BBA 403

EFFECTS OF ACETYLCHOLINE ON AXONAL CONDUCTION OF LOBSTER NERVE

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SUMMARY

Acetylcholine and related compounds have been tested for their effects on the electrical activity of lobster walking-leg nerve bundles. Changes in action and membrane potential were recorded with the "sucrose-gap" technique. ACh in concentrations of $5 \cdot 10^{-3}$–10^{-2} M decreases the membrane potential; the simultaneously recorded action potentials initially display an increased amplitude and a marked prolongation and elevation of the descending phase which appears oscillatory. As the depolarization progresses, the spike height decreases and the effects on the descending phase decline. Finally reversible block of conduction occurs. *d,l*-Acetyl-β-methylcholine (10^{-2} M) produces similar effects on the action potential but the membrane potential is only slightly depolarized. Choline, acetate and bromide in concentrations equal to those of ACh have no effect; in 10-fold higher concentrations only choline produces minor changes in the action potential.

Physostigmine (5 mM) produces effects similar to those seen with ACh while lower concentrations inhibit the action of ACh. Atropine and tetracaine reversibly block conduction and, like physostigmine, also inhibit the effect of ACh. Although curare was never demonstrated to block conduction, it does inhibit the action of ACh. Irreversible cholinesterase inhibitors (phospholine, Paraoxon) block conduction, Paraoxon much faster than phospholine. The membrane potential is decreased by 20–30 mV, and while the effects of these inhibitors on the membrane potential are reversible, conduction remains blocked. The difficulties of interpreting some of these actions on a nerve bundle are discussed.

INTRODUCTION

While it has been widely accepted that rapid and reversible conductance changes take place in the conducting membrane during nerve activity[1], the underlying molecular mechanism still offers many problems. NACHMANSOHN's theory suggests that the ACh system is essential for the control of ion movements. On the basis of a large amount of experimental data the role of the system in the elementary process is pictured in the following way: if a stimulus reaches the membrane, ACh is released

Abbreviation: ACh, acetylcholine

from a storage protein and reacts with an ACh receptor protein located in the membrane. This reaction produces the change of conductance observed with nerve activity, possibly by the removal of charged sites that impede ion movements. The free ACh is subsequently hydrolyzed by ACh-esterase. This second reaction permits the membrane conductance to return to its original value[2].

One of the chief objections to the theory has been the failure of externally applied ACh to affect electrical activity[3-5]. However, during the last few years a variety of observations have been reported in which ACh was found to affect axonal conduction[6-10]. This paper describes a new series of experiments in which a direct action of ACh and related compounds on the electrical activity of somatic nerve fibers of lobster has been obtained.

METHODS

The nerves of the 5th and 6th thoracic limb of the lobster (*Homarus americanus*) were used. A small bundle of fibers was dissected out of the main trunk. The length obtained was about 5 cm, and the diameter varied between 0.3 mm and 0.6 mm. The nerve bundle is surrounded by a connective-tissue sheath which is elastic and strong but rather thin. The individual axons are surrounded by a layer of Schwann cells while the extent of extramembranous lipid material is still uncertain at present[11]. Filtered natural sea water (pH 7.8) (0.5 ml 1.0 M Tris buffer was added per l) of the following composition (in μmoles/ml) was used: Na^+, 400; K^+, 8; Ca^{2+}, 7.4; Mg^{2+}, 70.3. The nerve bundle was mounted in a "sucrose-gap" apparatus designed for the recording of both membrane and action potentials[12,13]. The following compounds were found to affect electrical activity: ACh, mecholyl, physostigmine, tertiary phospholine, Paraoxon, tetracaine, atropine, and *d*-tubocurarine. Carbamylcholine was also tested but had no effect at concentrations as high as $5 \cdot 10^{-2}$ M. The solutions were freshly prepared before testing and adjusted to pH 7.8–7.9. The typical action potential obtained from these nerves with the sucrose-gap technique consisted of two elevations. The smaller and earlier one had a lower threshold, while with slightly higher stimulation the main component of the action potential responded. The experimental records include both components and their responses to the various compounds tested were qualitatively identical; however, in some cases the response of the smaller component proved to be less marked.

RESULTS

The action of ACh on membrane and action potential. When the sea water bathing the nerve contained 5 mM ACh, a 4–6 mV decrease in the membrane potential was observed within 6 min. The onset of the depolarization had a delay of approx. 30 sec. A part of this delay can be attributed to the time required for the complete change of solutions. On return to normal sea water complete repolarization occurs and has a slower timecourse than the depolarizing phase.

