Correlative histochemical study of skeletal muscle after suprasegmental denervation, peripheral nerve section, and skeletal fixation

George Karpati, M.D., and W. King Engel, M.D.

Mammalian skeletal muscle has been studied with histochemical techniques after various experimental procedures such as nerve section, neuronal regeneration, "foreign" and cross innervation, and tenotomy. The effects of these methods upon the muscle cell were produced by direct involvement of the lower motor neuron innervation or the length of the muscle or both.

In the present study, acute complete transection of the high thoracic spinal cord was performed to determine the indirect effects on the gastrocnemius and soleus muscles of adult and newborn guinea pigs. Histochemical and cytochemical changes in the muscles after cordotomy were compared with the effects of peripheral nerve section and skeletal fixation to elaborate on various aspects of the complex nerve-muscle interrelationship.

METHODS

Cordotomy. Nineteen adult (age three months) and 4 newborn female guinea pigs of the Hartley and NIH strains were anesthetized with intraperitoneal sodium pentobarbital, 25 mg. per kilogram (adults), or open mask ethyl ether inhalation (newborns). With the animal prone, an 8- to 10-mm. segment of thoracic spinal cord was exposed by removing the arch of the sixth or seventh thoracic vertebra and incising the dura mater. With fine dissecting scissors, the exposed cord was severed transversely at the T-6 or T-7 level. Careful hemostasis was obtained by packing absorbable gelatin sponge (Celfoam®) between the ends of the severed cord. The dura mater was not closed but the paraspinal muscles were sutured over the laminectomy site. In two adult and two newborn animals, subsequent to the cordotomy and during the same operation, a left sciatic neu resection was performed in our routine manner.

The cordotomized adult animals were sacrificed by an overdose of barbiturate at the following intervals after the procedure: three days (2), seven days (2), ten days (2), twenty days (2), thirty days (2), sixty days (2), ninety days (2), and one hundred and twenty days (1). The four adult cordotomy-sciatic neu rection animals were sacrificed at thirty days. Three newborn animals were sacrificed at forty-two days and 1 at seventy days postoperatively.

Controls consisted of completely normal animals matched for sex and age with the experimental animals and two additional guinea pigs which were subjected to a T-6 laminectomy without cordotomy one month prior to sacrifice. The operated animals were kept in cardboard boxes filled copiously with sawdust. Self-cannibalism of the feet of the hind extremities was prevented by wrapping them with padded...
tape. In the first week postoperatively, evacuation of the bladder was attained several times daily by gentle suprapubic massage. After the first week spontaneous evacuation reflexes developed.

**Skeletal fixation by "pinning."** In four adult and four newborn guinea pigs of the NIH strain under intraperitoneal sodium pentobarbital anesthesia (adults) or open mask ethyl ether inhalation (newborns), the right ankle joint was fixed with a stainless steel wire (used as the holding frame for Michael clips) or an ordinary 20-bore hypodermic needle in the following manner: The needle or wire was introduced to the medial malleolus and advanced with gentle rotation directed sharply down and posteriorly until it emerged at the lateral border of the calcaneus. The needle or wire thus transfixed the talus and calcaneus within the ankle joint and as a result the foot became firmly fixed in the neutral (90°) position. The animals were checked daily for possible dislodgment of the fixing agent and if undue mobility of the foot was found the needle or wire was replaced until firm fixation was attained. The adult animals were sacrificed with an overdose of barbiturate thirty days after the procedure and the newborn ones forty-two days postoperatively.

The medial (large) and lateral (small) heads of gastrocnemius and the soleus muscles were removed from the cordotomized and skeletal fixation animals at the time of sacrifice. A cylindrical 1-cm. long segment from the midportion of each muscle was mounted on chucks for transverse section in such a manner that on every chuck was placed the same kind of muscle from each side of the animal. Fresh frozen cryostat sections, 10 μ thick, were prepared in the routine manner and the specimens were stained with a battery of histochemical reactions detailed elsewhere.

