with type-specific FA conjugate and with type-specific precipitin antisera. This finding indicates that L forms after repeated passage *in vitro* may lose the type-specific M protein.

Discussion. The experiments of Freimer *et al*(1), which employed a modified Ouchterlony technique, demonstrated the diffusion of M protein from L-form colonies into the agar medium, thus suggesting a continuous production of M protein, which, in the absence of a bacterial cell wall, passes freely into the surrounding medium. The accumulation of an appreciable quantity of extracellular M protein and its absence in isolated protoplast membranes suggested than an insignificant amount of synthesized antigen persisted within multiplying L forms. The present data confirm the diffusion of M protein out of L-form colonies and into the agar. It is of interest, however, that M protein was readily detected in L-form colonies when examined by the immunofluorescent technique. It is unlikely that this finding is dependent upon a nonspecific cross-reaction. There is, for instance, no doubt that the type-specific fluorescein-labeled antisera employed here contained anti-M antibodies(4). Furthermore, specific fluorescence in the absence of significant heterologous staining was noted with 5 different types of L forms and homologous conjugated antisera.

Although the FA method employed in the experiments reported above was used to identify subcultures of L forms, it has now been employed successfully to identify original isolates. The FA procedure provides a simple and rapid method for direct detection of M protein in L-form colonies, affording an expedient method for identification and classification.

Summary. M protein was detected in growing L-form colonies of Group A streptococci by an immunofluorescent technique which employs type-specific fluorescein-labeled antibody. The type-specificity of the FA-staining reactions exhibited by the L forms was confirmed by parallel precipitin studies. These data suggest the feasibility of identifying the L-form colonies of Group A streptococci by detecting the M protein with the FA method.

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Received September 2, 1964. P.S.E.B.M., 1965, v118.

Abnormal Limb Development in *Rana catesbeiana.** (29797)

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The occurrence of limb abnormalities has been encountered in nature as a result of injury and has been well documented from defined experiments involving such representatives from the animal kingdom as the amphibians, crustaceans, and insects(1). In contrast to these abnormalities which in most cases were produced physically by cutting or breaking, certain teratogenic chemicals have proven to be effective in mammals and birds (2). Other approaches have been reported with urodele Amphibia using localized ultraviolet irradiation(3) and still another experi-

* This investigation was supported in part by Research Grant CA-04027 from Nat. Cancer Inst. and by GRSG-52 Grant, 1-GS-52 from U.S.P.H.S.

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mental procedure involved the implantation of anuran carcinomata(4).

Most of the early work, which made use of the Amphibia, was concerned mainly with the urodele *Amblystoma* and the abnormal conditions that could be produced in the anterior limb-forming region(1,5). On the other hand studies with anurans were confined mainly to the hindlimb region(6,7). The urodeles have an advantage over the anurans in this respect in that the anterior limb develops externally, and is not covered over by the outer epidermis of the body wall. Still another advantage in the use of urodeles in this kind of approach is the prolonged period of self differentiation or the ability to regenerate certain organs throughout the life cycle, which in the anurans ceases, for the most part, just prior to metamorphosis(8).

These findings are presented in order to focus attention on the extent to which the anterior limb primordium of an anuran, presumably during its restricted self differentiating period, can respond to external stimuli, and thus lead to abnormalities in the form of multiple limb formations.

Materials and methods. The results from these observations are purely fortuitous and were obtained in conjunction with another experiment concerned with the immune response in the larvae of *Rana catesbeiana*(9). Laboratory maintenance of this species has been previously described by Hildemann and Haas(10).

The operation involved bilateral thymectomy and removal of lymph glands at standard stage 25 when both organs are in close proximity to the developing anterior limb. Gross observations revealed that the limb primordium, at this stage, is undifferentiated as indicated by the lack of digital indentations. The removal of the lymph gland surgically involved cutting around and very near the limb bud. While no special precautions were taken to leave the bud undamaged, retrospectively, it can be assumed that it was probably left intact. Presumably, then, these observations result from accidental stimulation of the limb bud and more importantly the whole limb bud field.