During the intial phase of the depolarization a small but significant increase in pike height was usually observed but as the depolarization progressed the spike height subsequently decreased. Twice this concentration of ACh (10^{-2} M) increased the rate and magnitude of depolarization and finally blocked conduction within

10–20 min (Fig. 1). 1 mM ACh had no significant effect on either the membrane or action potential. A partial spontaneous repolarization was sometimes observed when the nerve was exposed to 5 mM ACh for prolonged periods (30–60 min). In these cases, switching to higher concentrations of ACh had no effect on the spontaneous recovery.

In addition to its depolarizing and spike-height reducing or increasing actions, ACh has a marked effect on the descending phase of the action potential. The main change produced by 5 mM ACh is a prolongation and elevation of the descending phase (Fig. 2). Exposure of the axons to slightly higher concentrations ($7.5 \cdot 10^{-3}$–10^{-2} M) also produced this prolonged and elevated descending phase of the spike but as can be seen in Fig. 1, the effect decreases as the nerve approaches block of conduction.

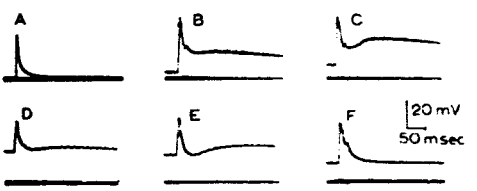


Fig. 1. Effect of 10^{-2} M ACh on the membrane and action potential of the lobster walking-leg nerve. A, control in sea water. B, C, D, after 2, 5 and 7 min exposure to ACh. Initially there is an increase in spike height while the descending phase is markedly elevated and prolonged. As the depolarization progresses the spike height decreases and the effect on the descending phase is attenuated. Note the oscillations associated with the descending phase of the spike in B and C. E, F, 3 and 7 min after returning to sea water; the spike height and membrane potential are increasing. Temperature, 22°; pH 7.7.

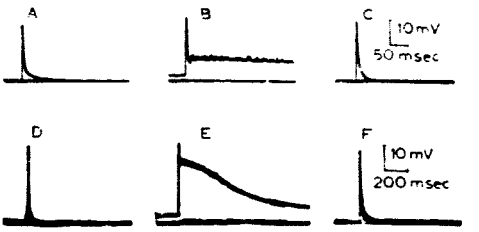


Fig. 2. Effect of 5 mM ACh on the membrane and action potential of the lobster walking-leg nerve. A, D, controls of 2 different preparations in sea water. B, E, after 6 and 9 min exposure to ACh (note the difference in the time scale). Accompanying the decrease in membrane potential, the descending phase of the spike is markedly prolonged and elevated; oscillations are seen. C, F, the effect is reversed after switching to sea water. Temperature, 22°; pH 7.7.

The elevated and prolonged phase always demonstrated oscillatory peaks (Fig. 2). The duration was usually extended for 1.5–2 sec while the average elevation of the descending phase of the spike was about one third of the original spike. These effects were all completely reversible and could be repeated several times on the same preparation.

To determine if the above effects were due to a specific action of ACh and not its hydrolytic products or accompanying anion, choline, acetate and bromide were tested in the same and 10-fold higher concentrations. Only choline had an effect: at $5 \cdot 10^{-2}$ M a small but significant increase in spike height was observed but there was no effect on the descending phase of the spike; 10^{-2} M choline had no effect. Thus the marked effects observed with 5 mM ACh appear to be due to a specific action of ACh.

Effects of mecholyl on membrane and action potential. *d,l*-Acetyl-β-methylcholine (mecholyl) is a specific substrate for ACh-esterase. Only the *d*-form is active[14,15]. Mecholyl, at 10^{-2} M, increased the duration of the descending phase of the spike. The onset of its effects is slower than that of ACh and concentrations lower than 10^{-2} M have no effect (Fig. 3). Occasionally a small depolarization is seen.

The effects of physostigmine on membrane and action potential. The effects of physostigmine ($5 \cdot 10^{-4}$–10^{-2} M) were similar to those of ACh, but the spike durations were usually not increased beyond 1 sec (Fig. 4). It was of interest to determine whether inhibitors of cholinesterase would potentiate the action of ACh by preventing its enzymic hydrolysis. At no time was physostigmine observed to significantly potentiate the action of ACh. In fact, physostigmine as low as $5 \cdot 10^{-5}$ M usually inhibited the action of 5 mM ACh.

The effects of irreversible inhibitors of ACh-esterase on the membrane and action potential. When *O,O'*-dimethyl-*S*-2-dimethylaminoethyl phosphonothiolate (tertiary phospholine) was added to sea water at a concentration of $5 \cdot 10^{-3}$–10^{-2} M, a slow

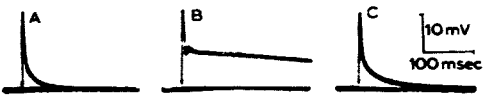


Fig. 3. Effect of mecholyl on the membrane and action potential of the lobster walking-leg nerve. A, control in sea water. B, after 15 min exposure to 10^{-2} M mecholyl. The descending phase of the spike is markedly prolonged and elevated; oscillations are present. There is no apparent depolarization. C, the effect reverses in sea water. Temperature, 22°; pH 7.7.

