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STAINING AMPHIBIAN PERIPHERAL NERVES WITH SUDAN BLACK B: PROGRESSIVE VS REGRESSIVE METHODS.—There are few comparative studies of the peripheral nervous system, especially in anamniotes. This may be attributed in part to the difficulty of reconstructing nerve tracts from serial sections (Freihofer, 1966). However, histological techniques for studying the peripheral nerves of whole cleared specimens have been available since the last century (Sihler, 1895). Recently, Freihofer (1966), Fraser and Freihofer (1971), and Freihofer et al. (1977) presented a series of improvements on Sihler's silver method, and Filipski and Wilson (1984, 1985) described a new method for staining peripheral nerves of whole cleared specimens using Sudan Black B (color index #26150, Sigma Chemical Co.).

Although the potential usefulness of these methods for studies of comparative and developmental neurobiology is great, the field has remained technologically limited. Freihofer's method is not only time-consuming but also unreliable, partly because it is very sensitive to fixation methods. The applicability of Filipski and Wilson's method is limited by variation among species and by variation among developmental stages within species in the quality of the result (Filipski and Wilson, 1984).

Previous studies using Sudan Black B in whole specimens (Filipski and Wilson, 1984, 1985), dissected specimens (Rasmussen, 1961), or sectioned material (McManus, 1946; Stilwell, 1957) have used a regressive staining procedure in which tissues are overstained in a saturated solution of Sudan Black B in 70% ethanol, followed by destaining in 70% ethanol or 0.5% KOH. Here, I present an alternative progressive method for staining peripheral nerves with Sudan Black B in which specimens are stained for a longer time in a 5% saturated Sudan Black B solution so that no destaining step is necessary. I compare the results of the progressive and regressive methods among anuran larvae of two species (*Xenopus laevis* and *Rana pipiens*) at several stages of development. Comparison of the two methods provides insight into some properties of Sudan Black B which previously have been overlooked, as well as an explanation for the variability in staining that occurs with regressive methods.

Materials and methods.—Ten larvae each of *R. pipiens* and *X. laevis* (Gosner stages 25–42), which had been fixed, then stored in 10% neutral-buffered formalin for more than one year, were skinned and eviscerated, soaked in distilled water for 72 h to remove the formalin, and macerated for 4–7 d in trypsin buffered with 30% saturated aqueous sodium borate (Taylor, 1967). Specimens were then stained with Sudan Black B using either the regressive method of Filipski and Wilson (1984) or the progressive method described below. After staining, all specimens were cleared in a distilled water : glycerin series (2:1, 1:1, 1:2) and stored in 100% glycerin in a dark place to prevent fading. All specimens were treated in the same way, except for the staining and destaining steps.

The progressive protocol differed from the regressive method in three respects: 1) the concentration of the staining solution; 2) the length of time in the staining solution; and 3) the destaining step. The differences between protocols are summarized below:

1) After maceration, specimens were immersed for 7–10 d in a 5% saturated solution of Sudan Black B in 70% ethanol (i.e., a saturated solution diluted 19:1 with solvent) for the progressive protocol, or for 1 min in a 100% saturated solution of Sudan Black B in 70% ethanol for the regressive protocol (Filipski and Wilson, 1984, 1985).

2) After staining, the specimens were rinsed gently in distilled water and cleared in a glycerin series without destaining for the progressive protocol, or destained first in 70% ethanol for less than 1 min and then in 0.5% KOH for less than 1 min for the regressive protocol.

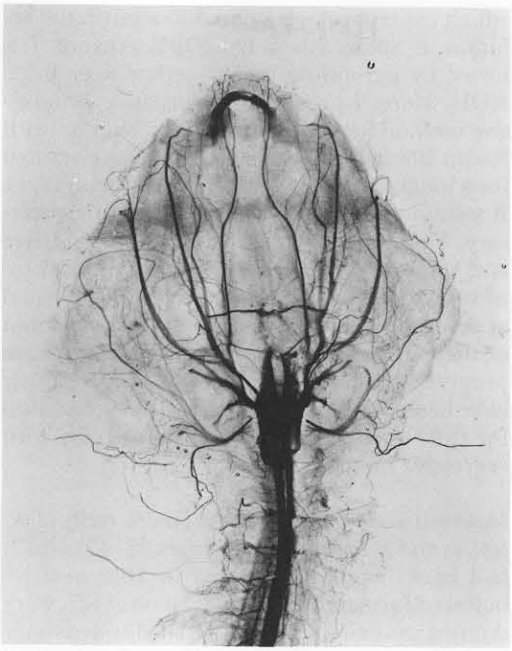

Fig. 1. Cranial nerves of a larval *Rana pipiens* (Gosner stage 35). Distal branches of each nerve are stained as darkly as proximal branches, and superficial nerve branches, such as the trigeminal innervating the nasal area, are stained as darkly as the auditory nerve inside the otic capsule. Head width = 11 mm.