From the cordotomized animals, the lumbar enlargement of the spinal cord was removed and mounted unfixed for transverse sectioning. From the cordotomized animals at least one sciatic nerve was removed, placed on cardboard, and mounted unfixed for longitudinal and transverse sections. The preparation of cryostat sections of 10-μ thickness was similar to the method described for the muscles. On the muscle and cord sections the following techniques were applied: hematoxylin-eosin, modified trichrome, DPNH tetrazolium reductase, menadione mediated alpha-glycerophosphate dehydrogenase, nonspecific esterase, modified periodic acid–Schiff for myelin, toluidine blue, cresyl violet, oil red O, and on a few specimens the Glees-Marsland silver method with luxol fast blue counterstain. Sections of peripheral nerves were stained with the modified trichrome method, DPNH-tetrazolium reductase, and in a few cases the Glees-Marsland silver method.

Muscle fiber diameters were determined from photographic prints (100 × magnification) of sections stained with the myofibrillar ATPase reaction. The smallest diameter of 100 fibers in the randomly selected field of the sections was measured and the results were graphed. Our preferred method of determining the histochemical type of a muscle fiber is the myofibrillar ATPase reaction and in this study the designation of a fiber type implies it was displayed with that reaction. The histochemical profile of the two basic fiber types has been detailed elsewhere.

**RESULTS**

**Clinical signs. Cordotomy.** On awakening from the anesthesia complete flaccid paralysis of the hindlegs was present. On locomotion, the paralyzed hindlegs were dragged in extension and "spontaneous" reflex movements remained scanty. An apparently dense analgesia to pinprick was demonstrable up to a level just below the cordotomy. Bladder paralysis during the first week postoperatively necessitated assistance in evacuation by suprapubic pressure. There was no arrest of spontaneous bowel evacuation. Three to four weeks after cordotomy, progressive spasticity of the hind extremities started to develop, with increasing contractures. As a result, by sixty days, the hind feet became firmly fixed in extreme plantar flexion which was not abolished under anesthesia before sacrifice. No definite clonus or exaggerated tendon stretch reflexes were elicitable. Plantar stimulation did not evoke movement of the toes.

Most adult cordotomized animals lost 10 to 20% of their body weight within the first two weeks postoperatively. Although the animals
HISTOCHEMICAL STUDY OF SKELETAL MUSCLE

SUMMARY OF THE PRINCIPAL HISTOCHEMICAL FINDINGS ONE MONTH AFTER THE THREE EXPERIMENTAL PROCEDURES

<table>
<thead>
<tr>
<th>Experimental model</th>
<th>Degree of atrophy</th>
<th>Estimated percent of Cytoarchitectural changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>(One month)</td>
<td>Gastrocnemius Type I</td>
<td>Type II</td>
</tr>
<tr>
<td>Normal</td>
<td>Adult 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Newborn 0 0 55-65 0</td>
<td></td>
</tr>
<tr>
<td>Cordotomy</td>
<td>Adult ++ ++ 35 +++</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Newborn + + 65 +</td>
<td></td>
</tr>
<tr>
<td>Skeletal fixation</td>
<td>Adult + + 3-5 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Newborn + + 35 0</td>
<td></td>
</tr>
<tr>
<td>Denervation</td>
<td>Adult + +++ 0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Newborn + +++ 35 0</td>
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</tbody>
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*Degree of atrophy and architectural changes are estimated arbitrarily. For more accurate assessment refer to Figure 1 and Figures 3, 4, and 7, respectively.

appeared to be lethargic for some days after the procedure, the degree of weight loss was greater than expected from their relatively normal dietary intake. Subsequently, a gradual regain of weight occurred so that when the more chronic animals were sacrificed their weight loss was not more than 5% of the preoperative weight.

The cordotomized newborn animals showed a relatively normal rate of growth above the level of the cordotomy and postoperative lethargy was not observed. The growth of the body and extremities below the cordotomy was symmetrically retarded. Atrophy of the muscles of the hind extremities rapidly occurred after cordotomy in both adult and newborn animals. By four weeks, the muscle mass was reduced to about one-fourth of the normal state. We have not observed a gradual regain of the lost muscle mass in later stages after cordotomy as reported in rats.20

Clinical signs. Skeletal fixation. The ankle joint around the pin or wire appeared enlarged in some animals due to accumulation of fibrous tissue. The joint itself became firmly fixed in the neutral position and the usual flexor or extensor reflex movements of the foot were not possible. Marked atrophy of the gastrocnemius and soleus muscles was evident at the time of sacrifice (four weeks). On the side of skeletal fixation, the muscle mass was reduced to about one-half that of the contralateral unoperated side.

