epithelial movement ceases the epithelium is again single-layered throughout. It has been suggested (Abercrombie, 1958) that in the case of fibroblasts, cells
in mitosis do not show contact inhibition to others of their kind. Mitosis does not take place in the epithelial cells until after cell movements have ceased, so this cannot be the explanation for the incomplete contact inhibition described here. It is thus clear that in *Arion* both mass movement and individual cell movement contribute to the spreading of the epithelium over the wound surface.

The initiation of movement in the epithelial cells begins in those closest to the wound and gradually extends outwards to more distant cells. This correlation between the distance of a cell from the wound margin and the time at which it commences to move may well prove to be due to the spreading outwards of some change at the epithelial cell and basement membrane interface, beginning at the wound margin and initiated by wounding. It is known that certain enzymes, e.g. acid phosphatase, decrease the adhesion of Landschutz ascites cells to glass, possibly by being adsorbed onto the cell surface and thus altering its adhesive powers (Ambrose and Easty, 1960). It is also known from the present investigation (p. 130), that there is a significant increase in the acid phosphatase content of the wound region at this stage in healing. It is possible that these factors may be correlated in *Arion* epithelium, also.

Certain facts revealed in the course of these experiments are of importance in connection with the cessation of epithelial movements.

(a) The time of cessation is earlier after single than after double wounding. Statistical evidence has been offered which indicates that this difference is significant.

(b) There is no significant difference in the rates of movement of cells after single and double wounding.

(c) Cessation is not, in *Arion*, due to contact inhibition caused by homologous cells.
There are several possible explanations why the epithelial cells may cease to move:
1. Accumulation of toxic materials at the wound surface.
2. Formation of a barrier by non-homologous cells.
3. Lack of oxygen or some other essential metabolite.
4. Lack of an activator substance.
5. Presence of an inhibitor.

The accumulation of toxic metabolic by-products is extremely likely in the initial stages of wound healing, for in Arion there is a temporary reduction in the blood supply of the wound region during the interval between excision and wound closure. During this interval, which lasts for several hours, it is possible that toxic materials might accumulate and affect epithelial movement. It is of interest to compare the partial wound closure by epithelial cells found in Arion with the method of closure of small wounds in vertebrates, where the period of initial capillary contraction is much shorter, and where toxic by-products can thus be removed more rapidly; here the cells do not stop moving precociously but continue moving until they touch homologous cells and close the wound completely, (Nouy, 1936; Lash, 1955).

The fact that the epithelial cells of Arion continue to move for longer periods after double wounding than they do after single wounding cannot be explained entirely on the basis that cessation is caused by the degree of toxicity of the cellular environment. Care was taken to make all the wounds inflicted as similar in relative position, size and shape as possible, and, where double wounding was involved, to make the wounds equal distances apart. It is thus unlikely that double wounds should together produce less toxic by-products than a single wound. It may well be that the accumulation of toxic materials plays some part in affecting the cessation of epithelial cell movement, but it is likely that some other factor is also involved.
Neither does the formation of a mechanical barrier by non-homologous cells provide an adequate explanation for the cessation of epithelial movement. The act of wounding always exposes such cells, as well as resulting in the presence of some debris (p. 77), and the migrating epithelium moves forwards initially without noticeable hindrance. Other authors, such as Lash (1955), have recorded that when epithelial cells encounter a barrier they either dislodge it or pile up against it; they do not stop moving. The mechanical barrier hypothesis can therefore be discounted.

The possibility that the epithelial cells are being starved of some essential metabolite, as a result of reduction in blood supply, may partly explain the cessation of their movement. In the case of double wounding, if the two wounds are competing for a limited supply of the metabolite, then it might be expected that movement would cease earlier than after single wounding when there could be no such competition. In fact the reverse was found, and it is thus clear that some further factor must be involved.

Some activator substance, released by wounding, might be responsible for epithelial cell movement. When the activator had been used up then cessation of movement would follow. This hypothesis can be dismissed on similar grounds for it does not explain why cessation occurs earlier after single than after double wounding.

A further possible explanation is that cessation of epithelial cell movement is caused by the presence of an inhibiting factor at or above the minimum functional level of concentration. Since the epithelium is normally non-mobile it can be assumed that the inhibiting factor, if one exists, is present before injury. One of the effects of injury is that the chemical environment of the wound region changes considerably (see p. 65). The changed conditions might well affect the action of the inhibitor and result in cell
mobilisation. Where two wounds are made in one animal the total amount of inhibitor lost will be substantially increased. If it is assumed that the uninjured tissue adjacent to the wound continues to produce inhibitor at its usual rate, and that this is in excess of the minimum required to cause cessation of epithelial movement, then the concentration of inhibitor reaching the wound region by transport in the blood or by diffusion will gradually increase until the functional minimum is attained when epithelial cell movement will cease. If the rate of inhibitor production by uninjured tissue remains constant when adjacent regions are injured, then it will take longer to replace the inhibitor destroyed on double wounding than to replace the smaller amount destroyed on single wounding, and consequently it would be expected that cell movement would continue longer. This is, in fact, the case. It is not suggested that this 'inhibiting factor' is entirely responsible for the cessation of epithelial cell movement. The absence of essential metabolites or the presence of toxic substances may also be involved.

The 'inhibiting factor' hypothesis is tentatively suggested as a possible explanation of the limited facts available on this aspect of wound healing in Arion.
B. ORIGIN OF SUB-EPITHELIAL BLASTEMA CELLS.

INTRODUCTION.

The blastemata of vertebrates are produced from local de-differentiated cells (Guyenot, 1927; Hertwig, 1927; Butler, 1933; et al.). Those of the lower invertebrates, e.g. planarians, coelenterates and annelids, are systemically recruited from non-local migratory cells, (Messing, 1903; Kanajew, 1926; Faulkner, 1932; Schotte, 1939; et al). Berrill (1935) believed that in tunicates both de-differentiated type-specific and non-differentiated pluripotent cells contributed to the regeneration blastema. Several conflicting opinions have been expressed as to the origin of blastema cells in molluscs. Techow (1910) believed that the blastema was immigrant; Hanko (1913) that it was indigenous; while Lange (1920) reported that in cephalopods both immigrant and indigenous cells contributed to blastema formation, the immigrant cells producing connective tissue and the indigenous cells forming the rest of the regenerate. Pande (1938) considered that the blastema cells of Helix aspersa originate from local undamaged cells which accumulate at the wound site. The conclusions of all four authors were based on histological observations on regeneration induced by experimental wounding.

There are several ways in which blastema formation could occur:

1. De-differentiated or non-differentiated cells may migrate to the wound area from some other region of the body, i.e. the blastema may be entirely immigrant.

2. Cells present within the wound region at the time of injury may de-differentiate and divide 'in situ' i.e. the blastema may be entirely indigenous.

3. The blastema may be formed partly from immigrant cells and partly from indigenous tissue.
In all three cases cell division within the blastema may add to its growth.

Evidence that the epithelial cells of the regenerate are immigrant (pp.199-207), and that the muscle cells are derived from indigenous cells (pp.90,95), has already been presented. It has also been suggested that the rest of the sub-epithelial tissue of the regenerate is probably derived from immigrant cells (p. 244). The aim of the experiments described below was to investigate the origin of the sub-epithelial blastema cells more thoroughly and to assess the importance of cell division in growth of the blastema.

Two techniques were used in this investigation: tissue culture and colchicine treatment.

1. Tissue culture.

Although the literature on the culture of embryonic organs is extensive, (see references cited in Willmer, 1935, and Paul, 1959), far less attention has been directed to the possibilities of blastema culture. Wound healing 'in vitro', especially the repair of microwounds and subsequent epithelial movements, have been studied by Bentley, (1936), Wilbur and Chambers (1942) et al., while liver regeneration has been observed 'in vitro' by Abercrombie and Harkness (1951). Sengel, (1960) succeeded in culturing undifferentiated caudal and cephalic blastemata of Dugesia lugubris for as long as 4 weeks, during which period differentiation of eyes, pharynx, muscle and pigment occurred. Little difficulty was encountered by Hill (1934) in the culture of mantle integument from pulmonate molluscs even when no particular care was taken to ensure that the cultures were aseptic. Pande (1958) using a modification of Shaffer's (1956) technique to culture blastemata from Helix aspersa found, however, that infection of the explant
occurred at an early stage. Care was therefore taken to maintain sterile conditions during blastema culture in the present investigation.

