On the 22nd day, at which time most of the treated animals were exhibiting the signs described above, the dose of streptomycin given to them was doubled, so that the animals received 36,000 μg/day. Within a few days the treated animals began to show improvement. The convulsive attacks and paralysis disappeared rapidly, the loss of equilibrium more slowly. Except for two animals which died of intercurrent disease on the 5th and 10th days after infection, the treated guinea pigs all remained well thereafter. After the 58th day, streptomycin treatment was stopped in one-half the group (4 animals), the remainder continuing on treatment. Those animals deprived of streptomycin showed a gradual return of paralysis of the hindquarters, which became progressively worse until time of death. All 4 animals died from the 98th to the 132nd day after infection (an average of 58 days after treatment was stopped).

TABLE 1

| Guinea pig No. | Days of treatment with streptomycin | Days of life |
|---|---|---|
| 1 | None | 21 |
| 2 | " | 17 |
| 3 | " | 92 |
| 4 | " | 19 |
| 5 | " | 19 |
| 6 | " | 19 |
| 7 | " | 18 |
| 8 | " | 19 |
| 9 | " | 22 |
| 10 | " | 21 |
| 1 | 5 | 5* |
| 2 | 10 | 10* |
| 3 | 58 | 132 |
| 4 | 58 | 127 |
| 5 | 58 | 109 |
| 6 | 58 | 98 |
| 7 | 173† | Living |
| 8 | 173† | " |
| 9 | 173† | " |
| 10 | 173† | " |

* Died of intercurrent disease.

† As of September 3, still living and being treated.

The 4 guinea pigs which were kept on treatment are still alive and well 173 days after infection.

All the treated animals responded to 5 per cent Old Tuberculin when tested intracutaneously 40 days after infection. The 4 remaining pigs are still skin positive at the time of writing this report, but the intensity of the reaction is declining. A summary of the time of death in relation to days of treatment is given in Table 1.

This experiment demonstrates that streptomycin administered intramuscularly or subcutaneously can effect inoculation tuberculosis of the brain in guinea pigs and cause improvement in the peripheral manifestations of such a lesion.

Previous experience in this laboratory has shown that an inoculum of attenuated or heat-killed tubercle bacilli equivalent to the infecting dose used in this experiment is not sufficient to produce skin hypersensitivity in guinea pigs. The results set forth in this paper, therefore, indicate that multiplication of the infecting organisms must have taken place in the guinea pigs despite the presence of streptomycin, and that it was only when the mechanisms of acquired immunity of the animal came into play that the disease was held under control by the drug. Further data on this subject will be reported from this laboratory (*2*).

The intracerebral method of infection offers a rapid means of testing the *in vivo* effect of antituberculosis drugs.

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## "Acid Phosphatase" Reactions in Peripheral Nerves[1]


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The methods developed by Gomori (*4*) to reveal phosphatases in tissues have produced striking differentiations and are important additions to histologic technique. There is, for example, no simpler or more specific method for demonstrating the axis cylinders of nerve fibers than the acid phosphatase method following acetone fixation. The interpretation of the reaction, however, has presented difficulties so far as the nervous system is concerned. Heinzen (*5*) compared the acid phosphatase activity in the central and peripheral stumps of a series of transected nerves by standard biochemical methods and found increased activity in both stumps as compared with normal nerves. Histologic sections prepared by the Gomori method, on the other hand, showed a feeble reaction in the peripheral stump at a time when both stumps exhibited great activity *in vitro*. He suggested that the peripheral stump had suffered greater loss in staining capacity than the central between the time of removal of the tissue and the mounting of the sections.

Bodian (*3*) found the acid phosphatase activity in central stumps of transected nerves twice that of normal nerves and that of peripheral stumps 6 times as great. These biochemically determined differences could not be seen in sections prepared by the Gomori method. Bodian and Flexner then showed "that every step in the preparation of the histological sections for histochemical study sharply reduces the phosphatase activity as determined with the biochemical method."