HISTOCHEMICAL FINDINGS

A summary of the principal findings is shown in the table.

Adult cordotomy. Gastrocnemius. Progressive decrease of diameters of both histochemical fiber types to about an equal degree was observed. In Figure 1, the atrophy of extrafusal muscle fibers is shown after cordotomy,

![Fig. 1. All sections show the adult gastrocnemius muscle stained with the myofibrillar ATPase reaction, 140 X. Under the photomicrographs the histograms of the corresponding muscle are shown, including the percent of each fiber type. [A] Normal. [B] One month after sciatic nerve resection. [C] One month after cordotomy. [D] One month after skeletal fixation.](image_url)
sciatic nerve section, and skeletal fixation. While after cordotomy and skeletal fixation a "mixed" atrophy is present, denervation caused severe preferential atrophy of the histochemical type II fibers. In later stages of cordotomy (at two and especially at three months) in all but one animal, a marked predominance of the type II fibers developed. It is not certain whether this change was due to a fallout of the type I fibers or many type I fibers changed to type II. The type II predominance was not considered due to sampling error because the entire cross section of the muscles was examined. The atrophy of muscle fibers did not progress significantly after six weeks post-cordotomy, and in some animals at three and four months the average fiber diameter was even greater than at one month. The cytoarchitectural changes of the muscle fibers were not prominent apart from a few fibers with increased central nuclei and some rounded hypertrophic fibers with snake coils (see below).

The relative activity of the studied enzymes in the muscle fibers was preserved to a large extent and the fiber types were readily discernible even four months after cordotomy. In contradistinction, the profound loss of the activity of most oxidative enzymes and amyllophosphorylase in early stages of denervation was quite striking (Fig. 2).2

Soleus. Marked architectural changes of the extrafusal muscle fibers were evident as early as seven days after cordotomy. These abnormalities consisted of fiber necrosis, progressive increase of the endomysial connective and adipose tissue, rounded fibers of larger diameter with or without snake coils, ringed fibers, increased number of central nuclei, vacuolization, and targetoid or central core-like fibers (Fig. 3). In some solei of animals sacrificed one month after cordotomy, the majority of the fibers contained snake coils or rings (Fig. 4) and in one animal sacrificed at ten days, the majority of the fibers contained central unstained regions resembling central core or targetoid fibers (Fig. 3C). No classic target fibers with three concentric zones were seen. It appeared that cytoarchitectural changes were less prominent in later stages of cordotomy than earlier. In areas of the muscle where the foregoing changes were not prominent, the normal histochemically uniform profile of fiber types in the soleus was altered such that a mosaic of both type II and type I fibers developed. This was observed to be present as early as twenty days after cordotomy. In contradistinction, one month after sciatic denervation the soleus still showed the normal uniform histochemical profile (that is, only type I fibers), and cytoarchitectural changes were virtually absent. Figure 5 shows the histochemical fiber type profile of the soleus one month after cordotomy, denervation, and skeletal fixation.

Infant cordotomy. Only the soleus muscles were closely examined. At time of sacrifice at age six weeks, all solei were still histochemically mixed (as they are in the normal newborn guinea pig) and contained in some cases even a greater percentage of type II fibers than the soleus of the normal newborn animal. The soleus of the neonatally denervated guinea pig also remained markedly mixed at six weeks but contained a lower percentage of and smaller diameter type II fibers than after cor-
Fig. 3. Soleus, 100 x. [A] Ten days after cordotomy, scattered necrotic fibers, myofibrillar ATPase. [B] One month after cordotomy, larger rounded fibers, some with snake coils and central nuclei, modified trichrome. [C] Three months after cordotomy, marked fatty replacement and vacuolated fibers, myofibrillar ATPase. [D] Fourteen days after cordotomy, every fiber in the field harbors targetoid or central core-like lesions, myofibrillar ATPase.