No daily observations were made inasmuch as the wound in the outer epidermal wall remained sutured throughout the experimental period. The anterior limbs erupted when the larvae approached metamorphosis and in some instances the tadpoles were assisted in freeing the severely deformed extremities.

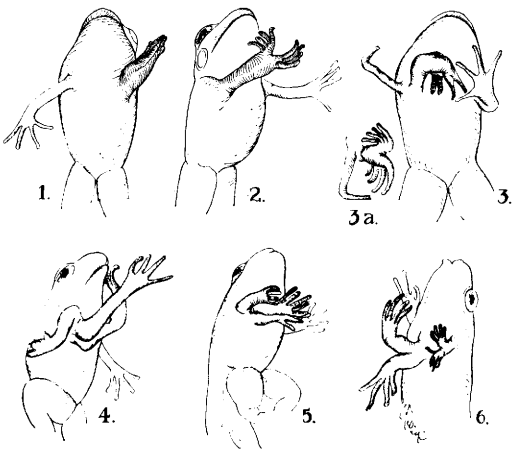

FIG. 1, 2. Abnormal duplicate limbs. Both pairs are mirror images.

FIG. 3, 4. Triplicate limbs showing abnormal digit number.

FIG. 5. Triplicate limb formation with normal digit number.

FIG. 6. Quadruplicate limbs with both pairs developed as mirror images.

Observations. A large number of cases would have been presented if most of them had not been eliminated after the experimental period and before the anterior limbs erupted. Six examples from approximately 100 animals that were discovered accidentally are described herein. Four (Fig. 1-4) reached metamorphosis so that the original drawings were made from live specimens, while 2 others (Fig. 5, 6), which died before metamorphosis, were preserved in formalin. Because histological descriptions of the differentiating limbs were not made, this aspect has been omitted.

While all of the examples are different in most respects, these structures were conveniently grouped into duplicate and triplicate formations (Fig. 1-5), with one quadruplicate exception (Fig. 6). Irrespective of the kinds of limb formed there were in all cases, with one exception, mirror images in one pair of each group. Another aspect of interest, which likewise appears to be accidental, is

the universal development of these limbs unilaterally and not on both sides. In no case did the control animals (sham thymectomized) develop abnormal appendages, which is due no doubt to the technically incomplete sham operation.

In spite of the abnormalities in the limbs, the digit number was normal except in 2 examples. In Fig. 3 one of the members of the triplicate group possessed a reduced lateral arm with only digital indentations (Fig. 3A). Likewise the lateral member of the triplicate combination in Fig. 4 developed not only abnormal blunt appendages in form but 7 in number. The pectoral girdle was affected in Fig. 1-4.

Discussion. Evidence has been presented indicating that supernumerary limb formation can be produced in the anuran, *Rana catesbeiana*. Presumably these abnormal anterior appendages were the indirect result of removing the thymus gland and lymph gland at stage 25 which involved cutting near the limb bud. Operating under the assumption that the limb forming area encompasses not only the bud but a whole region which may be referred to as the limb bud territory, then any disturbances that derange this cell community would perhaps lead to limb abnormalities. It is expected then that the degree of derangement would be reflected in the kinds of limb produced.

Inasmuch as anurans progressively lose the capacity to regenerate lost parts as they approach metamorphosis, then elucidation of the time when no supernumerary limbs can be stimulated to differentiate may give some evidence as to the time when regeneration of the whole limb may be possible. Dent(11) has analyzed the regenerative responses of the larvae and metamorphosed individuals of the South African clawed toad *Xenopus laevis*. From his findings it can be concluded that the younger the individuals are at the time of amputation of the hind bud, the more complete is the regenerated limb. It might be suggestive from these conclusions that the triplicate structures in the present study may have been derived from a younger limb bud than those which formed the duplicates. However, the present amount of data does not fully support such a contention.