We have studied the Gomori acid phosphatase reaction in peripheral nerves which had not been subjected to all the insults of histologic technique. Acetone-fixed nerves were teased in 80 per cent alcohol and transferred to the glycerophosphate-lead reagent buffered at pH 4.8 with molar acetate. After 2–18 hours at 38° C. they were washed thoroughly in distilled water and the lead visualized with ammonium sulphide.[2] Under these conditions the peripheral stump of a cat's sciatic nerve 16 days after transection appeared to the naked eye to be as intensely stained as the central stump. Under the microscope the normal fibers of the latter showed the usual precise staining of the axones; as in the normal controls, nuclei could rarely be demonstrated. Peripherally, where invasion of regenerating fibers had been prevented, all


[1] Aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of The University of Chicago.


[2] Rinsing with 1 per cent aqueous acetic acid before the sulphide did not materially alter the picture.

types of nuclei reacted strongly, as did the degenerating remains of axones and myelin. The neuroma presented the same picture with many nuclei and, in addition, had the regenerating fibers differentiated in all detail. When strands of these same nerves were extracted with distilled water either *before* or *after* the glycerophosphate-lead treatment, the nuclei were the irregularly over the larger myelinated fibers, thanks to the staining of small granules. Fig. 1 shows a Schwann cell after 1½ hours of incubation.

We have obtained this result in various normal nerves of the cat, rabbit, rat, guinea pig, macaque monkey, and man.[3] The promptness and regularity with which the Schwann cells and

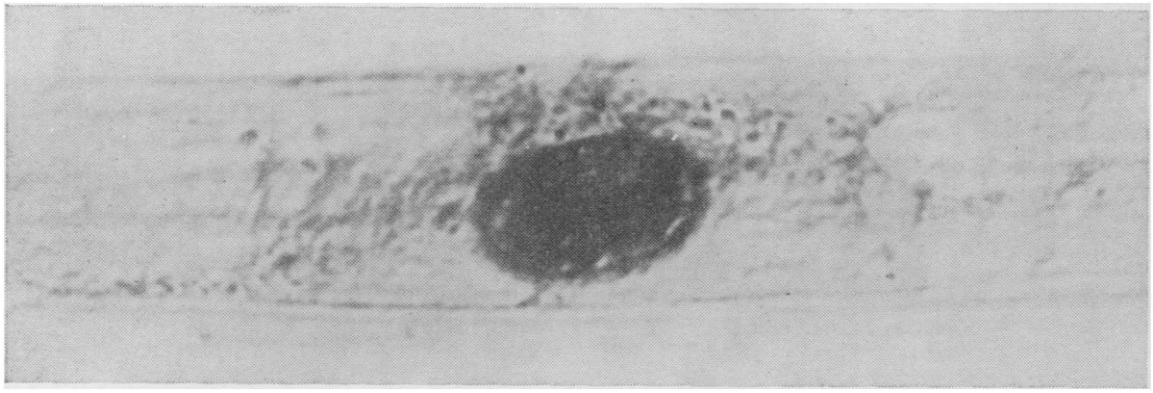

FIG. 1 Photomicrograph of cell of Schwann, viewed from above, showing stained granules in nucleus and cytoplasm. The cel appears irregularly branched. (From a bit of fresh sciatic nerve of a kitten placed directly into the glycerophosphate-lead reagent and incubated for 1½ hours. Photographed by R. D. Bensley trom a teased nerve mounted in glycerine. (× 2,000.)

first to suffer. After extractions of 16–24 hours, which left the axones stainable, the nuclei no longer reacted at all. There is thus a differential susceptibility to washing with water which appears to be greater in paraffin material cut into thin sections, such as Heinzen used, than it is in strands of teased fibers.