Blastemata were removed from slugs, wounded as described on p. 68, at different stages of growth and allowed to develop 'in vitro'. By removing the blastemata from the only possible source of immigrant cells, namely the injured animal, it is possible to assess the importance of cell migration to development of the regenerate. Counts of the number of nuclei present in a known area of the blastema were made at intervals during regeneration of isolated blastemata and of controls allowed to develop 'in situ'. These estimates of nuclear density gave some idea of the rate of cell division at different stages in the development of the isolated blastemata and of the relative importance of cell migration and cell division in blastema growth. This technique was used because mitotic figures are not readily visible in Arion blastemata and consequently the direct counting of such figures does not give a reliable estimate of cell division. The difficulty of observing mitotic figures in histological sections of somatic tissues is well known (Montagna, 1952).


The mode of action and possible uses of colchicine have been reviewed by Eigsti and Dustin (1955). The drug arrests mitosis at metaphase by acting on the spindle mechanism in such a way that no spindle fibres are formed and consequently no chromatid separation occurs. At metaphase nuclear chromatin is readily stainable and the drug is thus of use in detecting dividing nuclei.

Colchicine has been used relatively little in studies on wound healing and regeneration. Dustin and Chodkowski (1938) studied the effect of the drug on skin healing in rats. Rapid division of the endothelial cells of the blood capillaries was reported. The drug was injected 9 hours before fixation and since it would
presumably have arrested all attempted mitoses during that period at metaphase this may explain the high number of mitoses reported. Delcourt (1938) used colchicine to investigate division of Schwann cells after cutting peripheral nerves. He, also, found an increased number of mitoses but these he attributed to the influence of disintegration products of myelin. Thornton (1942) found that if larvae of *Amblystoma opacum* and *A. punctatum* were placed in a 1:1000 or a 1:5000 solution of colchicine at the moment of amputation all regeneration was suppressed. Luscher (1946) reported that treatment of Xenopus larvae with a 1:2000 solution of colchicine for 1 hour inhibited mitosis but did not stop cellular migration so that tail regeneration could proceed for a while (5 days) but later ceased, while larval growth was completely inhibited. Bernhard (1947) found that local application of 1:500 M colchicine for 20 minutes inhibited tail regeneration but not growth in the tadpole.

The fact that there is usually an apparent increase in the mitotic rate after application of colchicine makes it inadmissible to use counts of mitotic figures from treated specimens as an indication of the true division rate. If such counts are used comparatively, however, and the time between colchicine application and fixation of the material is constant in all experiments, then the drug can be used validly as an experimental tool.

In the course of this work colchicine is used as follows:

(a) To assess the importance of cell division at different stages in blastema development.

Colchicine was injected into wounded animals with developing blastemata a few hours before fixation to arrest all mitoses at metaphase. Counts of arrested nuclei were made for blastemata at different stages of development.
(b) To determine the effect of total mitotic arrest on blastema formation.

Each wounded animal was given a haemocoelic injection of colchicine immediately after operation. Further injections were given at 24 hour intervals to arrest mitosis completely.
METHODS.

1. CULTURE OF ISOLATED BLASTEMATA.

Slugs were wounded with operating forceps (p. 68) and kept at 15°C under the conditions described on p. 67. Blastemata were removed from randomly selected animals, at intervals of 24 hours, from 1 to 10 days after wounding and cultured at 15°C.

(a) Blastema removal.

Standard sterile precautions were maintained throughout this operation. Instruments were sterilised by dry heat at 140°C and then transferred to a beaker of 70% alcohol. They were rinsed in sterile distilled water immediately before use. The blastemata were removed under a dissecting microscope within a polythene cage to minimise contamination with dust. All working surfaces were cleansed with alcohol.

The mantle edge was removed from the un-narcotized specimen with fine scissors and rinsed quickly in a solution of 20 units of sodium penicillin G per ml. of sterile Holtfreter's saline. It was then transferred to a freshly-flamed, wax-lined operating dish containing a drop of sterile saline. The blastema tissue, which is always much paler than the heavily pigmented surrounding tissue, was dissected off with a cataract knife and transferred with a pipette to a sterile culture dish. Care was taken to dissect off only blastema material. Specimens with adherent surrounding tissue were discarded.

(b) Preparation of culture dishes.

The moist chamber method of organ culture in current use at the Strangeways Laboratory was adopted (Fell, cited in Atkins, 1959). Each culture vessel consisted of a watch glass, placed in a petri
dish, and surrounded by cotton wool saturated with a 0.9% solution of sodium chloride in sterile water to provide a moist environment. It has been found that when salt solution is used in place of distilled water there is less condensation of water droplets over the watch glass and therefore less danger of these droplets falling into and diluting the culture medium. The glassware was sterilised in a pressure cooker for 15 minutes at a pressure of 15 lbs. A little hot agar was poured into each watch glass and the petri dishes covered. The prepared culture dishes (Fig. 82) were stored temporarily in a refrigerator at 4°C until required.

(c) Blastema culture.

Fresh Arion blood was centrifuged to remove all blood cells and the supernatant plasma used as a culture medium. Samples of the plasma were examined microscopically to ensure that no cells were present. Since it seems probable that blood cells contribute to the blastema (p. 92) and the primary reason for using tissue culture was to remove the blastema from the source of immigrant cells, this precaution was essential. Two drops of plasma were placed 1 cm. apart on the surface of the agar in each watch glass. One blastema was placed in each plasma drop and the petri dish immediately covered. The culture dishes were kept in a constant temperature room at 15°C during the course of the experiments.

Batches of blastemata were removed from randomly selected wounded animals, at 24 hour intervals, from 1 to 10 days after injury, and cultured as described above. Samples of 5 blastemata were removed from each batch of cultures at 24 hour intervals from 1 to 8 days after culturing and fixed in Bouin. To avoid damage to the blastemata they were fixed still attached to the underlying agar. After embedding in paraffin wax and sectioning at 8µ the specimens were triple stained with Delafield's haematoxylin, eosin and alcian blue.
Fig. 82. Vessel used in culture of blastemata.

a., agar; b., blastema; b.p., blood plasma; c.w., cotton wool saturated with 0.9% saline; p.d., petri dish; w.g., watch glass.
(d) Blastema examination.

Blastemata which showed signs of infection or necrosis were discarded. In some instances the blastema cells had migrated over the surface of the agar; these specimens were also discarded. The degree of differentiation was noted and an estimate of the nuclear density, i.e. the number of nuclei per unit area, obtained. A calibrated graticule marked into squares was placed in the x10 eyepiece of a microscope fitted with a mechanical stage and the number of nuclei per field counted. The area of each field was $625 \mu^2$. This was repeated ten times for each blastema, and the means and standard errors calculated and plotted against blastema age. Where the standard error was greater than $\pm 1$, the sample size was increased to 20 and the means and standard errors recalculated. Changes in the nuclear density of control blastemata left to develop 'in situ' were also noted and plotted against blastema age.

2. COLCHICINE TREATMENT.

(a) To aid recognition of dividing nuclei.

Slugs were wounded in the manner described on p.68. At 3, 4, 5, 7, 8, and 10 days after wounding 5 specimens were selected and treated as follows:

(i) four were injected with 1:2000 colchicine in sterile Holtfreter's saline (i.e. 1 g./2000 ml).
(ii) one, the control, was injected with pure saline only.

0.05 ml. of fluid per 100 mg. body weight was injected directly into the haemocoele of each animal by inserting a hypodermic needle under the mantle edge and perforating the body wall at the junction of the mantle edge with the head integument.

After 5 hours the specimens were killed by immersion in cold Carnoy fixative and kept at 4°C during the fixation period.
hour. Paraffin wax sections of the material, cut at 5 μ, were either stained with Feulgen or triple stained with Delafield's haematoxylin, eosin and alcian blue.

The mitotic rate, i.e. the number of dividing nuclei per 1000 nuclei, was obtained for each specimen and plotted against age of the regenerate.

