

# Regeneration of amputated limb-buds in early rat embryos

By ELIZABETH M. DEUCHAR<sup>1</sup>

*From the Department of Anatomy, The Medical School,  
University of Bristol*

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## SUMMARY

Rat embryos, dissected from the uterus at 11½ days' gestation, have been used to study the regenerative powers of the embryonic limb. One forelimb-bud of each embryo was amputated, via an incision in the membranes. Embryos were subsequently grown in roller-bottle cultures for 44 h, then examined histologically. Twenty-nine out of 32 healthy embryos had formed limb-bud regenerates, 14 of which were of normal size and shape. Eight of them had a normal apical ectodermal ridge. It is concluded that at this stage of development there is sufficient versatility in the embryonic cells for a limb rudiment to be replaced by adjacent mesenchyme and epidermal cells. The implications of this finding are discussed in connexion with previous studies on the regeneration of appendages in vertebrate embryos.

## INTRODUCTION

It is well known that adult mammals and birds are incapable of regenerating either the limbs or the tail, and that in reptiles the tail is the only large appendage that can be regenerated, somewhat imperfectly (Goss, 1961; Bryant, 1970). On the other hand at stages prior to organ formation all early vertebrate embryos, including those of amniotes, are capable of replacing very large areas of tissue if these are removed, because their cells at this stage are very versatile and can adapt both in mitotic rate and in the course of their differentiation. Somewhere between these two extremes, therefore, in late embryonic stages, one might expect that developing appendages of amniotes should retain enough versatility in their cells to be able to regenerate.

Experiments on regenerative ability in amniote embryos have been limited by technical difficulties, and so far the results obtained have tended to suggest that there is little or no regeneration of developing appendages. For instance, in chick embryos the work of Hampé (1959) and Kieny (1964) on the regulative capacity of limb-buds has emphasized their inability to replace portions removed by surgery. In lizard embryos, Holder & Bellairs (1962) and Bellairs & Bryant (1968) carried out amputations of the limbs and tail *in vitro* and found no

<sup>1</sup> *Author's address:* Department of Biological Sciences, Hatherly Laboratories, University of Exeter, U.K.

evidence of regeneration except in the tail, and this not until just before hatching. Their negative results may have been due partly to the poor survival of the embryos under laboratory culture conditions, however. The pioneering study by Jolly (1974) on the regenerative ability of the tail-bud in rat embryos grown *in vitro* suffered even severer limitations: only epithelial healing occurred, no regeneration, and the embryos were frequently in poor condition when examined after 20–30 h in culture.

Probably the most comprehensive experiments on the regeneration of appendages in mammal embryos were those carried out by Nicholas (1926) on 14-day-old rat embryos *in utero*. He was able to amputate the forelimb at various levels in several hundred embryos which were then carried to term successfully. None of these showed true regeneration, though: the most that occurred, with more distal amputations, was the formation of a knob of undifferentiated or 'callus' cells at the amputated surface. This callus formation is typical of situations in which the limb is incapable of regeneration: e.g. in post-natal reptiles and mammals (see Bryant, 1970). Nicholas was not able to investigate limb regeneration in earlier stages of embryonic development, because these were too small to amputate *in utero* and at that time there were no methods available for culturing such stages *in vitro*.

The roller-tube culture method of New, Coppola & Terry (1973) makes it possible to maintain rat embryos of 9–14 days' gestation for periods of 2–4 days *in vitro*. This is sufficient time for one to expect that some regeneration as well as healing could occur after amputation experiments: indeed the axial tissues of 10- to 11-day embryos are reconstituted completely after damage, in as little as 20 h (Deuchar, 1975*a*). So in the work to be described here, the limb-buds of rat embryos have been amputated at 11 days' gestation and then the embryos allowed to continue development *in vitro* in roller-bottles for a further 2 days, the longest time that it was found possible to maintain them in good health after operations. The degree of regeneration occurring in the limb-buds in this time has been found to be considerable.