The water extractions provide an explanation of his discrepant results. They indicate that both enzymes and lead phosphate can readily be removed from the tissue. This would

the nuclei of other sheath cells react suggest that we are dealing with a highly specific reaction. There is no differentiation if glycerine is substituted for the glycerophosphate, if the fresh tissue is boiled for 5 minutes in distilled water,[4] or if NaF is added to the reagent in a concentration of .005 M. If the reactions in fresh tissue are indeed indications of intracellular phosphatase activity, there is no reason for concluding that the various observed granules are actual sites of enzymatic

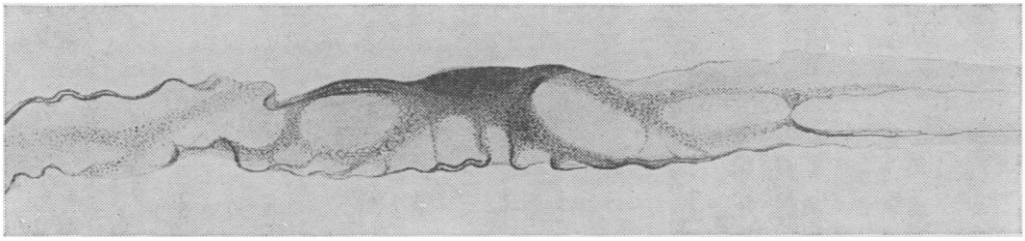

FIG. 2 Schwann cell in profile showing anastomosing ceil processes which, when viewed on edge. appear as dark lines. (From the same nerve as Fig. 1 but incubated for 23 hours Drawn by Agnes Nixon from a teased preparation in glycerine. (× 620.)

necessarily mean that both can move about freely within the tissue and that the Gomori method cannot be relied upon to furnish evidence as to the localization of acid phosphatase activity within the cell.

When acetone fixation was omitted and bits of fresh normal nerves were snipped off and placed directly into the glycerophosphate-lead reagent, the microscopic picture differed significantly in that the various types of nuclei were the dominant features. In the course of 25 minutes incubation at 38° C. an occasional nucleus appeared, and some cytoplasmic granules of the Schwann cells were sharply stained; a few nucleoli were darker than other structures. By 45 minutes practically all nuclei of sheath cells appeared filled with granules of uniform size, smaller than the nucleolus, which was darker. The cytoplasm of the Schwann cells could be followed as it spread

reactions. The reaction requires at least 25 minutes to attain microscopic visibility, and this affords ample time for the shifting of both enzymes and lead phosphate within the protoplasm. In this connection the findings of Owens and Bensley (7) on the drift and segregation of colloid particles in cells are particularly significant.

In the cat the cytoplasm of the Schwann cells develops a dark tinge in addition to the granules in the course of 18–24 hours of incubation. Cells such as those of Figs. 1 and 2 are present on every myelinated fiber midway between the nodes of Ranvier. The pictures resemble those obtained by Nemiloff

[3] We are indebted for the human material to D. B. Phemister and Alex Brunschwig.

[4] This method of destroying phosphatase activity was suggested to us by W. L. Doyle.

(*6*) in spinal root fibers of the cat stained intravitally with methylene blue. In our material it is occasionally possible to follow the cell processes to the nodes, where there is usually an accumulation of cytoplasm studded with fine granules. Various appearances like the spiny bracelets of Nageotte are sometimes differentiated. In time, the nuclei become opaque (cf. Fig. 2) through the enlargement and fusion of the granules.

Another phenomenon appears in the fresh tissue, and as promptly as the staining of the sheath cells. The ends of the axones react intensely where they were injured by pressure or by electrical changes induced by metal instruments in the fresh tissue. Similar reactions to injury occur with methylene blue in living animals (*2*). The lead sulphide staining at sites of injury always occurs after boiling fresh tissues and has nothing to do with phosphatases. In addition, the normal myelinated fibers assume a uniform yellow tint, both in fresh and boiled material. This is presumably due to a diffuse adsorption of lead. That it is not due to staining with sulphides is indicated by the fact that a similar uniform staining occurs if the lead is visualized by means of a freshly prepared unoxidized aqueous solution of hematoxylin.[5]

If dry ice is applied to a living nerve *in situ*, all axones are shriveled. When the frozen tissue is gradually thawed in chilled glycerophosphate-lead reagent and then incubated, all axones are as intensely stained as in acetone-fixed material. In addition, all nuclei react as in fresh tissue.

