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Induction of Regeneration of Forelimb of the Frog by Augmentation of the Nerve Supply.* (18508)

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The ability to regenerate extremities following amputation is observed in all stages of the life cycle of the salamander and other urodele amphibians but, in the case of frogs, it is restricted mainly to the tadpole (Barfurth, review by Polejaiev) (1,2) although occasionally a limited regeneration is seen in the adult (2,3). The loss in regenerative capacity occurs rather abruptly during metamorphosis (2), but it is not an absolute and irrevocable one for regeneration has been successfully evoked in limbs in which the wound area was subjected to mechanical and chemical irritations and alterations (2,4-8). The reason for the decline in regenerative power at metamorphosis is not known but a number of theories have been advanced which are re-

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1. Barfurth, D., *Arch. f. Entw. Mech.*, 1894, v1, 117.
2. Polejaiev, L. W., *Arch. d'Anat. Micr.*, 1936, v32, 437.
3. Thornton, C. S., and Shields, T. W., *Copeia*, 1945, v40.
4. Polejaiev, L. W., *Comp. Rend. (Doklady) Acad. Sci. URSS*, 1945, v49, 609.
5. Polejaiev, L. W., *Biol. Rev.*, 1946, v21, 141.
6. Gidge, N. M., and Rose, S. M., *J. Exp. Zool.*, 1944, v97, 71.
7. Rose, S. M., *J. Exp. Zool.*, 1944, v95, 149.
8. Rose, S. M., *J. Morphol.*, 1945, v77, 119.

viewed and evaluated by Polejaiev(2,5), Rose (7,9) and others. Salient among these views is the belief that some fundamental change occurs in the tissues or tissue interrelations which render extremities incapable of regeneration. The nature of the change has been speculated upon and the idea has been advanced that it involves increased "differentiation" or alteration in the "organizing power" of the limb. These theoretical explanations of regenerative loss in the adult frog must at this time be supplemented or, perhaps, partly substituted by another theory which has emerged from the results of experiments on the influence of the nerve in regeneration of the forelimb of the newt (Singer)(10-12).

In the newt, regeneration of the forelimb does not ensue if the number of nerve fibers normally available at the amputation surface is reduced beyond a critical threshold value through partial destruction of the contributing brachial nerves. Perhaps such quantitative requirements obtain also for the tadpole limb since it has been shown in experiments on the tadpole that nerves are essential for regeneration(13). Assuming that such threshold requirements do, indeed, exist, it is possible to advance the theory that loss in regenerative capacity of the frog's limb which occurs during metamorphosis is due, at least in part, to tissue or other changes which render the available nerve fibers numerically inadequate to evoke regeneration. If such changes do occur whereby the threshold nerve requirements for initiation of regeneration are no longer met by the existing nerve supply, then it would follow that regeneration should ensue if such threshold nerve requirements are satisfied by some experimental means. In experiments described here the normal nerve supply of the forelimb was augmented substantially by nerves deviated from the hindlimb. As the results demonstrate, regeneration of the forelimb of the post-metamorphic frog is readily evoked thereby.†

Procedures. Young adult frogs (*R. pipiens* —about 3 cm body length) were anaesthetized with ether and subjected to the nerve operation diagrammed in Fig. 1. The forelimb was amputated in about the middle of the upper arm. Then a slit was made in the skin of the thigh and a threaded needle drawn under the skin of thigh, body wall and upper arm until it emerged at the amputation surface (Fig. 1A). The sciatic nerve and its four major branches were freed by dissection to the level of the ankle or foot and fastened to the end of the thread as shown in Fig. 1B. The free end of the thread was seized and pulled until the remaining thread and attached nerves were drawn under the skin to the amputation surface of the forelimb (Fig. 1C). The distal ends of the nerves were resected where they emerged at the forelimb thus freeing them from the thread. The dissected and nerveless hindlimb was then amputated near the body wall and the skin sutured over its surface. The deviated nerves consisted of the sciatic, its two major branches the tibial and peroneal, and their two subdivisions, respectively the deep and superficial (sural) tibial and the lateral and medial peroneal nerves. Dissection of the latter four subdivisions was carried to the ankle or foot region in order to obtain a sufficient length of nerve for deviation. Deviation of the nerves was done in 21 animals. Nineteen others of the same stock were used as controls. In all controls the amputation level of the forelimb was the same as for the experimental ones. In 9 control animals the hindlimb of the same side was also amputated and the skin sutured over the cut surface but this variation yielded no difference in results and is, therefore, not mentioned further. Reference will also be made in the ensuing description to other experimental animals which were employed in the preliminary elaboration and survey of the experiments