#### MATERIAL AND METHODS

Pregnant female Wistar rats were killed 11½ days after mating, and the embryos were dissected from the uterus as described by New (1966). After reflecting Reichert's membrane and removing the loose edges of the vascular yolk sac that tended to adhere to the culture bottles, the embryos were operated on in a watch-glass in rat serum, under sterile conditions. Using watchmaker's forceps, a small slit was made in the membranes and through it one of the forelimb-buds was grasped and amputated at its base. The limb was removed from the operating dish and discarded. Care was taken to remove the whole of the limb-bud, leaving no projecting tags of tissue: if there was any doubt as to the completeness of the removal, the embryo was discarded from the results. The

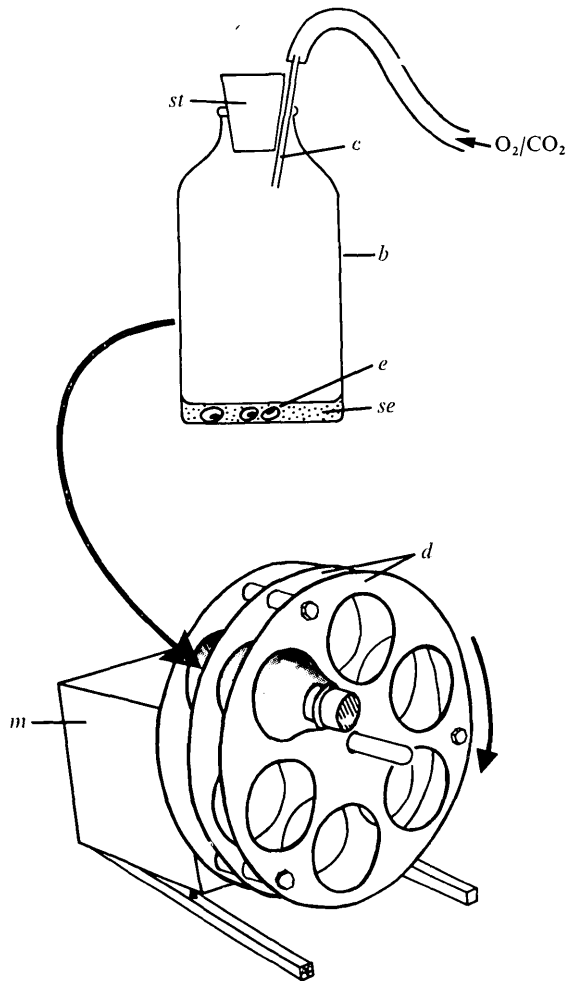


Fig. 1. Diagram of the culture apparatus. *b*, Bottle; *c*, capillary tube inlet for gas; *d*, rotating metal disc; *e*, embryo; *m*, motor; *se*, serum; *st*, rubber stopper.

unoperated forelimb on the other side of the embryo served as control in the histological comparisons, while in addition, control embryos whose membranes had been split but no amputation performed were cultured with each experimental group.

For culture, up to three embryos were placed in a sterile, stoppered bottle containing 3 ml rat serum (Fig. 1). Preliminary trials had shown that this was sufficient serum to keep the embryos alive for 2 days and to submerge them completely provided that they did not stick to the glass bottle during its rotation. Each bottle was gassed with a 95 %O<sub>2</sub>/5 %CO<sub>2</sub> mixture, via a metal capillary inserted between the rubber bung and the neck of the bottle, for 10 min. The bottles were then placed (see Fig. 1) on an L.E.C. rotating apparatus (Gallen-

kamp Co. Ltd) in the incubator at 37 °C. The bottles were examined at 3 h intervals during the first day of culture to check for possible adhesions of the embryonic membranes to the glass: any bottle in which this had occurred was shaken gently to dislodge the embryo and so ensure that it would remain submerged in the serum during the whole of each circuit on the rotator. It had previously been found that prolonged adhesion caused flattening, distortion and death of embryos.

After 44 h of culture, the embryos were examined under the dissecting binocular to check their general health and the external appearance of the limb-buds, then they were fixed in Bouin's fluid and afterwards stained *in toto* with 1% aqueous eosin before dehydrating in alcohols and clearing in methyl benzoate. A portion of the trunk of each embryo, including the forelimb-buds, was embedded and sectioned transversely at 8  $\mu\text{m}$ . The sections were stained with Ehrlich's haematoxylin and eosin.

## RESULTS

### (a) *External appearance*

All of the embryos which had not adhered to the culture bottles for periods of longer than 3 h were in reasonably good health at the end of the culture period. They had expanded to at least  $1\frac{1}{2}$  times their original linear dimensions, but were not as large as 13-day embryos obtained directly from the uterus. This deficiency of growth in culture as compared with development *in vivo* is probably due to nutritional deficiencies (cf. New, 1966; Payne & Deuchar, 1972). Some of the embryos also seemed anaemic, having very little red blood either in the membranes or in the embryonic blood vessels. It was evident that neither the chorio-allantois nor the yolk sac were maintaining normal numbers of blood cells. The heart was well developed, however, and was beating rapidly in embryos immediately after their removal from the incubator. Other morphological features of the embryos appeared normal too: the brain had enlarged and the cerebral hemispheres were prominent; pharyngeal arches were well developed; the trunk had closed ventrally; the unoperated limb-buds had expanded and there was a long tail. Fig. 2 shows the external appearance of an embryo at the end of the culture period. In many cases a regenerate was visible at the site of amputation of the forelimb, but this varied in different embryos, from a mere tag of tissue to a full-sized bud differing little from the control on the unoperated side. More precise study of these regenerates was made in histological preparations (see below).