Different concentrations of colchicine were used in trial experiments, viz:


It was found that though concentrations as low as 1:8000 caused metaphase arrest in some animals, the lowest concentration to cause metaphase arrest in all the trial animals was a 1:2000 solution and therefore this was used in subsequent work.

(b) Total mitotic arrest during healing.

30 animals were operated on in the manner cited on p. 68. Immediately after operation 10 specimens were isolated as controls. The 20 experimental animals were injected with 1:2000 colchicine in sterile Holtfreter's saline, while the controls were injected with pure saline only. The method of injection used was that described above, (p. 222). Each slug received a further injection every 24 hours throughout the experiment.

Three specimens were selected at random every 24 hours (two experimental and one control) and fixed for 1 hour in Carnoy at 4°C. Paraffin wax sections, cut at 8 μ, were stained with Delafield's haematoxylin, eosin, and alcian blue. Any variation in the normal wound healing pattern was noted, and the nuclear density obtained using the method described on p. 222.
RESULTS.

1. CULTURE OF ISOLATED BLASTEMATA.

67% of the cultured blastemata remained healthy throughout the culture period. Migration of cells occurred in 11% of the specimens, 10% became infected by bacteria, and 12% exhibited a phenomenon termed here 'aging'. In the latter case the nuclei degenerated, cellular detail was lost, pigmentation occurred and there was a pronounced increase in the quantity of collagenous material present (Fig. 83). Only healthy cultures were examined further. The results below were obtained after examination of 150 specimens.

(a) Development of isolated blastemata.

The isolated blastemata develop in much the same manner as controls left 'in situ'. Several interesting differences have, however, been found:

(i) When blastemata are excised from experimental animals the epithelium is intact. The cells of this epithelium usually disintegrate during culture (Fig. 84 a, b). The reason for this is not known, though it may be due to some nutritional deficiency of the culture medium. Bullough and Johnson (1951) have shown that to maintain epidermal mitoses 'in vitro' it is necessary to add glucose to the culture medium.

(ii) There is an increase in the concentration of nuclei per unit area in both isolated and control blastemata (Fig. 84 c) but the maximal and final nuclear densities are lower in isolated blastemata than in the controls.

(iii) Myoblasts of the isolated blastemata differentiate into muscle cells in the same way as in controls where the blastema re-
Fig. 83. T.S. of cultured blastema showing 'aging'.

(Specimen excised 7 days after wounding and cultured for 8 days).

a., traces of adherent agar;
c., connective tissue, showing high collagen content, nuclear degeneration, and loss of cellular detail;
p., heavy pigmentation.
mains 'in situ', but the regenerated muscle cells of the isolated blastemata do not become organised into a lattice and a ventral mat; instead they remain randomly distributed throughout the regenerate (Fig. 84 d).

(iv) Neither mucous nor protein glands have been found in isolated blastemata, though these have been successfully cultured for 15 days after initial wounding. Blastemata left to develop 'in situ' produce Type 3 mucous glands by the fourteenth day after injury (p. 116). It is of some interest that even when blastemata are removed 24 hours after injury and cultured, muscle fibres can differentiate.

(v) Calcium glands were detected in three specimens; of these, two were 15 days old and had been cultured for 5 days, and the third was 12 days old and had been cultured for 6 days, (Fig. 84 e).

(b) Changes in nuclear density during blastema culture.

Changes in nuclear density with blastema age are illustrated in Fig. 85 and are listed in Appendix 7, where the means and standard errors may also be found. In Fig. 85 the mean nuclear density is plotted against blastema age for each batch of cultures. To facilitate comparison the results obtained for all batches are plotted on the same graph.

The results obtained can be summarised as follows:

(i) The nuclear density of both isolated and control blastemata increases steadily to a maximum which is reached between 5 and 7 days after wounding.

(ii) After reaching its maximum the nuclear density decreases at first rapidly and then more slowly.

(iii) The maximum nuclear density is lower in isolated blastemata than in controls left to develop 'in situ'.
**Fig. 84. T.S.'s of cultured blastemata.**

(a) **Onset of blastema formation.** (Specimen excised 1 day after wounding and cultured for 1 day).

(b) **Increase in nuclear density.** (Specimen excised 1 day after wounding and cultured for 4 days).

b.s., blood space; b and p., blastema cells and phagocytes; c., collagen; c.t.e., exposed connective tissue; (epithelium has now completely degenerated); d.e., degenerating epithelium; d.m.f., degenerating muscle fibres; d.m.g., degenerating mucous gland; n.b.c., nuclei of blastema cells.
(c) **Peak of nuclear density.** (Specimen excised 6 days after wounding and cultured for 1 day).

*Stain: Delafield's haematoxylin and eosin.*

- **b.n.** increased number of blastema cell nuclei;
- **b.s.** small blood spaces of regenerate;
- **d.s.** dorsal surface, with epithelium already degenerating and connective tissue partly exposed.
(d) **Muscle fibres lacking orientation.** (Specimen excised 5 days after wounding and cultured for 3 days).

(e) **Developing calcium glands.** (Specimen excised 10 days after wounding and cultured for 5 days).

b.s., blood spaces of blastema; b.n., nuclei of blastema cells; c., abnormally high collagen content; c.g., calcium glands; e., epithelium (partly degenerate); d.s., dorsal surface without epithelial covering; m.f., muscle fibres apparently arranged at random; v.s., ventral surface without epithelial covering.
Age at which blastemata were cultured.

- Controls.
- 1 day.
- 2 days.
- 3 days.
- 4 days.
- 5 days.
- 6 days.
- 7 days.
- 8 days.

Variation of nuclear density with age of the regenerate for blastemata cultured 'in vitro' and controls left 'in situ'.

Fig. 85

Age of regenerate in days.

Nuclear density.
Fig. 86. Correlation between nuclear density of blastemata and the age at which they were excised and cultured.

(a) Cultured blastemata aged 7 days when examined.
(b) Cultured blastemata aged 9 days when examined.
(iv) The longer the blastemata remain 'in situ' the higher the maximum nuclear density attained.

(v) There appears to be some correlation between the nuclear density of a blastema at any given time and the age at which it was excised and cultured. Blastemata aged 7 and 9 days were examined and their mean nuclear density, obtained in the manner described earlier (p. 212), was plotted against the age of the blastema when excised and cultured. Correlation coefficients were calculated. The data are listed fully in Appendix 8 and the results shown graphically in Fig. 86, where the means, standard errors, and the best-fit lines are shown.

2. COLCHICINE TREATMENT.

(a) To aid recognition of dividing nuclei.

The mean mitotic rates for each sample are shown plotted against blastema age in Fig. 87. Since the sample size was small standard errors were not calculated but the individual rates are shown on the graph as well as the means. The data on which Fig. 87 is based are given in Appendix 9.

There is an increase in the mitotic rate during the initial stages of the healing process, which reaches a peak on the seventh day after wounding, and then falls quite rapidly. It should be noted that the mitotic rate given here is a measure of the nuclei attempting to divide during the five hour period between injection of colchicine and fixation of the tissue; it is not an estimate of the cells dividing at the moment of fixation.

The specimens used in obtaining these results showed regenerative progress at the normal rate for such tissues when kept at 19°C. By the third day after wounding demolition of damaged tissue was still in progress and the blastema was increasing in size. At 4 days the phases of demolition and repair still overlapped some-
Fig. 87. Variation of mitotic rate, measured after colchicine treatment, with age of regenerate.

- Rate for one animal (mean of 5 samples, each of 1000 cells).
- Mean rate for 4 animals.
what. After 7 days demolition was nearly complete and differentiation of the blastema had commenced: myoblasts had begun to differentiate into muscle cells. By 10 days increase in blastema size was slight and cell differentiation at an advanced stage.

(b) Total mitotic arrest during healing.

The initial stages of healing after total mitotic arrest are essentially similar to those in normal untreated tissue. At 2 days after injury demolition of damaged tissue is still in progress and the wound contains much debris and phagocytic cells.