9. Rose, S. M., *Annals N. Y. Acad. Sci.,* 1948, v49, 818.

10. Singer, M., *J. Exp. Zool.,* 1946, v101, 299.

11. Singer, M., *J. Exp. Zool.,* 1947, v104, 223.

12. Singer, M., *J. Exp. Zool.,* 1947, v104, 251.

13. Schotté, O. E., and Harland, M., *J. Exp. Zool.,* 1943, v93, 453.

† These theoretical interpretations as well as initial successful results with two animals were presented by the author for the first time in the discussion of a paper given by Rose at the AAAS meetings in Boston in 1946. They are mentioned by Rose in his later review(9).

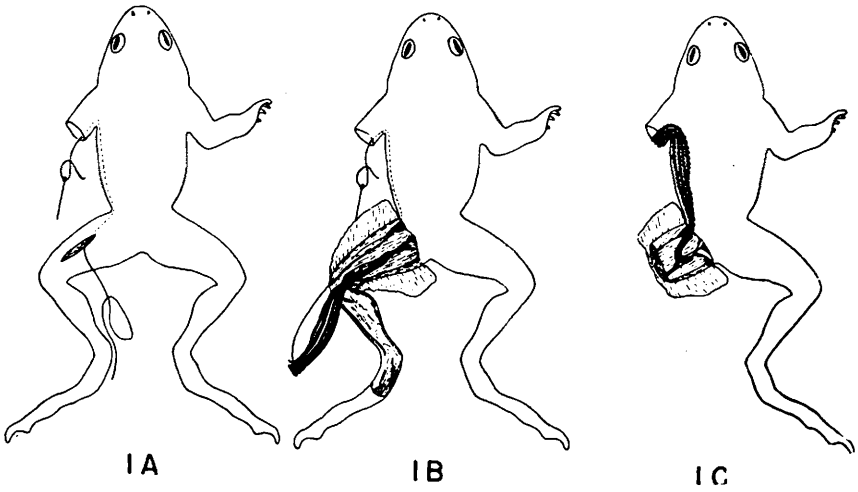

FIG. 1A, B, C.
Diagram of the successive operative procedures used in deviating the sciatic nerve and its branches to the amputation surface of the forelimb.

described herewith. All operations were confined to the left side. Animals were kept in aquarium tanks containing dilute NaCl solution (0.15%) rather than tap water to avoid infections and the tanks were tilted slightly to provide an amphibious environment. Animals were force-fed 2 or 3 times a week with ground beef dipped in cod liver oil and bone meal according to the recommendation of Rose(7). The amputated forelimb was observed periodically for signs of regeneration.

Results. Three of 19 control animals showed some limited signs of forelimb regeneration after 6 to 8 weeks. These growths were quite small (approximately 0.75 mm long in one case and less than 0.50 mm in the other 2), appeared later than those described for experimental animals and were restricted to a small part of the amputation surface. The appearance of an occasional regenerate among the controls emphasizes the fact that regenerative capacities of the post-metamorphic frog are latent. Signs of occasional spontaneous regeneration, particularly of more distal levels of the frog's limb, have been observed before(2,3).

Twenty of the 21 animals in which nerves were deviated showed a regenerative response of the forelimb, and the regenerate in 16 of these cases was larger than in the positive control ones. At 8 weeks following operation most of these 16 were between 2 and 3 mm in length and continuing to grow. Initial experiments of nerve deviation with other animals showed that lengths of 6 mm or more could be eventually attained. Generally, the first gross signs of regeneration appeared about 3 weeks after amputation but in some instances as early as 2 weeks. The rate of growth was greatest in the period immediately following growth initiation.

