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Influence of neural extracts on myoblast development

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Although skeletal muscle type myoblasts can undergo growth and differentiation to striated contractile muscle in the absence of neural influences, the continued existence and further differentiation of muscle is dependent upon innervation, indicating neuronal regulation of these processes. Also, the repair of damaged muscle is improved in the presence of nerve. As a part of our program to study these trophic influences, we are investigating how early in the development of muscle sensitivity to trophic influences appears.

Determinations of the rates of increase in DNA, RNA, and protein in primary cultures of rat myoblasts or in cultures of a pure strain of myoblasts (L_6) reveal that extracts of brain or of sciatic nerve increase the amount of protein and DNA synthesis. The active factor has a high molecular weight.

The second messengers, cyclic AMP and GMP, which are synthesized in response to sympathetic amines and acetylocholine, respectively, cause increases in protein synthesis.

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Histological techniques for cranial bone studies

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The preparation of bone specimens for histologic study has been considered to be difficult because of the variation in the tissue reaction to the fixative and the decalcification solutions. The ideal fixative for bony structures is subject to widely divergent opinions and limited to specific bones. In the course of this study, the following fixative solutions were tested: Heidenhain's Susa, Zenker's, Bouin's, Gilson's, Stieve's "Sublimatgemisch," Stieve's "Tripiform" or "Pikrinsaurgemisch," and buffered formalin following the Lillie formula.

Preliminary findings on 16 specimens indicated that the most satisfactory fixation was achieved by a solution containing trichloracetic acid. These include: Stieve's Tripiform, Heidenhain's Susa, and Stieve's Sublimatgemisch. Gilson's solution was found to dissolve the small cranial bones before fixing them to a stage for satisfactory cytologic study. Any solution containing acids and mercuric chloride had an action of destroying the skull lamellae. The other named solutions produced either

poor fixation or made the decalcified bone difficult to section.

Decalcification of all test specimens was carried out with 5% formic acid after fixation and storage in 70% ethyl alcohol. The tissues in the decalcifying solution were placed in a low vacuum oven maintained at 56 C. The solution was changed at 24-hour intervals and tested for the presence of calcium by means of 1 ml. of 5% ammonium oxalate in 5 ml. of the used decalcification solution. When the tested solution no longer caused a white precipitate to appear, the specimens were washed overnight in running tap water and double-embedded according to the Peterfi method.

The embedded tissues were sectioned on a rotary microtome with a knife tilt from 45 to 50 degrees. Three hundred and fifty slides have been prepared and representative sections have been stained with hematoxylin and eosin (both Harris and Meyers and Phloxine B-Eosin), Verhoeff's elastin stain, Masson's trichrome, and others.

Because of the complicated and variable morphology in different areas, the Masson trichrome was found to be the best initial stain. The prevalence of connective tissues and reticulin has initiated studies with variations of the van Gieson and Wilder's reticulin stains. The vascularization of the suture areas and the numbers of mitotic figures seen in many preparations also indicate further work in preparations demonstrating glycogen storage and nuclear detail. There have been no difficulties with staining preparations because of fixation or decalcification methods.

Suture areas from the basal occipital (sphenoid), frontal parietal, parietal occipital, parietal temporal, and sphenoid ethmoid portions of the squirrel monkey's crania have also been prepared for separate histologic comparison. For uniformity, some 39 blocks of tissue fixed in 10% formalin have also been decalcified in formic acid and double-embedded for sectioning. Approximately 800 slides have been prepared with serial numbered sections in each case. Alternate slides have been stained with the detailed Masson technique, leaving the other slides for stain experimentation.

Stain technique

1. Deparaffinize slides through three absolute alcohols.
2. Place in 0.25% celloidin in ether-alcohol for 10 minutes.
3. Air-dry slides by swinging in air a few seconds.
4. Harden in 80% alcohol for 10 minutes.
5. Hydrate through 70% alcohol to distilled water. (This will prevent albuminized slides from dropping sections in alkaline solutions.)
6. Place sections in Bouin's solution for 24 to 58 hours.
7. Wash in running tap water until all yellow color leaves sections.
8. Proceed with standard Masson technique using Regaud's hematoxylin, Biebrich scarlet-acid fuchsin, phosphotungstic-phosphomolybdic acid, and aniline blue.
9. Dehydrate and clear in two changes each of 95%, absolute alcohol, and xylene.
10. Cover, using synthetic mounting medium.