It is not until 3 days after injury that the first effects of colchicine treatment become recognisable. In specimens injected with colchicine, neither dorsal nor ventral epithelia have succeeded in migrating over the injured surface as a continuous sheet of cells. Breaks in the continuity of the epithelia are noticable and the cells appear very attenuated. The wound area is disorganised, phagocytes are plentiful and no blastema tissue has yet been produced (Fig. 88). In control specimens healing has proceeded normally; the epithelial layers have migrated over the wound surface as a continuous sheet of tissue and blastema formation has begun.

After 5 days the experimental specimens have begun to show some blastema formation (Fig. 89a) though phagocytosis is still in progress. In control specimens the blastemata are larger, myoblast orientation has commenced, and the wound has already been cleared of much debris (Fig. 89b).

By 6 days after wounding the effect of the drug has become much clearer. The uninjured dorsal and ventral epithelial layers, in experimental animals, are now continuous; the cells are not noticeably attenuated, as are those of control animals, and there is no sign of cell division. Phagocytosis and cell demolition
Fig. 38. T.S. wound region of specimen showing mitotic arrest. (3 days after wounding.)

b.v.e., break in ventral epithelium; d.e., dorsal epithelium, lacking continuity; p., phagocytes; s.e.s., sub-epithelial space; u.t., uninjured tissue; w.r., wound region.
Fig. 89. T.S.'s of wound regions 5 days after wounding.

(a) EXPERIMENTAL. Specimen treated with colchicine and showing mitotic arrest.

(b) CONTROL. Specimen not treated with colchicine.

b., slight blastema formation; b', blastema formation well advanced; d.z., demolition zone containing much debris; d'.z', demolition zone, from which most of the debris has already been cleared; p., phagocytes; u.t., uninjured tissue.
are still in progress but blastema formation is retarded. It is usual, at this stage, in specimens untreated with colchicine, to find a ventral block of undifferentiated myoblast cells constituting the bulk of the blastema. No such cells are present after colchicine treatment: damaged muscle cells are removed, but apparently not replaced, (Fig. 90).

After 8 days an incomplete blastema, which appears to be composed entirely of connective tissue elements, has been produced in colchicine-treated animals. The covering epithelial cells are still not attenuated, (Fig. 91). There is still much debris in the wound area and phagocytosis is still in progress. No myoblasts are present. In control specimens, injected with saline, some myoblast formation has occurred. In both experimental and control animals collagen has been laid down.

The longest period for which animals injected daily with colchicine remained alive was 10 days. Animals injected daily with similar volumes of saline had only the same length of life so it can be assumed that the drug was not the prime cause of death. The actual cause of death has not been investigated but the severity of the experimental method should not be ignored.

By 10 days after wounding the experimental animals showed healing but little replacement of excised tissue. No replacement of the damaged muscle mat and lattice occurs, but a small 'connective tissue' blastema is produced. Some epithelial nuclear division is attempted, but in only one case has an anaphase nucleus been seen, indicating that the degree of mitotic arrest achieved, though not complete, is yet very high. The epithelium is of a mature type, containing numerous gland cells.

To summarise, in animals showing almost complete mitotic arrest wounding by excision is followed by rapid healing and only limited replacement of the tissue removed.
Fig. 90. T.S. wound region of specimen showing mitotic arrest.  
(6 days after wounding).

d., debris and connective tissue cells in region where, in animals not treated with colchicine, it is usual to find myoblast cells;  
d.e.h., dorsal epithelium of head;  
d.z., demolition zone;  
v.e., ventral epithelium of wound region.
Fig. 91. T.S. wound region of specimen showing mitotic arrest.
(8 days after wounding).

d., debris; d.e.h., dorsal epithelium of head;
i.b., incomplete blastema, composed of connective tissue elements and not containing myoblasts;
v.e., ventral epithelium of wound region.
An estimate of the nuclear density in the wound region was made for colchicine treated animals, and the results plotted against blastema age (Fig. 92 and Appendix 10). It can be seen that there is a slight increase in nuclear density initially, reaching a maximum on the fifth day after wounding, and that the nuclear density decreases as healing progresses. Also shown on the same graph for comparison are the nuclear density changes of blastemata developing 'in situ', whose cells can divide freely and into which cells can migrate, and of isolated, untreated blastemata, whose cells can still divide, but into which there can be no further migration of cells.

The following features are of note.

(i) The maximum nuclear density is reached earlier in blastemata from colchicine treated animals than in either isolated blastemata or in those which remain 'in situ' on untreated animals.

(ii) By the tenth day after wounding the nuclear density of blastemata from colchicine treated animals is appreciably higher than that found in isolated blastemata, and only slightly below that of blastemata allowed to develop 'in situ' on untreated animals.

These facts would appear to indicate that of the two types of cell present in Arion blastemata, namely immigrant and indigenous cells, the former are the most numerous.
Fig. 92. Graph showing nuclear densities of blastemata in the following conditions:

(a) left in situ, (where both cell division and cell migration can occur);
(b) after colchicine treatment, (no cell division possible);
(c) isolated blastemata, (no cell migration possible).
SUMMARY AND DISCUSSION.

These results bring to light several interesting facts concerning blastema formation and development.

1. The origin of gland cells.

The epithelial cells of the blastemata usually disintegrate during culture. After 4 days in culture the epithelial cells of all specimens examined had disintegrated. This fact is of considerable value in considering the origin of the gland cells.

It has already been suggested (pp. 57) that the mucous and protein glands of A. hortensis are probably epithelial in origin while the calcium glands arise from connective tissue cells. This view was based on histological evidence. It is interesting to find, therefore, that in isolated blastemata, though glands rarely regenerate, those which do are calcium glands. Since the epithelial layer had degenerated it can be inferred that they arose from some tissue other than epithelial. Neither mucous nor protein glands have been found in isolated blastemata. This does not prove that these glands are epithelial in origin, but when this fact is considered in relation to the histological evidence already presented (pp. 123, 124), the possibility that this is so seems more credible.

The culture medium used in the course of this work was cell-free Arion blood plasma. If the culturing medium could be improved so that the epithelial cells also persisted, it would be possible to use blastemata growing in such a medium as controls. If, in conditions favourable to epithelial growth, mucous glands differentiated, then their origin could be determined conclusively. Such an experimental programme is, however, beyond the scope of this thesis.

In the absence of such a control it might be argued that the
non-development of mucous and protein glands is an effect of the culture conditions and not caused by the absence of an epithelium. It is merely claimed that these results support those given earlier with regard to the origin of the gland cells and indicate lines for further research.

2. Development of muscle fibres.

The differentiation of muscle fibres from myoblasts occurs in what appears to be the same manner both in isolated blastemata and in controls left 'in situ'. When the blastema remains 'in situ', however, muscle fibre differentiation is accompanied by a characteristic arrangement of the fibres so that the excised muscle mat and lattice are replaced. This characteristic arrangement of the fibres appears first at the edge of the blastema adjacent to the uninjured tissue and spreads centripetally into the rest of the regenerate (p. 112). When blastemata are removed and cultured 'in vitro' the myoblasts differentiate into muscle fibres but do not become arranged into a muscle mat and lattice. This would appear to indicate that the stimulus for the characteristic orientation of muscle fibres is provided by adjacent uninjured tissue and is not inherent in the blastema.

Even when blastemata are removed at the 24 hour stage and cultured muscle fibres can differentiate. It would be of interest to remove younger blastemata but so far attempts have proved unsuccessful, due in part to their small size and fragile nature.

3. Changes in nuclear density.

Perhaps the most significant fact resulting from blastema isolation is the change in nuclear density recorded during culture.

That the nuclear density of isolated blastemata shows a steady increase initially has two possible explanations:
(a) the blastema cells may be aggregating, thus increasing the number of nuclei per unit area;
(b) the number of nuclei present may be increasing.
The literature indicates that the former explanation is unlikely to be correct. Organ culture, of which the culture of blastemata is one aspect, often results not in aggregation but in outward migration of cells, which must be discouraged (Fell, cited in Atkins, 1959). In 11% of the blastemata examined in the course of this work outward migration of cells had occurred (p. 224). Although re-aggregation of dissociated cells has frequently been reported (Holtfreter, 1947; Grover, 1961), no reference to closer aggregation of already associated cells in culture has been found. The second explanation, that the number of nuclei present has increased, thus seems the more likely of the two.