The first signs of morphogenesis appeared 8 to 10 weeks after operation and subsequently showed tremendous variations among the animals in extent and rate. In some instances a bend appeared in the regenerate suggesting elbow formation; or, a distal flattening occurred which was reminiscent of hand formation as seen in newt regeneration. Another sign of differentiation was the appearance of irregularities over the distal end of the regenerate which anticipated finger formation. Examples of regenerates in various conditions of differentiation are shown in Fig. 2 and 3 which may be contrasted with the non-regenerating control of Fig. 4. In general, regenerates were quite heteromorphic as compared to those of the salamander.

Finally, preliminary observations of histological sections through regenerates revealed extensive histogenesis of limb tissues. Cartilaginous skeletal elements having articulating

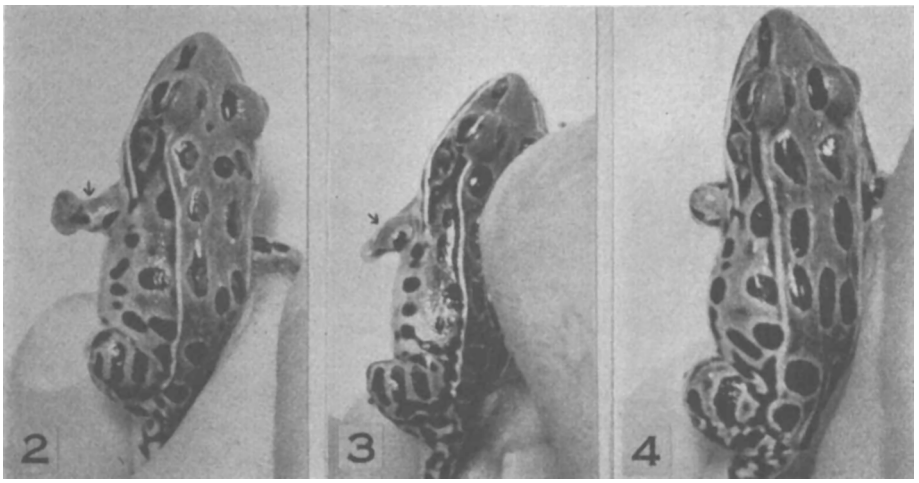

FIG. 2, 3, 4.
Representative results of experiments. Fig. 2 and 3 show regenerates of 8 weeks, stimulated by the augmented nerve supply, whereas Fig. 4 shows a non-regenerating control. Arrows in Fig. 2 and 3 point to original amputation level. (1½×).

surfaces, muscle, tendon and other connective tissue elements were present in various stages of development.

Discussion. The results show that regeneration of the forelimb of the frog may be successfully evoked by an augmented nerve supply. It is reasonable to conclude therefrom that the nerves normally available at the amputation surface are quantitatively inadequate to yield a regenerative response. Alternative explanations seem less likely; for example, that the sciatic nerve possesses the peculiar quality of stimulating forelimb regeneration not shared by nerves of the forelimb, or that potentialities of growth stimulation, latent within the nerve, are somehow called forth when the nerve is deviated to the forelimb. Assuming that the nerve action is non-specific and the essential effect of nerve deviation was to raise the fiber quantity above the threshold, then it is evident that loss of regenerative capacity which occurs at metamorphosis is the result of some anatomical or physiological change which renders the available nerve fibers quantitatively an ineffective stimulus of regeneration. Theoretically the anatomical or physiological change may have its locus in the nerve, in other tissues of the forelimb, or, indeed, in both.

These results re-emphasize an aspect of nerve function which is little appreciated, namely the ability to stimulate growth of another structure(14). As shown experimentally here, this quality of the nerve is not lost at the time of metamorphosis but is retained in the adult and under appropriate circumstances may be expressed in organ regeneration.

Summary. The ability of the amputated forelimb of the tadpole to regenerate declines and in most cases is lost completely at the time of metamorphosis. However, the present report shows that regeneration can be evoked experimentally in the adult frog if the number of nerve fibers available at the amputation surface of the forelimb is augmented by deviation of the sciatic nerve from the hindlimb. The theoretical considerations which led to these experiments emerged from the study of the influence of quantity of nerve fibers in regeneration in salamanders and are discussed here.

14. Sidman, R., and Singer, M., *Am. J. Physiol.*, 1951, in press.

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