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Effect of Salicylic Acid on the Early Development of the Chick Limb-Bud

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SYNOPSIS. Transplanted hind limb-buds of chick embryos were treated with salicylic acid. Treatment with this chemical caused reduction in length, weight and volume of the developing limb-buds. A side effect observed was an apparent loosening of the and examined with regard to length, weight, and volume.

Mitochondria are organelles found in all aerobic organisms in which the oxidation of pyruvic acid to carbon dioxide and water takes place.

Several workers have isolated mitochondria to study their properties and find their role in embryogenesis. Carey and Greville (1959a,b) found that the mitochondria obtained from embryonic preparations appeared to be more fragile than their adult counter parts. McAlpino (1960) reported a marked increase in phosphate activity (which is related to mitochondria) in the ectodermal tips of the developing limb-buds of rat embryos. These observations and others (Bosund, 1957; Charnock, and Opit 1962a) show an essential mitochondrial activity during early development.

Salicylates are thought to be inhibitors of mitochondrial activity, which may act by disrupting or uncoupling the link between oxidation and phosphorylation. Brody (1956) was the first to show that salicylates in a concentration above 3 mg/100 ml. can decrease the P:O (phosphorylation: oxidation) ratio of rat liver and kidney mitochondria oxidizing a variety of substrates. The actual site of salicylate activity has not yet been found; but Charnock and Opit (1962b) have suggested that the locus of the action of salicylate in uncoupling oxidative phosphorylation is at the level of the mitochondrial membrane. It was therefore considered desirable to observe the effect of Salicylic acid on the early development of the chick limb-buds and to find if Salicylic acid has another effect in addition to its suggested uncoupling factor.

METHODS AND MATERIALS

The hind limb-buds of white leghorn chick embryos at stage 19 of Hamilton and Hamburger, were extirpated and transplanted singly onto the chorioallantoic (CA) membrane of host embryos of stage 35 of Hamilton and Hamburger. Immediately after transplantation, 0.08 ml. of salicylic acid were dropped on top of the transplanted buds with a micro-dropper. There were seven groups of transplants corresponding to seven concentrations. The host eggs were again incubated at 38° C. The transplanted limb-buds were recovered at intervals of 6 hours, 1, 2, 3, 4, and 5 days after operation

basal membrane. The above observations are discussed in relation to the different hypotheses of the ectoderm-mesoderm interaction. The physiological role of the basal membrane, as well as the possible implications of the basal findings to the question of limb development and morphogenesis, are also discussed.

INDEX DESCRIPTORS: Salicylic acid, chick limb bud, ectoderm-mesoderm interaction, limb bud growth.

Some of them were then fixed in 4 per cent neutral formalin and others in Helly's fluid, washed in tap water, dehydrated, and embedded in paraffin. The sections were cut at 4 μ by AP-121 Microtome, and stained either by Novelli's method for demonstration of mitochondria or with eosine and haematoxylin (E and H). Controls were done with each group of experimental concentrations in which 0.08 ml. of Pannet-Compton solution were added to the transplant.

For thin sectioning, limb-buds were fixed in chilled gluteraldehyde (4°C) for 4 hours, and post fixed in osmium tetroxide (in phosphate buffer) for 45 minutes. After dehydration with graded alcohols (35, 70, 90 per cent and three changes of absolute alcohol) the tissues were transferred to the Araldite mixture, the Araldite was changed twice or thrice during this time. The sections ($\frac{1}{2}$ - $\frac{3}{4}$ μ thick) were cut by glass knives on LKB ultra-microtome, and were stained with acid fuchsin.

OBSERVATIONS

The group of limb-buds treated with 250 μ gm/ml and 200 μ gm/ml concentration showed a pronounced growth inhibition during the whole time of transplantation, and especially during the first 4 days of transplantation, compared to the controls (Figure 1). The groups of limb-buds treated with the concentrations of 170, 150, and 135 μ gm/ml also showed growth inhibition but to a lesser degree and in the period of the first 3 days of transplantation (Figure 2). The groups of limb-buds treated with the concentration of 125 and 100 μ gm/ml did not show any significant growth inhibition; (Figure 3) and the growth of the group treated with 100 μ gm/ml was very similar to the controls. The only difference between the groups of limb-buds treated with the last two concentrations and the control was less growth in the first day (Figure 3).