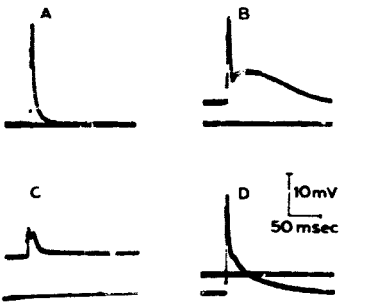


Fig. 4. Effect of physostigmine on the membrane and action potential of the lobster walking-leg nerve. A, control in sea water. B, C, after 2 and 12 min exposure to 5 mM physostigmine. Initially as the membrane potential decreases the descending phase of the spike is prolonged and elevated. With increasing depolarization the spike height and the effect on the descending phase are attenuated. D, after 8 min in sea water the spike effects are almost completely reversed while the membrane potential appears to be hyperpolarized. Temperature, 22°; pH 7.7.

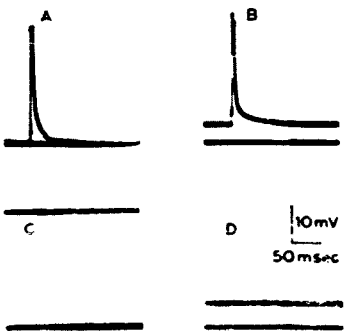


Fig. 5. Effect of phospholine on the membrane and action potential of the lobster walking-leg nerve. A, control in sea water. B, C, after 6 and 57 min exposure to 5 mM phospholine. Conduction block did not occur until 57 min and is accompanied by a very large depolarization. D, after 90 min in sea water the depolarization has substantially decreased but conduction is not restored. (Even after 3 h there was no demonstrable conduction.) Temperature, 22°; pH 7.7.

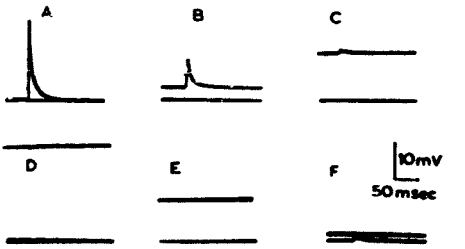


Fig. 6. Effect of Paraoxon on the membrane and action potential of the lobster walking-leg nerve. A, control in sea water. B, C, D, after 1, 2 and 6 min exposure to 5 mM Paraoxon. Conduction is rapidly blocked (in 2 min) and accompanied by a large depolarization. E, F, repolarization in sea water occurs with a small hyperpolarization, but even after 90 min (F) there is no significant return of conduction. Temperature, 22°; pH 7.7.

decrease in spike height and membrane potential was observed; conduction was nearly blocked after 1 h exposure (Fig. 5). After the return to normal sea water the depolarization was almost completely reversed but at no time was conduction restored (Fig. 5). $5 \cdot 10^{-3}$ M Paraoxon (*p*-nitrophenyl-diethylphosphate) produced the same type of effect but was more potent, conduction block occurring in 2 min (Fig. 6).

Effect of local anesthetics on membrane and action potential. Tetracaine in concentrations as $7 \cdot 10^{-5}$ M decreases the spike height slightly; a higher concentration (10^{-3} M) blocks conduction within 5 min. Tetracaine in high concentration (>0.1 mM) was also noted to decrease the membrane potential. Concentrations between $1-5 \cdot 10^{-5}$ M do not significantly depolarize or decrease spike height but a slight increase in the spike duration was usually observed. This was a generalized broadening of the spike process; there did not appear to be a specific effect on the descending phase of the spike as had been seen with ACh.

When fibers are exposed to 5 mM ACh in combination with $7 \cdot 10^{-5}$ M tetracaine, the changes regularly caused by this concentration of ACh are greatly inhibited (Fig. 7). After the removal of tetracaine by rinsing with sea water, ACh is effective again. Sometimes higher concentrations of tetracaine are necessary to completely inhibit the ACh effect and in these cases reversibility is not as complete.

Effects of curare on membrane and action potential. Curare in concentrations of 10 mg/ml increased the spike height and prolonged the descending phase of the spike. A small depolarization was also noted at this concentration. All these effects are reversible (Fig. 8). 5 mg/ml produced smaller but identical effects except for the absence of a depolarization. 2.5 mg/ml curare had no effect by itself but it partially inhibited the action of 5 mM ACh (Fig. 9). After washing the nerve with sea water, ACh is as active as it had been before the addition of curare.