There are two possible explanations of how the number of nuclei per unit area may increase:
(a) cells may migrate into the blastema;
(b) cells already within the blastema may divide.
Since tissue culture removes the blastema into a cell-free medium the increased number of nuclei must then be the result of division.

After reaching a maximum, the nuclear density of the isolated blastemata reaches a level somewhat below that of control blastemata left in situ. Several possible explanations can be advanced for this decrease:
(a) cells may be migrating outwards from the blastema;
(b) the rate of cell division may have decreased, and cell growth may have resulted in the nuclei becoming spaced further apart;
(c) the tissue may be aging and the nuclei disintegrating.
The first and third possibilities are unlikely, for all blastemata in which migration, aging, or necrosis could be detected were excluded from these observations. In the absence of further evidence
explanation (b) seems the most likely.

The maximum nuclear density reached in isolated blastemata is lower than that of controls left 'in situ'. Furthermore, the longer the blastemata remain 'in situ' the higher, in general, the maximum nuclear density attained when they are subsequently cultured. These facts seem to indicate that the nuclear density is not solely an expression of the rate of nuclear division. Some other factor must normally effect nuclear density and the number of cells constituting the blastema. If blastema cells were increased simply by division of the initial cells without the addition of any immigrant cells, then the time after wounding at which the blastema was cultured should have no effect on the nuclear density, yet such an effect is clearly shown. If, however, migration of cells into the blastema occurred concurrently with division of those cells already there, then the longer the blastemata remained 'in situ' before culturing the more cells they would amass. The latter explanation is in line with the experimental results described earlier, (p. 231).

It should not, however, be forgotten that the act of blastema removal which immediately precedes culturing may have a traumatic effect on the blastemata and affect their growth rate at least temporarily.

It might be thought that the blood plasma used as a nutrient medium was directly responsible for the fall in nuclear density, for as Willmer (1935) pointed out, blood plasma is a non-growth-promoting medium, and cells kept in it often show differentiation and lose the power to divide. It should be noted that:

(a) The use of a growth-promoting medium, e.g. blood plasma plus embryo extract, would have been inadmissible for this investigation because such a medium has the property of artificially stimulating cells to divide and inhibits differentiation.
It was essential that the tissue be allowed to develop and differentiate normally.

(b) Blastemata cultured before the time at which nuclear density was expected to reach a maximum showed an increase in nuclear density at or above the rate shown by controls left 'in situ'. This would surely not be found if the culture conditions were inhibiting cell division.

That cell migration and cell division both contribute to formation of the blastema is given support also by the fact that in blastemata of any selected age there is a correlation between the nuclear density and the age at which the blastemata were cultured. In general, the later the time of culturing the higher the nuclear density found. The only satisfactory explanation for this is that cells migrate to the blastema during the time it is left 'in situ' on the experimental animal and that these cells increase the total number of cells capable of dividing. Considered together the results obtained from tissue culture work indicate that in Arion hortensis both cell migration and cell division contribute to blastema formation.

4. Changes in the mitotic rate during regeneration 'in situ'.

The purpose of the injection of colchicine several hours before fixation into animals with developing blastemata was to arrest any nuclei attempting to divide during injection and fixation at the metaphase, i.e. at the stage at which they could most readily be detected. This somewhat artificial device was used because difficulty had been encountered earlier in detecting dividing nuclei.

The increase in the mitotic rate during the initial period of wound healing closely follows the increase in nuclear density which also occurs during this period. In both cases a maximum
is reached at the seventh day. The closeness of this parallelism indicates that cell division plays a significant part in increasing the number of cells constituting the blastema.

5. Progress of healing after complete mitotic arrest.

Further experiments with colchicine, in which the drug was injected daily in an attempt to arrest all mitoses after wounding, also yielded interesting results. If mitosis is blocked completely then any growth of blastema tissue must be the result of cells migrating to the wound region. The results of histological studies of the wound site have been given elsewhere (p. 234). The significance of these results will now be considered.

(a) The demolition phase of healing progressed normally. This phase involves cell lysis and the removal of tissue debris, functions requiring the presence of an abundance of phagocytes, such as are normally present in the blood. If migration of blood cells into the tissue can occur then there is no obvious reason why this process should not progress normally. The cessation of mitosis should have little effect on this aspect of healing, as has been found experimentally.

(b) The epithelial tissue failed to migrate over the injured surface as a continuous sheet. Usually, in specimens untreated with colchicine, the attachment of the epidermis to the basement membrane loosens, and the cells stretch out over the wound, becoming extremely attenuated (p. 77). Whether or not complete epithelial coverage can be obtained in this way depends on the size of the exposed surface. Usually the wound is partly sealed by blood corpuscles. The epithelial cells divide, cell growth follows, and complete coverage is eventually obtained. Presumably the inability in this case, of the epithelial cells to divide, imposed a limit on the amount of
epithelial tissue available for wound coverage. As the cells of the colchicine treated specimens stretched out over the injured surface breaks appeared in the epithelial sheet.

(c) Damaged muscle fibres gradually disintegrated but the excised muscle tissue was not replaced. This would seem to indicate that muscle tissue cannot be replaced by migrating cells but is dependent entirely on the division of indigenous tissue. If such division is arrested, then muscle tissue cannot be replaced.

(d) A small blastema composed entirely of connective tissue elements developed. This could only have been produced by cells migrating to the wound region from the rest of the body and accumulating there.

(e) Of all the sections examined, in only one was a cell found whose nucleus was at anaphase. This indicates that the degree of metaphase arrest achieved, though not quite complete, was virtually so.

6. Changes in nuclear density of specimens showing complete mitotic arrest.

That there is still a slight increase in the nuclear density even after mitotic arrest indicates clearly that the migration of cells plays a role in blastema formation. The reaching of maximum nuclear density earlier in colchicine treated specimens than in untreated specimens probably indicates that during normal development the rate of cell division continues to increase after the rate of cell migration has begun to decrease.

By the tenth day after wounding the nuclear density of colchicine treated specimens, where there is no cell division, was appreciably higher than that of cultured blastemata, where there is no cell migration, and only slightly below that of untreated blastemata left 'in situ', where there is both cell division and cell migration. This would appear to indicate that cell migration
contributes the majority of the cells constituting the blastema.

To summarise:
1. Both immigrant cells and indigenous tissue contribute to blastema formation.
2. The epithelial and connective tissue components of the blastema are produced by immigrant cells, which normally divide on reaching the wound site.
3. The myoblast component is derived by the de-differentiation and division of indigenous muscle tissue.
4. The majority of the blastema cells are immigrant.
5. Though cell division is essential for the replacement of excised tissue, healing can occur when mitosis has been arrested.
VI. FINAL CONCLUSIONS.

1. The mantle edge consists of:
   (i) a dorsal glandular epithelium;
   (ii) a zone of connective tissue, consisting of
       (a) a dorsal pigmented region,
       (b) a median vascular region, through which ramifies a
           lattice of smooth muscle fibres;
       (c) a ventral region containing a mat of muscle fibres;
   (iii) a ventral glandular epithelium, (pp. 21, 22).

2. There are three main categories of cutaneous glands: calcium,
   protein, and mucous glands. The latter may be further sub-divided
   into four distinct types, of which one is restricted to the
   ventral surface. The histology and histochemistry of these
   glands is described on pp. 23-35, and 54-56.

3. There is no free uroporphyrin in the mantle edge of Arion hortensis
   (p. 36), as is found in A. ater. The black pigment present is
   a melanin.

4. The small blood spaces are lined by a thin endothelium (pp. 37, 38).

5. There are three types of blood cell and six of connective tissue
   cell, three of the latter being similar in appearance to the
   blood cells, (pp. 37-42). It is thought that blood cells can
   pass through the walls of the blood spaces.

6. The muscle fibres of the mantle edge are arranged as a ventral
   mat, consisting of horizontally arranged fibres, some at right
   angles to and some parallel to the long axis of the body, above
   which is a three-dimensional lattice-work composed of six groups
   of fibres and through the meshes of which the blood spaces
   ramify (pp. 42-47). The fine structure of the muscle fibres
   is described on p. 48.

7. Provisional wound closure is effected by blood cell agglutination
and by migration of the adjacent uninjured epithelial cells over the injured surface (p. 87).