In all groups of transplanted limb-buds (except for those treated with the first two concentrations) the growth (length, weight, and volume) tends to become normal after the third day of treatment. In the groups treated with the last two concentrations the growth is very close to the normal. In all the transplanted limb-buds there was no significant difference in growth during the first 6 hours of transplantation compared to the controls. After this the growth behavior differed according to the concentration.

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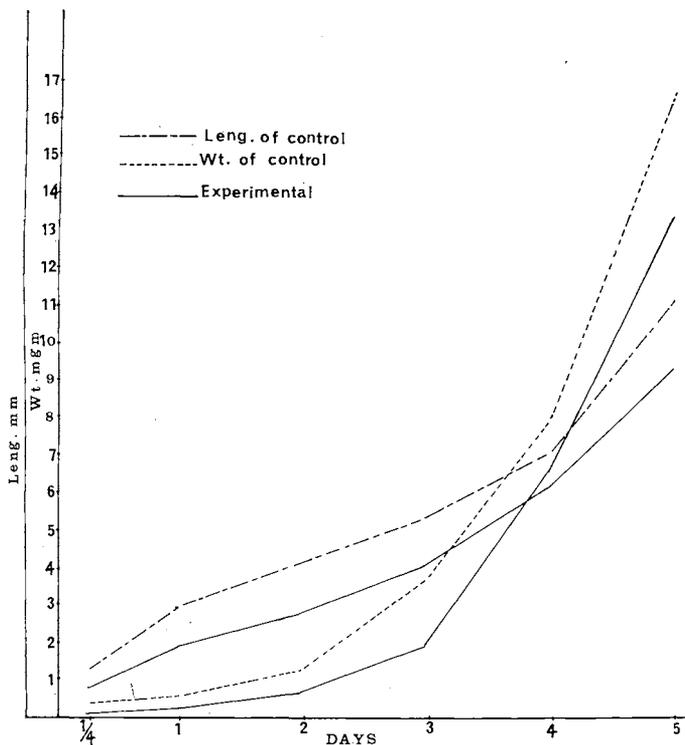


Fig. 1. Growth of transplanted limb-buds treated with 250 $\mu\text{gm/ml}$ of salicylic acid.

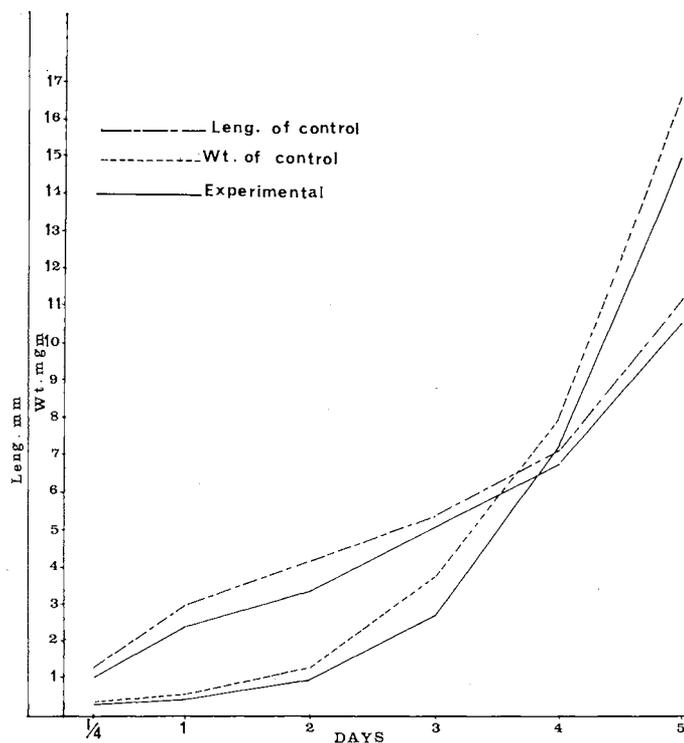


Fig. 2. Growth of transplanted limb-bud treated with 170 $\mu\text{gm/ml}$ of salicylic acid.

In the untreated limb-buds (Figure 4) the basal side of the ectoderm is sharply demarcated from the mesoderm. It was observed that in Salicylic acid treatment with the higher concentrations (250 $\mu\text{gm/ml}$ and 200 $\mu\text{gm/ml}$) the basal membrane looked loose under the light microscope, and it was easy to separate an almost intact ectoderm from the examined transplanted limb-buds in the period of the third day of transplantation.