Although the limb develops internally, the usual outer epidermis is present and presumably during a certain limited period, blastema formation may follow amputation. If it is possible to observe regeneration in the anterior limb of *Rana catesbeiana* this kind of finding would be especially interesting in light of the results obtained by Goss(12,13) in the urodele amphibian *Triturus viridescens*, a form which possesses the power to regenerate numerous lost parts throughout the life cycle. He found that skinless amputated anterior limbs would not regenerate if inserted immediately into the body cavity. But if some time elapsed between amputation and insertion, then the degree of regeneration was commensurate with the amount of delayed time. The conclusions formulated were that epidermal wound healing and initiation of blastema formation prior to insertion into the body cavity, were indispensable prerequisites for regeneration.

This report does not answer the fundamental questions as to how the stimulus of cutting acts on this highly plastic system or how the cells move about into new locations. But it does suggest that once separated then rapid healing must inevitably occur in order to give rise to complete limbs. Or, in those cases where fusion occurred then it might be inferred that separation of the primordium was not complete and that regrowth and proliferation occurred faster than differentiation. Thus, it would be informative to be able to correlate a carefully defined surgical procedure with a particular kind of abnormality.

Summary. Preliminary evidence has been presented which indicates that in the larval anuran, *Rana catesbeiana*, stimulation of the anterior limb bud field by cutting leads to the formation of supernumerary limbs. The abnormal appendages were conveniently categorized as duplicate or triplicate structures. In most instances 2 members of each of the above 2 classes were mirror images. These findings are discussed in relation to others dealing with similar systems.

The author is grateful to Miss Gwynne Gloege for

executing the original drawings, and to Dr. Richard J. Goss, Brown Univ., Providence, R. I., for critically reading the manuscript.

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Received September 2, 1964. P.S.E.B.M., 1965, v118.

Sexual Dimorphism of Mouse Submaxillary Glands and Its Relationship to Nerve Growth Stimulating Protein.* (29798)

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The biological activity of mouse submaxillary gland (MSG) protein from the mature male mouse was shown to be 4 to 7 times more concentrated than from the adult female MSG(1). This sexual difference may in some way be associated with the sexual dimorphism of the MSG. Intercalated ducts connect the acini with the serous tubules. The serous tubules hypertrophy with the administration of testosterone and the diameter, tubular index (T.I.), is a good measure of the degree of masculinization of the gland (2). This dimorphism has also been associated with several physiological differences: concentration of amylase(3), protease(4), iodine(5), and oxygen consumption(6). The structure can be changed to that of the opposite sex by castration of the male, or by testosterone injection of the female(2) and this has been associated with a reversal in the nerve growth protein content(7). The structure of the female gland has been shown to undergo a "spontaneous masculinization" during pregnancy and lactation(8). In this investigation we studied "spontaneous masculinization" as expressed in the dimorphism of the serous tubules of the female MSG and correlated these observations with a concomitant "masculinization" or increase in the production of nerve-growth-stimulating protein.

Materials and methods. Female Swiss white mice were sacrificed by chloroform inhalation at various life stages: prepubescent (3-6 weeks) and mature virgin (6-12 weeks) various stages of pregnancy and lactation, and several months after lactation. Some animals, representing pregnant stages, were in their first pregnancy and some were not. Immediately after sacrificing, their submaxillary glands were excised. One-half of one lobe was fixed in acetic acid-alcohol-formalin or in 10% formalin, sectioned and stained with hematoxylin and eosin. The diameter of the secretory tubules was measured in microns and recorded as the tubular index (T.I.)(8). The remainder of the gland was homogenized in isotonic saline buffered at pH 7.2, and centrifuged at 15,000 $\times$ g for 10 minutes. All steps were done at 4°C. The supernate was decanted, analyzed for total protein by the Lowry method(9), and assayed at 10-fold

* This investigation was supported by research grants NB 0-3979 and HD 00162 from Nat. Inst. Health.

† Recipient of National Science Foundation Postdoctoral Fellowship, 1963.