8. The migratory epithelial cells contain less glycogen than stationary epithelial cells, indicating that glycogen is being metabolised, possibly as a source of energy, (p. 149).

9. The epithelial cells generally move over the injured surface as a coherent sheet, though sometimes individual cells slip out of the sheet and move at a rate different to that of the rest of the sheet, (pp. 78, 202, 203).

10. The epithelial cells are mobilised in an orderly fashion beginning with those nearest to the injured surface and gradually extending outwards to more distant cells, (p. 202).

11. The mobile cells appear, in fixed specimens, to be detached from the basement membrane, (p. 78).

12. It is suggested that the epithelial cells of uninjured tissue are stationary because of the production of an inhibitor by that tissue. Loss of this factor on injury results in epithelial cell movement, and this movement ceases once the inhibitor again reaches a functional level (pp. 211-213). It is recognised that other subsidiary factors may also be involved in the process, (p. 211).

13. Blood loss is reduced by muscle contraction. The muscle fibres of both lattice and mat are involved and act in such a way that they decrease the size of the lattice meshes, thus constricting the blood spaces and so reducing the blood supply of the wound region, (pp. 81, 86). This reduction in blood supply is only temporary and ends once the wound is provisionally sealed, when the blood spaces become dilated, (p. 92).

14. It is suggested that the dilation of the blood spaces which follows provisional wound closure, (p. 92), may, as shown by other
authors for vertebrates, increase the volume of blood reaching the wound and the number of phagocytes carried to it, thus increasing the rate of demolition and removal of waste, (p. 186).

15. The demolition phase of the healing process is effected by cytolysis and phagocytosis, (pp. 185, 186).

16. Acid phosphatase activity is greatest when cell degeneration is occurring (pp. 130-132).

17. The demolition and differentiation phases overlap, a condition not found in vertebrate regeneration, (p. 91).

18. Final wound closure is effected by the epithelial cells, which extend over and cover the agglutinated blood cells, (p. 104).

19. Cell division, which marks the onset of the repair phase of regeneration, begins precociously in the nerve sheath cells at 24 hours after injury (p. 90), and reaches a maximum for the blastema tissue as a whole by the seventh day after injury. Epithelial cells appear to divide as required, keeping pace with growth of the rest of the regenerate.

20. The blastema is formed from certain local cells which first de-differentiate and from more distant cells which migrate into it. It increases in size by division and growth of these cells (pp. 187-192).

21. The migratory cells of the blastema are of the following types: epithelial, blood, connective tissue and nerve sheath cells (p. 92).

22. Differentiation commences in the blastema tissue adjoining the uninjured tissue and extends forwards from the postero-ventral regions (p. 112).

23. Regenerated muscle fibres are functional by the twelfth day (p. 114) and collagen fibres, thought to increase the tensile strength of the wound, are most abundant in those regions where muscle
fibres have re-formed (p. 114). De-differentiation of local uninjured muscle fibres produces the myoblasts from which the muscle fibres of the regenerate develop (p. 95).

24. Mucous glands arise from the epithelium (p. 119), while calcium glands originate in the connective tissue (pp. 123-124). The origin of the protein glands is not known.

25. The cytoplasmic R.N.A. content decreases as differentiation progresses (p. 167). The glycogen store of the blastema is exhausted after differentiation (p. 158).

26. It has been shown conclusively that both immigrant and indigenous cells contribute to blastema formation (pp. 245, 246, 248, 249).

27. The majority of the blastema cells are immigrant (p. 240, 241).

28. Cell division is essential for the replacement of excised tissue, though healing without extensive regeneration can occur when mitosis has been arrested (pp. 237, 247).
VII. APPENDICES.
Appendix 1(a). Frequency distributions: changes in area of blood spaces during early stages of healing.

(1 unit of area = 625 \( \mu^2 \)).

1. At site of injury: 1 hour after wounding.

<table>
<thead>
<tr>
<th>Area</th>
<th>Frequency</th>
<th>Sample size</th>
<th>Arith. Mean in ( \mu^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-0.5</td>
<td>4</td>
<td>20</td>
<td>631</td>
</tr>
<tr>
<td>0.6-1.0</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1-1.5</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6-2.0</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.1-2.5</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. In distant uninjured tissue: 1 hour after wounding.

<table>
<thead>
<tr>
<th>Area</th>
<th>Frequency</th>
<th>Sample size</th>
<th>Arith. Mean in ( \mu^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-0.5</td>
<td>3</td>
<td>20</td>
<td>681</td>
</tr>
<tr>
<td>0.6-1.0</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1-1.5</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6-2.0</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.1-2.5</td>
<td>2</td>
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<td></td>
</tr>
</tbody>
</table>

3. In uninjured tissue adjacent to wound:
   (a) 1 hour after wounding.

<table>
<thead>
<tr>
<th>Area</th>
<th>Frequency</th>
<th>Sample size</th>
<th>Arith. Mean in ( \mu^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-0.5</td>
<td>13</td>
<td>20</td>
<td>306</td>
</tr>
<tr>
<td>0.6-1.0</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1-1.5</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

   (b) 1 day after wounding.

<table>
<thead>
<tr>
<th>Area</th>
<th>Frequency</th>
<th>Sample size</th>
<th>Arith. Mean in ( \mu^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-0.5</td>
<td>11</td>
<td>20</td>
<td>375</td>
</tr>
<tr>
<td>0.6-1.0</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1-1.5</td>
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</tr>
</tbody>
</table>
Appendix 1(a); cont.

(c) 2 days after wounding.

<table>
<thead>
<tr>
<th>Area</th>
<th>Frequency</th>
<th>Sample size</th>
<th>Arith. Mean in $\mu^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-0.5</td>
<td>3</td>
<td>20</td>
<td>562</td>
</tr>
<tr>
<td>0.6-1.0</td>
<td>10</td>
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<td></td>
</tr>
<tr>
<td>1.1-1.5</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6-2.0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(d) 3 days after wounding.

<table>
<thead>
<tr>
<th>Area</th>
<th>Frequency</th>
<th>Sample size</th>
<th>Arith. Mean in $\mu^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-0.5</td>
<td>3</td>
<td>20</td>
<td>631</td>
</tr>
<tr>
<td>0.6-1.0</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1-1.5</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6-2.0</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Appendix 1(b). Change in area of blood spaces of regenerate with age

<table>
<thead>
<tr>
<th>Time after injury, in days</th>
<th>Sample size</th>
<th>Arith. Mean in $\mu^2$</th>
<th>Standard error in $\mu^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>20</td>
<td>312</td>
<td>$\pm$ 19</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>312</td>
<td>$\pm$ 19</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>375</td>
<td>$\pm$ 25</td>
</tr>
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<td>7</td>
<td>20</td>
<td>312</td>
<td>$\pm$ 25</td>
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<tr>
<td>14</td>
<td>20</td>
<td>312</td>
<td>$\pm$ 19</td>
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<td>21</td>
<td>20</td>
<td>375</td>
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<td>35</td>
<td>20</td>
<td>375</td>
<td>$\pm$ 19</td>
</tr>
<tr>
<td>42</td>
<td>20</td>
<td>437</td>
<td>$\pm$ 19</td>
</tr>
<tr>
<td>Tissue from uninjured specimen</td>
<td>20</td>
<td>500</td>
<td>$\pm$ 13</td>
</tr>
</tbody>
</table>
Appendix 2(a). Variation in lattice fibre diameter (Groups 1 and 2), from uninjured tissue adjacent to the wound, with time after wounding.

<table>
<thead>
<tr>
<th>Time after wounding</th>
<th>Sample size</th>
<th>Arith. Mean</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour</td>
<td>20</td>
<td>1.60</td>
<td>± 0.13</td>
</tr>
<tr>
<td>1 day</td>
<td>20</td>
<td>1.40</td>
<td>± 0.13</td>
</tr>
<tr>
<td>2 days</td>
<td>20</td>
<td>1.10</td>
<td>± 0.08</td>
</tr>
<tr>
<td>3 days</td>
<td>20</td>
<td>0.87</td>
<td>± 0.07</td>
</tr>
<tr>
<td>From uninjured tissue</td>
<td>20</td>
<td>0.84</td>
<td>± 0.26</td>
</tr>
</tbody>
</table>

(1 unit of diameter = 1.25 μ).