### (b) *Histology*

Trunk sections from 32 operated embryos which appeared in good health at the end of the culture period were used for histological study. Table 1 summarizes the condition of the regenerated limb-buds in these.

Only three of the 32 embryos had failed to form a new limb-bud at the site of

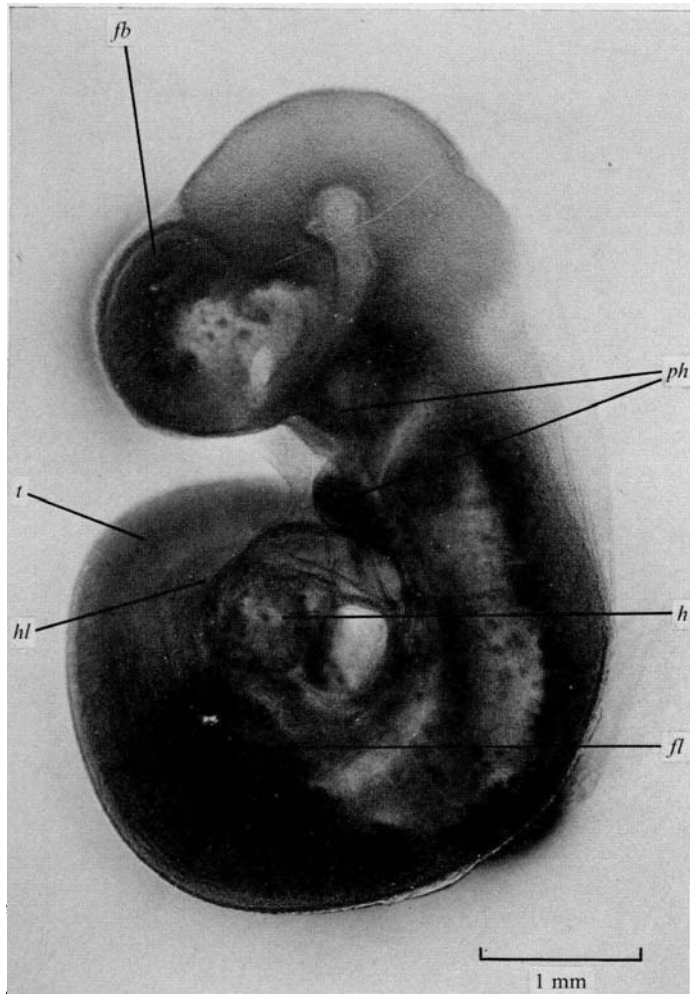


Fig. 2. External appearance of embryo at end of culture period, after removal of membranes. *fb*, Forebrain; *h*, heart; *fl*, forelimb-bud; *hl*, position of hind limb-bud, obscured by heart; *ph*, pharyngeal arches; *t*, tail.

amputation. In these three, the rest of the embryonic tissues seen in section appeared dead and partially disintegrated, so evidently their failure to regenerate could be attributed to general ill-health. Two of these embryos had adhered to the glass bottles several times during the culture period, so that their non-viability was probably due to distortion and desiccation.

In 27 of the 29 embryos that had regenerated limb-buds, the bud was covered with an intact ectodermal epithelium, showing that there had been proliferation and complete healing of the epidermis, as well as proliferation of mesoderm cells to form a protuberance beneath it (cf. Fig. 3). In two embryos the epidermis