In the case of the lower concentrations the loosening was less significant and it was more difficult to separate the ectoderm (Figure 6). In the groups receiving 150 $\mu\text{gm/ml}$ and 100 $\mu\text{gm/ml}$ concentrations there was no loosening except after six hours treatment. Whether any change has occurred in the chemical constitution of the basal membrane is not known.

DISCUSSION

During the past twenty years, much experimental work has been directed to the study of ectoderm-mesoderm interaction in limb morphogenesis in avian embryos. It has been debated whether the ectoderm or the mesoderm of the limb-bud is the site of developmental factors. Balinsky (1956) suggested that both mesoderm and ectoderm take an active part in the development of an induced as well as normal limb.

Recently two different hypotheses have been formulated to account for the development of limbs in tetrapod vertebrates. In both hypotheses the mesoderm is claimed to be the essential site for limb development. The area of disagreement is in the role played by limb-bud ectoderm, particularly the apical ridge. An increasing body of evidence has accumulated which

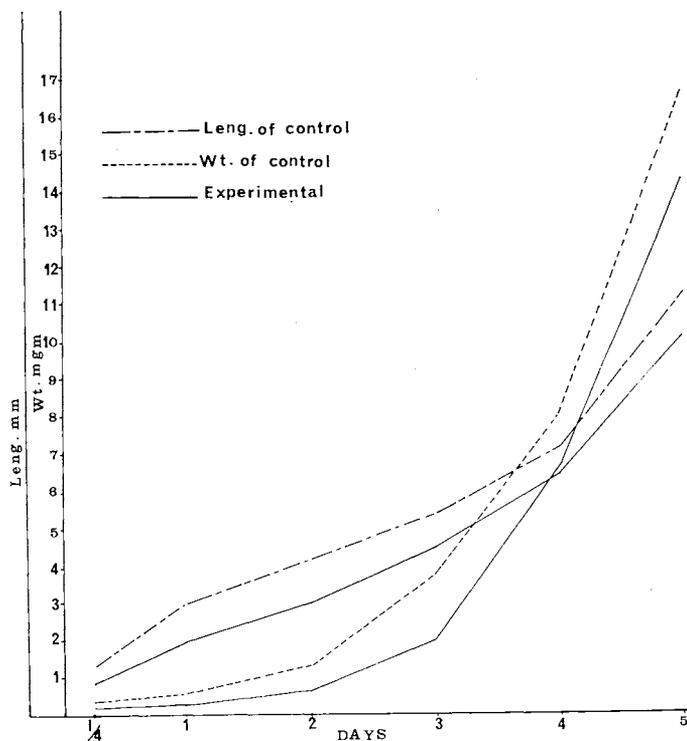


Fig. 3. Growth of transplanted limb-buds treated with 100 $\mu\text{gm/ml}$ of salicylic acid.

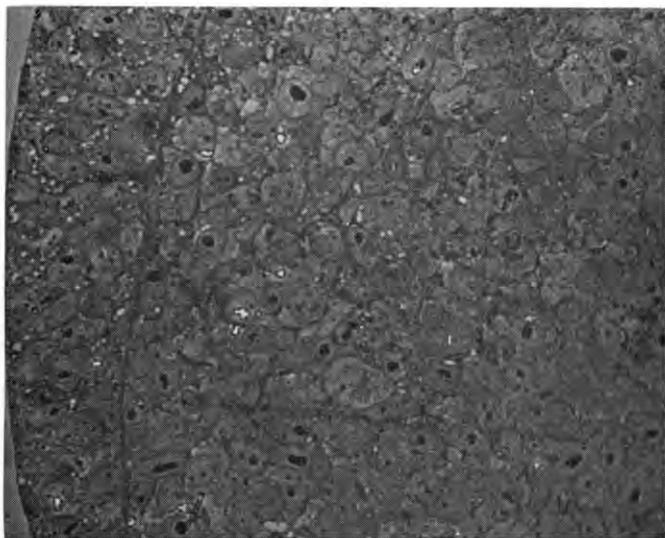


Fig. 4. Portion of sagittal section of untreated limb-bud recovered after one day; section at $1/2 \mu$ and stained with acid fuchsin. X 960.