Appendix 2(b). Variation in acute angles between lattice fibres (Groups 1 and 2), from uninjured tissue adjacent to the wound, with time after wounding.

<table>
<thead>
<tr>
<th>Time after wounding</th>
<th>Sample size</th>
<th>Arith. Mean in degrees</th>
<th>Standard error in degrees</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour</td>
<td>20</td>
<td>47</td>
<td>± 2</td>
</tr>
<tr>
<td>1 day</td>
<td>20</td>
<td>44</td>
<td>± 2</td>
</tr>
<tr>
<td>2 days</td>
<td>20</td>
<td>52</td>
<td>± 2</td>
</tr>
<tr>
<td>3 days</td>
<td>20</td>
<td>54</td>
<td>± 2</td>
</tr>
<tr>
<td>From uninjured tissue</td>
<td>20</td>
<td>54</td>
<td>± 5</td>
</tr>
</tbody>
</table>
Appendix 3(a). Frequency distributions: Changes in diameter of fibres of ventral muscle mat during early stages of healing.

(1 unit of diameter = 1.25 μ).

1. In uninjured tissue adjacent to wound:
   (a) 1 hour after wounding.

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Frequency</th>
<th>Sample size</th>
<th>Arith. Mean in μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-0.5</td>
<td>0</td>
<td>20</td>
<td>1.4 ± 0.06</td>
</tr>
<tr>
<td>0.6-1.0</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1-1.5</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6-2.0</td>
<td>7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(b) 1 day after wounding.

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Frequency</th>
<th>Sample size</th>
<th>Arith. Mean in μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-0.5</td>
<td>0</td>
<td>20</td>
<td>1.3 ± 0.05</td>
</tr>
<tr>
<td>0.6-1.0</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1-1.5</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6-2.0</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(c) 2 days after wounding.

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Frequency</th>
<th>Sample size</th>
<th>Arith. Mean in μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-0.5</td>
<td>0</td>
<td>20</td>
<td>1.2 ± 0.06</td>
</tr>
<tr>
<td>0.6-1.0</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1-1.5</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6-2.0</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(d) 3 days after wounding.

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Frequency</th>
<th>Sample size</th>
<th>Arith. Mean in μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6-1.0</td>
<td>16</td>
<td>20</td>
<td>1.0 ± 0.02</td>
</tr>
<tr>
<td>1.1-1.5</td>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. From distant, uninjured tissue.

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Frequency</th>
<th>Sample size</th>
<th>Arith. Mean in μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6-1.0</td>
<td>18</td>
<td>20</td>
<td>0.9 ± 0.03</td>
</tr>
<tr>
<td>1.1-1.5</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix 4. Variation of delay in epithelial cell movement with distance of cell from nearest wound edge.

<table>
<thead>
<tr>
<th>Wound type</th>
<th>Delay in minutes</th>
<th>Distance (μ)</th>
<th>Wound type</th>
<th>Delay in minutes</th>
<th>Distance (μ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td>120</td>
<td>900</td>
<td>Single</td>
<td>45</td>
<td>300</td>
</tr>
<tr>
<td>Single</td>
<td>30</td>
<td>120</td>
<td>Single</td>
<td>60</td>
<td>360</td>
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<td>Single</td>
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<td>210</td>
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<td></td>
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</tbody>
</table>

N = 41
r = 0.7235
p < 0.001

Equation of "best-fit" line:

\[ Y = 34 + 0.10X \]
Appendix 5. Variations of rate of movement of epithelial cells with original distance from wound edge.

<table>
<thead>
<tr>
<th>Wound type</th>
<th>Distance in μ.</th>
<th>Rate in μ/ hour.</th>
<th>Wound Type</th>
<th>Distance in μ.</th>
<th>Rate in μ/ hour.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td>900</td>
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<td>300</td>
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<td>Single</td>
<td>270</td>
<td>40</td>
<td>Single</td>
<td>150</td>
<td>60</td>
</tr>
<tr>
<td>Single</td>
<td>300</td>
<td>69</td>
<td>Single</td>
<td>450</td>
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</tr>
<tr>
<td>Single</td>
<td>120</td>
<td>45</td>
<td>Single</td>
<td>300</td>
<td>40</td>
</tr>
<tr>
<td>Single</td>
<td>450</td>
<td>72</td>
<td>Double</td>
<td>600</td>
<td>84</td>
</tr>
<tr>
<td>Double</td>
<td>585</td>
<td>76</td>
<td>Double</td>
<td>210</td>
<td>48</td>
</tr>
</tbody>
</table>

\[ N = 38 \]

\[ r = 0.3256 \]

\[ p < 0.05 \]

Equation of "best-fit" line:

\[ y = 0.03x + 66 \]
Appendix 6. Variation of time of cessation of epithelial cell movement with original distance from nearest wound edge.

<table>
<thead>
<tr>
<th>Wound type</th>
<th>Distance in μ.</th>
<th>Time in minutes</th>
<th>Wound type</th>
<th>Distance in μ.</th>
<th>Time in minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
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<td>165</td>
<td>Single</td>
<td>270</td>
<td>135</td>
</tr>
<tr>
<td>Single</td>
<td>810</td>
<td>135</td>
<td>Double</td>
<td>90</td>
<td>165</td>
</tr>
<tr>
<td>Double</td>
<td>450</td>
<td>165</td>
<td>Double</td>
<td>60</td>
<td>180</td>
</tr>
<tr>
<td>Double</td>
<td>15</td>
<td>180</td>
<td>Double</td>
<td>150</td>
<td>180</td>
</tr>
<tr>
<td>Double</td>
<td>360</td>
<td>165</td>
<td>Double</td>
<td>750</td>
<td>180</td>
</tr>
<tr>
<td>Double</td>
<td>570</td>
<td>180</td>
<td>Single</td>
<td>300</td>
<td>165</td>
</tr>
<tr>
<td>Single</td>
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<td>165</td>
<td>Single</td>
<td>270</td>
<td>150</td>
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<td>Double</td>
<td>210</td>
<td>195</td>
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</tr>
</tbody>
</table>

\(N = 23\)

\[ r = 0.3214 \]

\[ p > 0.1 \]

Equation of "best-fit" line:

\[ Y = 175 - 0.008X \]
## Appendix 7. Variation of nuclear density with age of regenerate for blastemata cultured 'in vitro' and controls left 'in situ'.

<table>
<thead>
<tr>
<th>Age at culturing, (in days)</th>
<th>Days 'in vitro'.</th>
<th>Age when examined, (in days)</th>
<th>Sample size</th>
<th>Mean nuclear density</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>2.4</td>
<td>± 0.3</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>10</td>
<td>1.7</td>
<td>± 0.3</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>4</td>
<td>10</td>
<td>1.9</td>
<td>± 0.4</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>5</td>
<td>20</td>
<td>5.4</td>
<td>± 0.5</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>6</td>
<td>20</td>
<td>4.2</td>
<td>± 0.6</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
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<td>10</td>
<td>4.0</td>
<td>± 0.6</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>8</td>
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<td>± 0.3</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>9</td>
<td>20</td>
<td>2.0</td>
<td>± 0.2</td>
</tr>
<tr>
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<td>9</td>
<td>10</td>
<td>10</td>
<td>1.8</td>
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<td>10</td>
<td>1.7</td>
<td>± 0.5</td>
</tr>
<tr>
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<td>2</td>
<td>4</td>
<td>10</td>
<td>3.0</td>
<td>± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>6</td>
<td>10</td>
<td>5.6</td>
<td>± 0.5</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>7</td>
<td>10</td>
<td>2.2</td>
<td>± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>8</td>
<td>10</td>
<td>0.9</td>
<td>± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>10</td>
<td>10</td>
<td>0.8</td>
<td>± 0.3</td>
</tr>
<tr>
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<td>10</td>
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<td>20</td>
<td>2.7</td>
<td>± 0.4</td>
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<td>± 0.3</td>
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<td>10</td>
<td>0.6</td>
<td>± 0.2</td>
</tr>
<tr>
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<td>7</td>
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<td>10</td>
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<td>± 0.2</td>
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<td>5.4</td>
<td>± 0.4</td>
</tr>
<tr>
<td>4</td>
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<td>10</td>
<td>4.3</td>
<td>± 0.4</td>
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<td>7</td>
<td>20</td>
<td>2.7</td>
<td>± 0.4</td>
</tr>
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</table>
## Appendix 7. (cont.)