Fig. 4. Soleus, three months after cordotomy, "hypertrophic" fibers with snake coils and rings, 300 x. [A] Myofibrillar ATPase. [B] DPNH-tetrazolium reductase. [C] Amylophosphorylase.
adult soleus, myofibrillar ATPase reaction, 140 ×. Under the photomicrographs, the histograms of the corresponding muscle are shown, including the percent of each fiber type. [A] Normal. [B] One month after sciatic nerve resection. [C] One month after cordotomy. [D] One month after skeletal fixation.

Fig. 5. Adult soleus, myofibrillar ATPase reaction, 140 ×. Under the photomicrographs, the histograms of the corresponding muscle are shown, including the percent of each fiber type. [A] Normal. [B] One month after sciatic nerve resection. [C] One month after cordotomy. [D] One month after skeletal fixation.

dotomy (Fig. 6). The most prominent cytochemical abnormality seen after neonatal cordotomy was the presence of dark staining centers of many muscle fibers with most oxidative enzyme reactions, including succinic dehydrogenase (Fig. 7). As these collections show a positive reaction with succinic dehydrogenase reaction they cannot be regarded as mitochondrial aggregates; they were not very dissimilar from sarcoplasmic masses, though they were not as sharply distinct from the rest of the fiber as are sarcoplasmic masses.

Spinal cords and peripheral nerves of cordotomized animals. The anterior, lateral, and dorsal white columns revealed no demyelination (Fig. 8). This seemingly paradoxical finding is considered in the discussion. The large and small motor neurons of the anterior gray horn of the lumbosacral spinal cord revealed no pathologic alterations with the methods used (Fig. 9). There was no demyelination evident with the PAS reaction on unixed tissue, and there was no gliosis seen with the oxidative enzyme reactions. In the sciatic nerves, no abnormalities were noted.

Adult skeletal fixation. Gastrocnemius. Atrophy of both histochemical fiber types developed but to a lesser extent than at an identical time following cordotomy (Fig. 1). On the contralateral "normal" side it was estimated that the diameter of many muscle fibers was greater than normal. Cytoarchitectural changes such as snake coils and coarsened intermyofibrillar network pattern with the oxidative enzyme reactions were few and were also present on the contralateral side. The relative activity of the studied dehydrogenases and amylophosphorylase was diminished only slightly in both fiber types.

Soleus. At the time of sacrifice, one month after operation, a number of type II fibers appeared in the solei on the operated side in all animals (Fig. 5), the percentage of type II fibers varying between an estimated 3 to 5%. Architectural abnormalities of muscle fibers were similar in nature and extent to those described in the gastrocnemius.

DISCUSSION

Comments on the experimental models employed in this study and related experiments in the literature. In the present cordotomy experiments, at least three factors acting upon the studied muscles should be considered: [1]
Fig. 6. Entire cross section of soleus muscles, myofibrillar ATPase, 22 x. [A] Normal newborn. [B] Six weeks after neonatal sciatic nerve resection. [C] Six weeks after neonatal cordotomy. [D] Six weeks after neonatal skeletal fixation. [E] Normal six weeks old, printed slightly darker than the others because it contains only light fibers, 15.5 x.
not been entirely satisfactory.\footnote{32,33} Even after "isolating" a segment of the lumbosacral cord by the method of Tower,\footnote{23} fasciculations develop in the hindleg within two weeks.\footnote{24} Skeletal fixation by pins or wires is usually effective in preventing shortening of muscle fibers during contraction, but the possibility of developing isometric tension is not eliminated. In addition, the possibility of direct damage to the investigated muscle by the fixing agent exists. Application of plaster cast is the least effective for production of muscle inactivity.\footnote{25} It should be pointed out that absolute inactivity of the extrafusal muscle fibers is not even achieved after peripheral nerve

Fig. 7. Soleus, six weeks after neonatal cordotomy. [A] DPNH-tetrazolium reductase, the majority of the fibers contain "dark centers," 110 ×. [B] Modified trichrome, note that the "dark centers" (single arrows) are unassociated with central nuclei, which are subsarcolemmal (double arrows), 900 ×.

complete lack of volitional activity, [2] a certain amount of abnormal segmental reflex activity, and [3] spasticity and permanent shortening of the muscle fibers due to contracture. Whether the net effect of these factors is an overall decrease of the muscle fiber activity or not cannot be determined without physiologic studies. The effect of skeletal fixation on muscles is different from that of cordotomy in at least three major respects: [1] The possibility of direct transneuronal effect of the upper motor neuron upon the lower one remains, whereas it is abolished after cordotomy. [2] There is no overt evidence of pathological segmental reflexes. [3] Significant shortening of the muscle fibers is obviated.