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The structures of cranial bone sutures

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There are few studies on cranial bone sutures which have utilized modern histologic techniques. One of the most informative studies on the structure and the development of mammalian cranial sutures was done by Pritchard, Scott, and Girgis in 1956.[1] Their primary concern was the development of the suture, so there was limited discussion of the adult structure. However, this report provided us with a starting point for our investigation.

Much of the initial work on this project has been the development of suitable histologic methods. This is reported in a paper by Popevec, Biggert, and Retzlaff.

Previously reported studies on cranial bone mobility[2-4] (Eighteenth Annual National Osteopathic Research Conference, March 15-16, 1974) were based on physiological studies using the squirrel monkey, Saimiri sciureus. Histologic studies are being performed on the same animal.

It is of particular interest to note that in the 10 adult monkeys from which the bone tissue was removed there was no evidence of suture ossification.

The general pattern of the suture was similar to that reported by Pritchard et al.[1] In each sample studied the sutures displayed five distinct layers of cells and fibers between the articulating edges of the bones. The outermost layer is a zone of connective tissue which bridges the

suture and is designated the sutural ligament. The next layer consists of osteogenic cells. These two layers appear to be continuous with that of the periosteum of the skull bones. This modified periosteal layer, the sutural ligament, is found on both the outer and the inner surfaces of the suture. The space between the ligaments is loosely filled with fibrous connective tissue.

The reticular connective tissue portion is seen in the space with extensions into the sutural ligament. This may provide an inner and outer binding structure which serves to hold the sutures but still permits some movement of the skull bones.

In addition to the connective tissue seen in the central space, blood vessels and nerve fibers are evident. The function of these nerve fibers is not known but it is possible that they may be involved in the physiological effects of cranial therapy.

The question whether suture obliteration, by ossification, ever occurs in man cannot be answered by this study. We do know that the sutures between the parietal bone and adjacent bones in the adult squirrel monkey show no evidence of closure in the specimens we have studied.

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The role of innervation on the development of involuntary muscle

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The study of the trophic influences that motor nerve fibers exert on skeletal muscle cells has been a field of considerable interest to neurobiologists. More recently, this interest has been extended to the interactions between sympathetic nerves and their effector organs, including smooth muscle. Previous work in our laboratory has shown that, following removal of the presumptive nervous system in amphibian embryos, *skeletal* muscle fails to differentiate or be maintained.

Recent ultrastructural work, however, indicates that under similar conditions of nervelessness, both cardiac and smooth muscle can differentiate fully and persist in the differentiated state. This differentiation includes not only the morphological maturation of the individual muscle fibers, but also the formation of the specialized and characteristic intercellular contacts between adjacent fibers, i.e., sites of close membrane apposition. Thus, as evaluated ultrastructurally, involuntary muscle shows considerably less trophic dependence on its innervation than voluntary muscle.

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Design and testing of an instrument for microcirculatory flow evaluation

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There presently exists no method which is clinically practical for the rapid determination of blood perfusion rate at a preselected point in tissue. We sought to develop a method which could be applied rapidly to determine relative blood perfusion rates in superficial tissues. We were primarily interested in a technique which would produce minimal trauma and which thus might be applied to human subjects in a clinical examination room setting without elaborate or expensive equipment.

The principle we selected upon which to base the design of a blood flow probe was that of thermal dilution—in essence, a variation upon the principle of the hot-wire anemometer. In this kind of instrument a temperature sensor exposed to the flowing medium is heated by some external energy source and the equilibrium temperature, which is a function of the flow rate, is measured. In our design, a thermistor operating in the self-heating mode served as a flow rate transducer. A constant current circuit maintained a current of 1.0 milliampere through the thermistor and the variation of voltage across the thermistor was proportional to changes in rate of fluid flow in the vicinity of the thermistor bead. The bead was inserted into the tissues of test animals via a small gauge needle. Response time of the thermistor probe assembly to temperature changes was a fraction of one second, so that rapid microvascular changes could be recorded.

This instrumentation method was tested by applying vasoactive drugs to hamsters and rats while recording from inserted thermistors. Both normal animals and hamsters of a strain carrying hereditary muscular dystrophy were used, the latter as a preliminary test of the hypothesis that some forms of muscular dystrophy may involve impaired microvascular control mechanisms. It was demonstrated that the thermistor probes responded sensitively to drug-induced vasoconstriction. These studies also give a preliminary indication of possible vasomotor differences between normal and dystrophic hamsters.