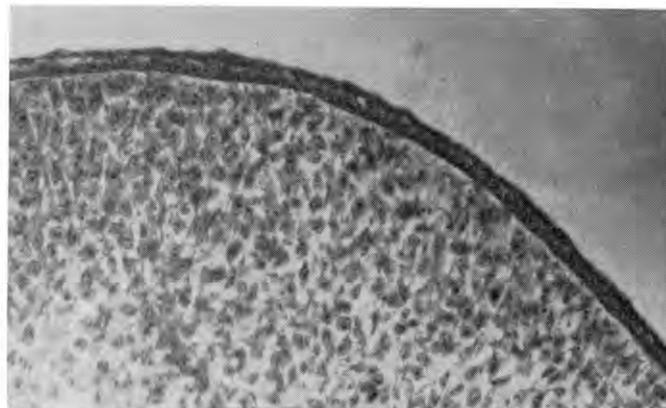


Fig. 6. Portion of sagittal section of limb-bud treated with 170 $\mu\text{gm/ml}$ of salicylic acid recovered after one day; section at 3μ and stained by Novelli's method. X 1110.

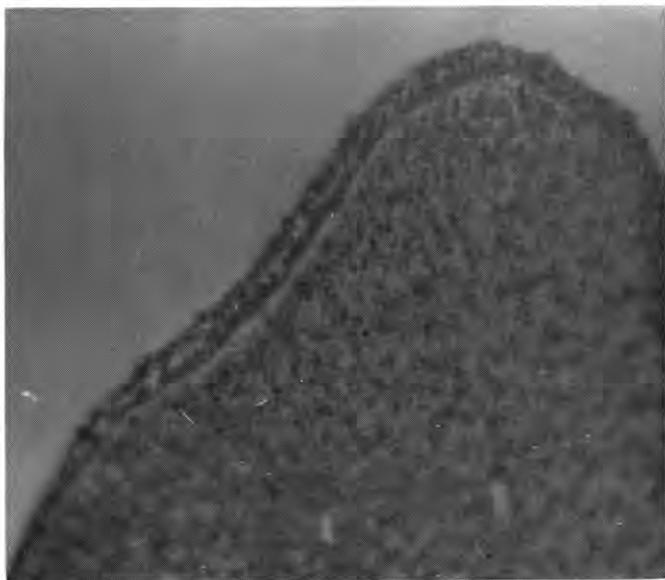


Fig. 5. Portion of sagittal section of limb-bud treated with 250 $\mu\text{gm/ml}$ of salicylic acid recovered after two days; sectioned at 3μ and stained by Novelli's method. Note the loosening of the basal membrane. X 1170.

indicates an active participation of ectoderm in limb development (Zwilling, 1961; Amprino, 1962; DeHaan and Ebert, 1964) especially the thickened portion which is morphologically prominent in birds. According to our observation of

loosening in the basal membrane, special attention must be given to this seemingly important intermediate zone between the ectoderm and mesoderm in the limb-bud of the chicken. It is observed that any effect on this is reflected on the limb-bud as growth inhibition. The above observation would also stress the importance of ectoderm (not just a covering), since the loosening of the basal membrane and its tendency to stay with the ectoderm reflects inhibition of growth.

In various publications the term basal membrane is applied to various structures, in this paper the basal membrane is defined as the zone present at the dermo-epidermal junction. In electron microscopy the term is usually applied to a continuous membrane covering the basal membrane cells (Sjostrand, 1953; Jurand, 1965).

It has been suggested that the basal membrane composed mainly of tropocollagen fine filaments which are either embedded in an amorphous matrix of the same density (probably mucopoly-saccharides), or are very closely compacted so that the individual fiber can not be resolved (Fawcett, 1966).

Although the exact function of the basal membrane is still uncertain, it is believed to be a diffusion barrier (Balinsky, 1956, 1957). Caesar and Edward (1957) suggested a protection function from too rapid ion concentration changes.

It is not known if salicylic acid acts on the tropocollagen in the basal membrane, but it interferes with the metabolism of collagen (Bellamy, 1963). It affects the biosynthesis of macopolysaccharides in connective tissues (Bostrum, 1955, 1963). The loosening of this membrane observed in our experiments may be attributed to this effect, or it might suggest an effect of salicylic acid on other components not yet known. The inhibition of growth by salicylic acid disappears especially in the groups treated with 100-170 $\mu\text{gm/ml}$ concentrations (Figures 1, 2, 3). This disappearance may be explained by diffusion of salicylic acid into the surrounding tissue. It is also likely that it affects an interaction between the ectoderm and mesoderm through this membrane. Its diffusion and reduction of its effectiveness allows a reformation of an "interacting substance" in this membrane, so a normal or very close to normal growth is maintained again.

More biochemical studies must be done to determine the precise make-up of the basal membrane, and the exact mode of action of salicylic acid on this membrane.

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