Animal models designed to study the effects of complete inactivity upon skeletal muscle without disturbing its lower motor neuron have
Fig. 9. Motor neurons of the anterior gray horn of the spinal cord at about the L-4 level are of normal appearance, 140 X. [A] DPNH-tetrazolium reductase. [B] Modified trichrome.

section, for soon after denervation spontaneous fibrillations of muscle fibers develop.26

Interpretation of the experimental results in the adult gastrocnemius. Our studies revealed that, in the histochemically mixed gastrocnemius muscles of adult animals, a greater atrophy sets in after cordotomy than after skeletal fixation, but in each instance both histochemical fiber types atrophy at about the same rate. The lesser degree of atrophy after skeletal fixation may be due to any of the factors cited above. The permanent shortening of the muscle fibers occurring in chronic cordotomized animals is probably a major factor, based on previous studies showing that “inactivation” of the muscle in a shortened state causes greater total weight and protein loss than at normal length.27,28 In contradistinction to the “mixed” atrophy after cordotomy or skeletal fixation, denervation causes a preferential atrophy of the type II fibers. This suggests that the type II fibers are more dependent on long-term neuronal regulatory influences than the type I fibers.10,20 It was also remarkable that the activity of the studied oxidative enzymes and amylrophosphorylase did not appreciably decrease in the muscle fibers after cordotomy or skeletal fixation, whereas early loss of these enzymes in denervation is characteristic.2

Interpretation of the experimental results in the newborn soleus. A comparison of the behavior of soleus after cordotomy and skeletal fixation is of special interest. In a previous study it was observed that the soleus of the newborn guinea pig is a markedly mixed muscle, containing about 55 to 65% type II fibers, and by six weeks of age it becomes completely uniform, containing only type I fibers.8 It was also shown that neonatal denervation prevents the soleus from becoming uniform, while neonatal tenotomy of the soleus does not. In another study it was shown that, six months after sciatic nerve section in the adult guinea pig, the soleus became “mixed,” which was interpreted as a reversal to the infantile histochemical profile.10

After neonatal cordotomy, the soleus at six weeks of age remained histochemically “mixed,” and even a larger percentage of type II fibers was present than in the normal newborn or six weeks after neonatal denervation. Neonatal skeletal fixation had a similar effect, but at six weeks of age the percentage of type II fibers remaining in the soleus was much less. It has been postulated that the changing histochemical profile of the developing soleus muscle of the guinea pig is a function of the influence of the maturing motor nerve on that muscle.8 If this were the case, one must surmise that both cordotomy and skeletal fixation interfere with this neuronal function. Cordotomy could interfere with the postulated “maturation” factor of the developing nerve by either [1] directly preventing descending cerebrospinal influences on the lower motor neurons50 or [2] the reduced muscular activity which could in turn alter the activity and metabolism of the lower motor neuron resulting in decrease or change of its “muscle maturation factor.” Skeletal fixation could have influenced the nerve only by the latter mecha-
nism. Such a “reverse” trophic effect of muscle fibers upon the lower motor neuron has been postulated. The normal morphological appearance of the spinal motor neurons after cordotomy would not absolutely exclude a functional defect of them, and the possibility that the upper motor neuron is a major stimulator of the maturational influence in the lower motor neuron cannot be disregarded. In fact, we think it a very likely possibility.