<table>
<thead>
<tr>
<th>Age at culturing, (in days)</th>
<th>Days 'in vitro'.</th>
<th>Age when examined, (in days)</th>
<th>Sample size</th>
<th>Mean nuclear density</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
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<td>10</td>
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<td>± 0.6</td>
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<td>± 0.3</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
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<td>10</td>
<td>0.9</td>
<td>± 0.3</td>
</tr>
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<td>12</td>
<td>10</td>
<td>0.9</td>
<td>± 0.2</td>
</tr>
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<td>1</td>
<td>6</td>
<td>20</td>
<td>6.3</td>
<td>± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>7</td>
<td>20</td>
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<td>± 0.4</td>
</tr>
<tr>
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<td>9</td>
<td>10</td>
<td>2.2</td>
<td>± 0.4</td>
</tr>
<tr>
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<td>5</td>
<td>10</td>
<td>10</td>
<td>1.7</td>
<td>± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>11</td>
<td>10</td>
<td>2.4</td>
<td>± 0.2</td>
</tr>
<tr>
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<td>7</td>
<td>12</td>
<td>20</td>
<td>0.9</td>
<td>± 0.2</td>
</tr>
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<td>1</td>
<td>7</td>
<td>20</td>
<td>6.7</td>
<td>± 0.5</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>8</td>
<td>20</td>
<td>3.0</td>
<td>± 0.5</td>
</tr>
<tr>
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<td>3</td>
<td>9</td>
<td>10</td>
<td>2.4</td>
<td>± 0.5</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>10</td>
<td>10</td>
<td>1.7</td>
<td>± 0.5</td>
</tr>
<tr>
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<td>5</td>
<td>11</td>
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<td>1.6</td>
<td>± 0.3</td>
</tr>
<tr>
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<td>6</td>
<td>12</td>
<td>10</td>
<td>0.7</td>
<td>± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>8</td>
<td>10</td>
<td>4.0</td>
<td>± 0.3</td>
</tr>
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<td>3</td>
<td>10</td>
<td>10</td>
<td>1.2</td>
<td>± 0.2</td>
</tr>
<tr>
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<td>4</td>
<td>11</td>
<td>10</td>
<td>1.0</td>
<td>± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>12</td>
<td>10</td>
<td>0.9</td>
<td>± 0.3</td>
</tr>
<tr>
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<td>1</td>
<td>9</td>
<td>20</td>
<td>3.2</td>
<td>± 0.5</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>10</td>
<td>20</td>
<td>2.9</td>
<td>± 0.3</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>13</td>
<td>10</td>
<td>1.6</td>
<td>± 0.3</td>
</tr>
</tbody>
</table>
Appendix 7. (cont.).

Controls left 'in situ'.

<table>
<thead>
<tr>
<th>Age when examined, (in days)</th>
<th>Sample size</th>
<th>Mean nuclear density</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
<td>2.1</td>
<td>± 0.2</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>2.4</td>
<td>± 0.5</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>2.2</td>
<td>± 0.4</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>2.5</td>
<td>± 0.4</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>4.2</td>
<td>± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>6.0</td>
<td>± 0.3</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>6.6</td>
<td>± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>7.8</td>
<td>± 0.4</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>5.2</td>
<td>± 0.8</td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>4.7</td>
<td>± 0.4</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>3.6</td>
<td>± 0.4</td>
</tr>
</tbody>
</table>
Appendix 8. Correlation between nuclear density of blastemata and the age at which they were excised and cultured.

(a) Cultured blastemata aged 7 days when examined.

<table>
<thead>
<tr>
<th>Age when cultured, (in days)</th>
<th>Mean nuclear density.</th>
<th>Sample size.</th>
<th>Standard error.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.0</td>
<td>10</td>
<td>± 0.9</td>
</tr>
<tr>
<td>2</td>
<td>2.2</td>
<td>10</td>
<td>± 0.9</td>
</tr>
<tr>
<td>3</td>
<td>2.7</td>
<td>20</td>
<td>± 0.4</td>
</tr>
<tr>
<td>4</td>
<td>2.7</td>
<td>20</td>
<td>± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>4.6</td>
<td>20</td>
<td>± 0.5</td>
</tr>
<tr>
<td>6</td>
<td>6.7</td>
<td>20</td>
<td>± 0.5</td>
</tr>
</tbody>
</table>

Equation of "best-fit" line: $Y = 2.2 + 0.6X$

$N = 60; \quad r = 0.7408; \quad p < 0.1$

(b) Cultured blastemata aged 9 days when examined.

<table>
<thead>
<tr>
<th>Age when cultured, (in days)</th>
<th>Mean nuclear density.</th>
<th>Sample size.</th>
<th>Standard error.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.8</td>
<td>10</td>
<td>± 0.3</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>3</td>
<td>0.6</td>
<td>10</td>
<td>± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>1.6</td>
<td>10</td>
<td>± 0.3</td>
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<td>10</td>
<td>± 0.4</td>
</tr>
<tr>
<td>6</td>
<td>2.4</td>
<td>10</td>
<td>± 0.5</td>
</tr>
<tr>
<td>8</td>
<td>3.2</td>
<td>20</td>
<td>± 0.3</td>
</tr>
</tbody>
</table>

Equation of "best-fit" line: $Y = 1.25 + 0.17X$

$N = 90; \quad r = 0.7310; \quad p < 0.05$
Appendix 9. Variation of mitotic rate, measured after colchicine treatment, with age of regenerate.

<table>
<thead>
<tr>
<th>Age of regenerate (in days)</th>
<th>Mitoses / 1000 cells</th>
<th>Mitotic rate for each animal</th>
<th>Mean mitotic rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>5,3,2,2,3.</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
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<td>5,2,2,2,4.</td>
<td>3.0</td>
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</tr>
<tr>
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<td>2,3,3,4,2.</td>
<td>2.8</td>
<td>2.85</td>
</tr>
<tr>
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<td>4,0,3,3,3.</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5,6,6,4,6.</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
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<td>5,2,5,5,5.</td>
<td>4.4</td>
<td>4.40</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
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</tr>
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<td>5,5,7,4,7.</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
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<td>5,6,6,6,5.</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
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<td>5.30</td>
</tr>
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<td>5,5,4,4,7.</td>
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<td></td>
</tr>
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<td>7</td>
<td>5,4,6,6,4.</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
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<td>7,7,5,6,3.</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
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<td>7,7,6,5,4.</td>
<td>5.8</td>
<td>5.55</td>
</tr>
<tr>
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<td>7,8,5,6,3.</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2,2,4,5,2.</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4,4,2,3,3.</td>
<td>3.2</td>
<td></td>
</tr>
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<tr>
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<td>4,3,2,2,2.</td>
<td>2.6</td>
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</tr>
</tbody>
</table>
Appendix 10.  Comparison of nuclear densities of blastemata in the following conditions:

(a) left 'in situ'; (for data see Appendix 7);

(b) after colchicine treatment, resulting in mitotic inhibition;

<table>
<thead>
<tr>
<th>Age of regenerate, (in days)</th>
<th>Mean nuclear density</th>
<th>Sample size</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2.2</td>
<td>20</td>
<td>± 0.2</td>
</tr>
<tr>
<td>5</td>
<td>5.6</td>
<td>20</td>
<td>± 0.3</td>
</tr>
<tr>
<td>6</td>
<td>5.3</td>
<td>20</td>
<td>± 0.4</td>
</tr>
<tr>
<td>8</td>
<td>4.7</td>
<td>20</td>
<td>± 0.2</td>
</tr>
<tr>
<td>10</td>
<td>4.0</td>
<td>20</td>
<td>± 0.3</td>
</tr>
</tbody>
</table>

(c) isolated blastemata; (for data see Appendix 7).
VIII. ACKNOWLEDGEMENTS.

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