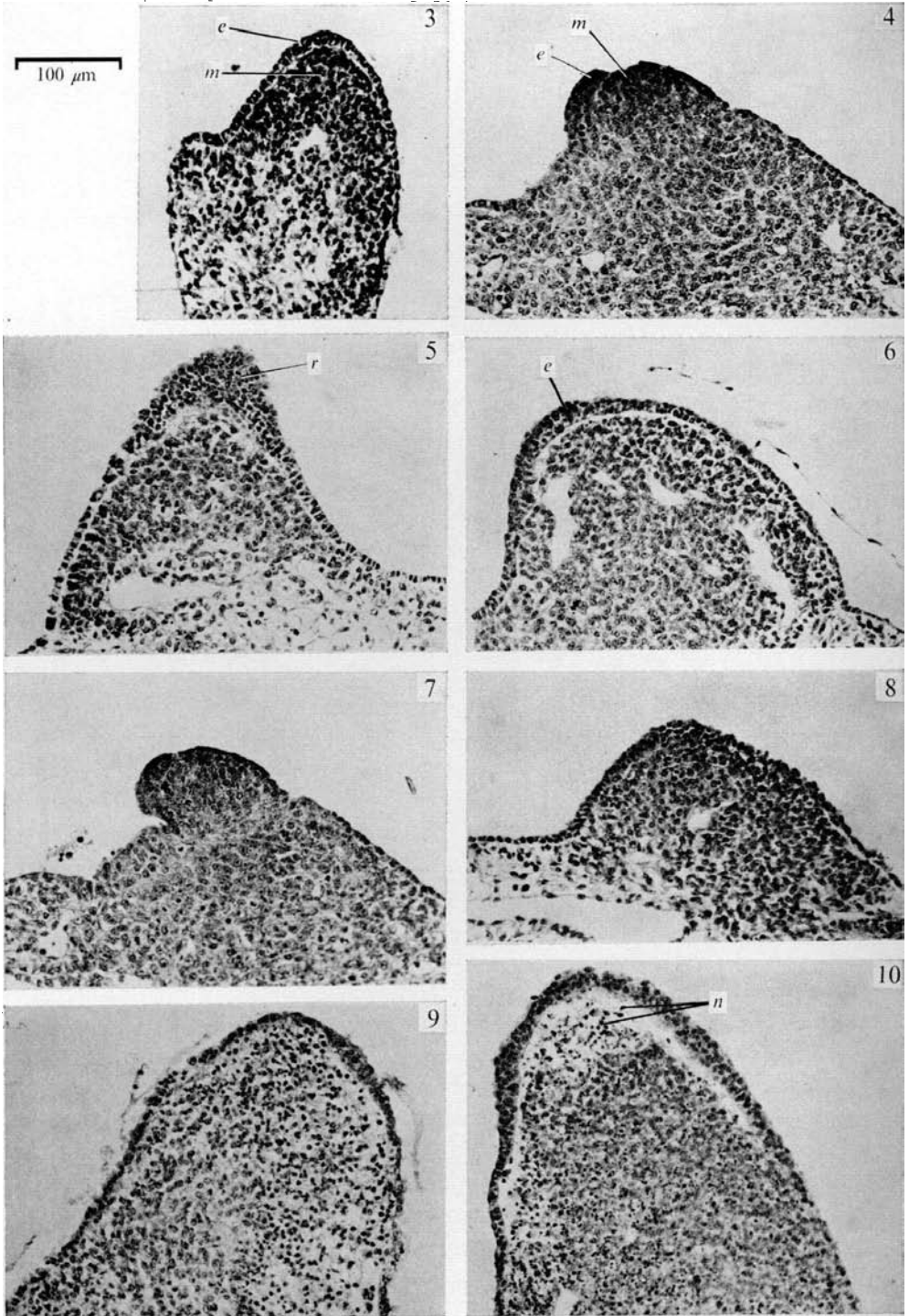


Table 1. Results of limb-bud amputations

Morphology	Histology	
	Ectoderm	
Regenerate present	29	Epithelial healing complete 27
No regenerate	3	Epithelium incomplete 2
Normal size	14	Normal apical ridge 8
Small size	15	Incipient apical ridge 7
Normal shape	15	No apical ridge 17
	Mesoderm	
Conical	6	Normal rounded cells 16
Rounded	4	Loose mesenchyme cells 11
Stump	2	Loose and some necrosis 2

was not completely healed however (cf. Fig. 4). These two embryos had also adhered to the glass bottles for short periods during culture, and it is possible that the epidermis was damaged in this way.

In the control, unoperated forelimb-buds of these embryos, there was still no differentiation of the mesoderm; this consisted simply of densely packed, rounded cells with occasional capillary vessel rudiments between them. The epidermis, however, had thickened to form three or four layers of columnar cells at the apex of the limb (Fig. 5): this is the apical ectodermal ridge, typical of the limb-bud of all vertebrate embryos, whose presence is essential for normal limb development. Of the limb-buds regenerated after amputation, eight showed a normal apical ridge (Table 1): seven others had a slight thickening of the epidermis which looked like the beginnings of a ridge (Fig. 6) and the other 17 had a single-layered epidermis with no apical ridge at all.

The shape of the regenerated limb-bud was abnormal in about half the embryos examined (cf. Table 1). In two cases it was little more than a stump (Fig. 7), in six embryos it formed a short cone (cf. Fig. 8) and in four embryos it

#### FIGURES 3-10

All to same magnification (see scale at top left).