Interpretation of the histochemical fiber type changes in the adult soleus. In the soleus of the adult guinea pig there appeared many type II muscle fibers twenty to thirty days after cordotomy, whereas after sectioning the sciatic nerve that muscle remained histochemically uniform at one month and only became mixed after three to six months. Skeletal fixation in the adult guinea pig resulted in the appearance of only about 5% type II fibers in the soleus. These results suggest that the appearance of type II fibers in the soleus of adult guinea pigs could not be related solely to “reduced” skeletal muscle activity, since nerve section is believed to produce the greatest degree of reduced activity yet resulted in the slowest appearance of type II fibers. It may be suggested that normally in the adult guinea pig soleus, the influence of the upper motor neurons upon the lower motor neuron-muscle complex determines the uniform histochemical profile (all type I fibers) of that muscle and thereby “prevent” it from becoming “mixed.” Thus, when the influence of the upper motor neurons is eliminated (that is, after cordotomy) the “uniform” histochemical profile of the soleus is rapidly lost and it becomes “mixed.” By contrast, when the lower motor neurons are interrupted (that is, after denervation) the soleus muscle is deprived of both the upper and lower motor neuron influence and the conversion to a “mixed” pattern is delayed, suggesting that the denervated soleus is less susceptible to the lack of the suprasegmental influences acting through its motor nerve than if that motor nerve is intact. Also to be considered is the possibility that certain characteristics (for example, frequency, pattern, and so forth) of the efferent neuronal impulses established after cordotomy and skeletal fixation, and the fibrillatory activity after denervation, may be major factors in producing the histochemical changes.

Comments on the cytoarchitectural changes in the soleus after cordotomy and on cord pathology. Greater cytoarchitectural changes occurred in the soleus than the gastrocnemius muscles after cordotomy. In a study of tenotomy in cats similar more severe involvement of the soleus muscle fibers was shown. The cause for the greater susceptibility of the soleus (which is normally all type I fibers in the adult guinea pig) to these changes is not obvious. The fact that the type I fibers in the gastrocnemius did not show these changes in either cordotomy or tenotomy suggests that histochemically similar type I fibers in the two muscles react differently to the same insult or that the insult affected the gastrocnemius and soleus in subtly different ways.

Some of the lesions observed in the guinea pig soleus after cordotomy were identical with lesions usually labeled as “myopathic.” The fact that such myopathic-like changes could be present after a lesion of the central nervous system would emphasize the need for caution in interpreting “myopathic” changes in skeletal muscle pathology. It is uncertain whether such lesions occur in human beings after upper motor neuron involvement, since only muscles other than soleus have been studied in human beings with corticospinal tract involvement. The absence of demyelinization and fiber destruction in the descending tracts of the spinal cord after cordotomy is of special interest. In a study of the pyramidal tract of the guinea pig, Reveley found that at the fourth lumbar segment only a tiny crescentic area of the posterolateral part of the dorsal column showed degeneration with the Marchi method after motor cortex ablation. Although this method has been criticized for the accurate mapping of neuronal pathways after injury, it is reasonable to assume that long descending tracts of the spinal cord in many small rodents terminate in the upper portions of the spinal cord and their effect is transmitted by propriospinal interneurons to the lower spinal levels. Such anatomical arrangement has not been precisely delineated but could be responsible for the finding of the present study.
SUMMARY

Changes which occurred in the gastrocnemius and soleus muscles of adult and newborn guinea pigs after high thoracic cordotomy were studied with histochemical techniques and compared to the effects of peripheral nerve section and skeletal fixation. In the adult gastrocnemius, moderate atrophy of both histochemical fiber types developed after cordotomy and to a lesser extent after skeletal fixation. In contrast, denervation resulted in preferential atrophy of the type II fibers.

The normal newborn guinea pig soleus is histochemically “mixed,” containing about an even number of type I and II fibers, and by six weeks of age it becomes “uniform,” containing only type I fibers. Neonatal cordotomy, sciatic nerve section, and skeletal fixation impaired this normal histochemical change such that by six weeks of age the soleus remained “mixed.” In the adult soleus, thirty days after cordotomy, about 50% type II fibers appeared and, after skeletal fixation, only about 5%. Sciatic nerve section in the adult guinea pig resulted in the appearance of type II fibers in the soleus only after 3 to 6 months. In the soleus muscle of adult animals, cordotomy was followed by “myopathic-like” changes (necrosis, increased endomyosial connective and adipose tissue, vacuoles, ringed fibers, and “snake coils”). These findings indicate the caution necessary in interpreting such changes as specifically “myopathic.”

The pathogenic factors possibly playing a role in the studied experimental models were discussed.

REFERENCES


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