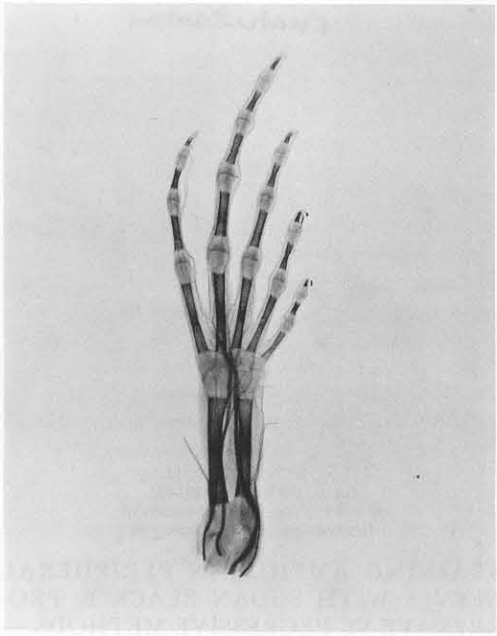

Fig. 2. Left hind foot of a larval *Rana pipiens* (Gosner stage 39). Limb bones are stained dark black, while cartilages are lightly stained. Along each digit, four small nerves can be seen, two on the left and two on the right. Length of longest digit = 6.5 mm.

Results and discussion.—In the regressive method, the entire specimen was black when the staining step was complete. Differentiation of nervous and non-nervous tissues did not occur until the destaining step. The degree of destaining varied greatly, even among the nerves of a single specimen. For all specimens stained with the regressive method, the cranial, brachial, and sciatic nerves showed the most uniform destaining. The greatest variation in rate of destaining was found among the nerves in the tail.

Complete destaining of caudal and superficial nerves occurred very rapidly (after only a few seconds in either 0.5% KOH or 70% ethanol), prior to differentiation of anterior and deeper nerves. After less than 30 sec in 70% ethanol, when the anterior nerves of the tail became differentiated, all stain had been lost from the posterior third of the tail and from the hind leg distal to the tibiofibula. Destaining occurred on contact with either 70% ethanol, 50% ethanol, or 0.5% KOH, beginning superficially and progressing to deeper tissues with increasing time in the solutions. Specimens were completely destained after less than 2 min in any of these solutions. Excessive destaining occurred even if the alcohol (50 or 70%) destaining step was omitted entirely from the protocol, and also if a mixture of 0.5% KOH and glycerin was substituted for the glycerin : distilled water series.

In contrast, in the progressive procedure, after 7–10 d in a 5% saturated solution of Sudan Black B in 70% ethanol, nervous and non-nervous tissues were well differentiated so that no destaining was necessary. Superficial and deeper nerves were simultaneously well differentiated (Fig. 1). If specimens had not been sufficiently macerated before staining, the nerves were not as well differentiated. These specimens were soaked in distilled water to remove the ethanol and then returned to the maceration solution until the non-nervous tissue cleared.

Because of the longer staining time in the progressive method, Sudan Black penetrated the specimen more thoroughly. As a result, fat-containing limb bones became darkly stained (Fig. 2). This serves as a reminder that Sudan Black is a general stain for neutral fats, including myelin (McManus, 1946). Bones and cartilages of

the skull and vertebral column, and other nonfatty tissues which are immune to trypsin digestion, such as unmyelinated nerves, cartilage, and notochord (Taylor, 1967), became lightly stained (Figs. 1, 2). Some advantages of the progressive method are that counterstaining of bone or cartilage to provide anatomical reference points may not be necessary in some cases, and that nerves of embryos and larvae that are not yet myelinated can also be studied under a dissecting microscope. A possible disadvantage is that longer time periods may be required for staining larger specimens.

At all developmental stages, the nerves of larval *R. pipiens* stained more clearly than the nerves of larval *X. laevis* when the regressive method was used. In addition, larger larvae stained more uniformly than smaller larvae regardless of species or developmental stage. Unlike the regressive method, the progressive method gave reliable results for specimens of all 28 amphibian species studied to date (including larvae of 26 anuran species from 15 families and larvae and/or adults of three salamander species from two families), over several developmental stages (Gosner 25–42).Museum specimens stored in 70% ethanol or 10% neutral buffered formalin gave equally good results.

The following variations in protocol were made:

1) The enzyme hyaluronidase, which digests all tissues except nerves, was substituted for trypsin in the maceration step. Specimens macerated with hyaluronidase were inferior to trypsin macerated specimens because important anatomical reference points were destroyed.

2) Maceration in 0.5% KOH before the staining step reduced stain intensity. The same result occurred if the specimen was treated before staining with 3% H_2O_2 to remove pigment from the skin. Hydrogen peroxide appears to damage nerves and inhibits stain uptake (Freihofer et al., 1977), KOH destroys the myelin sheaths (Freihofer, 1966), and both agents promote disarticulation of specimens (Fraser and Freihofer, 1971).

3) Immersion of larval anuran specimens in 70% ethanol, 50% ethanol, or 0.5% KOH after staining caused destaining of superficial nerves on contact, and complete destaining of small specimens in less than 2 min. KOH should be omitted from all stages of the protocol, including the glycerin series. Accidental destaining by immersion in KOH or ethanol could often be reversed by repeating the progressive staining step before placing the specimens in glycerin.

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