In 1906 R. R. Bensley (*1*) showed that the supposed histochemical test for organically bound phosphorus was nothing more than a staining reaction. The Gomori phosphatase reactions likewise involve substitutions; the substance visualized is not the substance sought. Adsorption and diffusion may play significant roles. Until it has been shown that they do not, the specificity of the reactions must remain in doubt. It may be that both boiling and fluorides do more than merely inhibit enzymatic activity.

Certain findings indicate that the histologic acid phosphatase reactions are unreliable: (1) Both enzymes and lead phosphate can be leached out of, and hence moved about within, fixed tissues. This deprives the method of value for the localization of enzymatic activity. (2) All nuclei and the Schwann cell cytoplasm react promptly when fresh nerves are placed directly into the glycerophosphate-lead reagent. This does not occur after acetone fixation. (3) The axones of fresh myelinated fibers do not react unless they have been injured. Axones shriveled by freezing or acetone react strongly except at the nodes of Ranvier. (4) The reaction of injured tissue after the destruction of enzymatic activity shows that under certain conditions the lead of the reagent can be specifically adsorbed.

We are continuing our efforts to obtain differentiations in peripheral nerves with reagents which simulate the glycerophosphate-lead reagent but which contain no phosphate.

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[5] We owe this test to R. R. Bensley.

# Polydactyly and Limb Duplication Occurring Naturally in the Tiger Salamander, *Ambystoma tigrinum*


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Few cases of polydactyly and limb duplication occurring in nature in *Ambystoma* have been reported in the literature (*8*). On the other hand, limb duplications following transplantation procedures in the laboratory are very well known from the work of Harrison and his students. Such anomalies may result from a variety of operative treatments, including the division of the limb rudiment (*11*), orthotopic and heterotopic limb transplantations (*2, 3, 5, 7, 9, 12*), and induction of supernumerary limbs by implantation of foreign tissue (*1, 4, 6*). The experimental production of limb duplications by methods other than transplantation techniques is still an unexplored field.

TABLE 1

Polydactyly and Limb Duplications in *Ambystoma tigrinum**

| Animal | Left hind limb | Right hind limb | Remarks |
|---|---|---|---|
| 2 | Normal | 8 digits | Metamorphosed |
| 3 | 6 digits | 6 " | |
| 4 | Normal | 6 " | |
| 5 | Main limb with 6 digits; *extra limb* with 12 digits | Main limb normal; small *extra limb* with 2 digits | |
| 6 | 8 digits | 8 digits | |
| 7 | Normal | 7 digits plus 1 very small bud | Questionable mirror-image symmetry |
| 8 | Main limb with 7 digits; *extra limb* with 7 digits | 8 digits | Questionable mirror-image symmetry |
| 9 | 6 digits | 6 " | |
| 10 | Normal | 8 " | |
| 11 | 6 digits | 6 " | |
| 12 | 9 " | 9 " | |
| 13 | Normal | 8 " | |
| 14 | Main limb normal; *extra limb* with 7 digits | 7 " | |
| 15 | 6 digits | 8 " | |
| 16 | 9 " | Main limb and *extra limb* fused with a total of 14 digits | |
| 17 | 9 " | 6 digits plus 1 small bud | Metamorphosed |
| 18 | 6 " plus 2 buds | 6 digits | " |
| 20 | Normal | 6 " | |
| 21 | 6 digits | Normal | |

* Animals 2 through 18 collected as larvae, 1946 (117–144 mm. in length); 20 and 21 collected as adults, 1947.

The anomalies reported here in the tiger salamander, *Ambystoma tigrinum*, represent the first recorded case of mass polydactyly occurring naturally. The abnormal animals were collected in Muskee Lake (altitude, 8,300 feet) approximately 15 miles west of Boulder, Cclorado. This and the surrounding lakes have been extensively studied in recent years, but the first polydactylous individuals were found in October 1946 (17 larvae) and in April and May 1947 (2 adults). The per-

[1] Now at the University of Illinois, Department of Zoology.