Fig. 3. Transverse section of regenerated limb-bud with complete epidermal healing. *e*, Ectoderm; *m*, mesoderm.

Fig. 4. Transverse section of limb-bud which did not have complete epidermal healing.

Fig. 5. Control limb-bud with well-developed apical ectodermal ridge (*r*).

Fig. 6. Slightly thickened apical ectoderm (*e*) in a regenerated limb-bud.

Fig. 7. Stunted regenerate.

Fig. 8. Short, conical regenerate.

Fig. 9. Regenerate showing loose, mesenchymatous mesoderm.

Fig. 10. Regenerate with some necrosis (*n*) among mesoderm cells.

was a small, rounded protuberance. As many as 15 embryos had a regenerated bud of quite normal shape, closely resembling the control bud on the unoperated side (cf. Fig. 5). When sizes of regenerated and control limb-buds were compared, it was again found that about half the regenerates were abnormal: these were all smaller than the bud on the control side. Fourteen embryos had buds of normal size (Table 1).

Bearing in mind that the regenerated limb mesoderm could have formed by dedifferentiation and proliferation of adjacent mesenchyme of the body wall, or perhaps by migration of cells from adjacent somites (cf. Vasse, 1974), the shape and density of the cells in regenerated and control limb-buds were compared and evidence of mitoses or migration was looked for in mesoderm near the regenerates. There was no evidence to suggest that the cells of the regenerates originated from nearby somites. On the other hand, there were indications that some of the regenerates had arisen by proliferation of blastema-like cells, perhaps derived from mesenchyme at the wound site. While 16 of the regenerates had rounded, compact mesoderm cells like those in the control limb-bud of the opposite side, in 13 other regenerates the mesoderm was noticeably looser and more like mesenchyme than in a normal bud at that stage. Two of these showed necrosis of some cells (see Figs. 9, 10).

#### CONCLUSIONS

It is unfortunate that the limited survival time of embryos in culture does not allow one to see whether the regenerated limb-buds are capable of forming normal, differentiated limbs. Since Hampé's (1959) and Kieny's (1964) work indicated an inability of chick limb-buds to form complete limbs if partly amputated at stages equivalent to the 11½-day-old rat embryo, it would indeed be interesting if the developmental potentialities of these regenerated rat limb-buds proved to be superior to those of the chick. What is clear from the present results is that a new limb-bud of apparently normal morphology can be regenerated in rat embryos, even under the limited growth conditions imposed by culture *in vitro*. Moreover, more than 25 % of the regenerates showed a normal apical ridge, and another 25 % had evident beginnings of a ridge (cf. Table 1), so these buds certainly possessed the components known to be necessary for normal development. Possibly the 17 regenerates that had no apical ridge at 13 days would have formed a ridge later if the culture period could have been prolonged. The present findings are very incomplete, and in future work it is hoped to follow the stages of the regeneration process in more detail, with particular attention to the origin of the cells that form the new bud. It may be possible to test the powers of further differentiation in regenerated limb-buds by isolating them subsequently in organ cultures so that they may be maintained *in vitro* for a further 4 days, by the method of Aydelotte & Kochhar (1972).

We still know relatively little about the regenerative ability of appendages



of vertebrates at embryonic stages of their development. The present findings in rats, however, fall in line with those of Zwilling (1942) in the chick embryo, since he showed that when the whole tail-bud is removed it will regenerate. Work on amphibians too (Münch, 1938; Deuchar, 1975*b*) has shown that in these the whole tail-bud can be regenerated at embryonic stages. Bouvet (1971) on the other hand found in trout embryos (by experiments similar to those of Hampé and Kieny on the chick) that parts of the fin were deficient if parts of the limb-bud were amputated. One may conclude, therefore, that sufficient versatility remains in cells adjacent to appendage rudiments in amniote embryos for these to replace the whole bud, but that it may not be possible for the component cells of a mutilated appendage rudiment to reorganize and replace lost parts. Embryonic regeneration can probably not be regarded as fully comparable with the elaborate events of demolition, dedifferentiation and blastema-formation (Needham, 1952) that characterize regeneration in adult appendages. But it has at least been noted in over 50 % of the regenerated limb-buds described above that mesenchyme cells appeared to have undergone some dedifferentiation to form rounded cells like those of the original bud (cf. Fig. 9 and Table 1).

I should like specially to thank Margaret Jolly for her hard, though unrewarding work on regeneration in the tail-bud of rat embryos, which was the main stimulus to the present study; also for her lively interest and discussions. I am indebted to Fiona Parker for technical assistance and to Anne Featherstone for photography and art work.

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