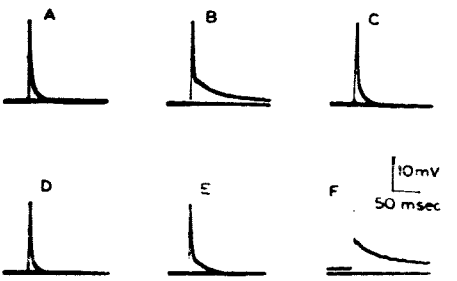


Fig. 7. Antagonism of the action of ACh on the membrane and action potential of lobster nerve by tetracaine. A, control in sea water. B, after only 2 min exposure to 5 mM ACh the descending phase of the spike is already moderately prolonged and elevated; the effect reverses in sea water (C). The nerve is then bathed in $7 \cdot 10^{-5}$ M tetracaine and after 12 min there is a small reduction in spike height (D). The nerve is bathed in sea water containing $7 \cdot 10^{-5}$ M tetracaine and 5 mM ACh. Even after 9 min (E) there is only a small change in the descending phase of the spike (compare with B). The nerve was then rinsed for 10 min in sea water and exposed again to 5 mM ACh; after only 3 min (F) the descending phase of the spike is markedly prolonged (compare with E). Because of the short exposure to ACh in B a depolarization was not yet apparent but it was seen in F. However, there is no depolarization with tetracaine and ACh at E despite the long exposure. Temperature, 23°; pH 7.7.

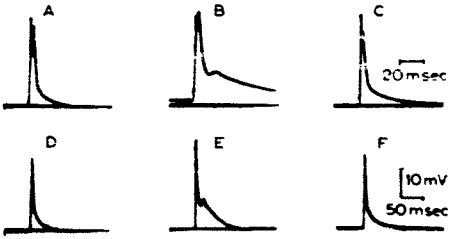


Fig. 8. Effects of curare on the membrane and action potential of the lobster walking-leg nerve. A, D, controls of 2 different preparations in sea water. B, after exposure to 10^{-3} M curare for 12 min; there is a small depolarization and the descending phase of the spike is elevated and prolonged. E, after exposure to 2.5 mM curare for 20 min; there is no depolarization but the spike height is increased and the descending phase prolonged. C, F, the effects reverse in sea water. Temperature, 24°; pH 7.7.

Effects of atropine on membrane and action potential. Atropine, at concentrations of 5 mM and 10^{-2} M, prolongs the descending phase of the spike, reduces the spike height, depolarizes and finally blocks conduction (Fig. 10). At 1 mM, atropine had no effect on the spike but it completely inhibits the action of 5 mM ACh. The axons respond to ACh again only after prolonged washing with sea water (Fig. 11).

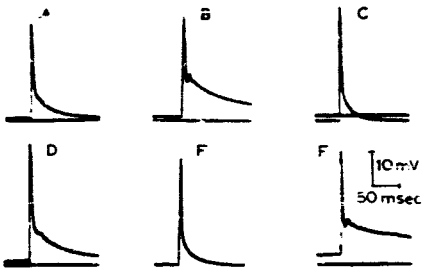


Fig. 9. Antagonism of the action of ACh on the membrane and action potential of lobster nerve by curare. A, control in sea water. B, after 2 min exposure to 5 mM ACh; there is a small depolarization, a slight increase in spike height and the descending phase of the spike is elevated and prolonged. The effect was reversed in sea water. After exposure to 2.5 mM curare for 15 min there is an increase in spike height (C). The nerve was then rinsed in sea water containing 2.5 mM curare plus 5 mM ACh; D shows the effect after 9 min (compare with B). E, the effect is reversed in sea water. Finally F shows the effect of 5 mM ACh without curare after 3 min exposure (compare with D). Temperature, 23°; pH 7.7.

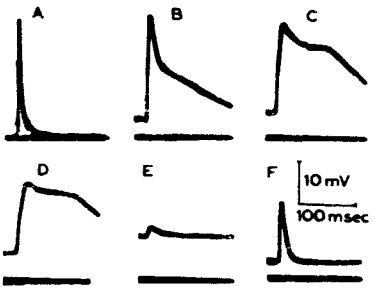
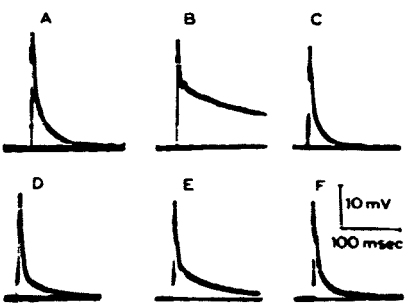


Fig. 10. Effect of atropine on the membrane and action potential of the lobster walking-leg nerve. A, control in sea water. B, C, D, after 3, 7 and 12 min exposure to 5 mM atropine; the membrane potential is progressively reduced and the descending phase of the spike is elevated and prolonged while the spike height decreases and is nearly blocked after 15 min (E). In sea water the effects are beginning to reverse (F). Temperature, 22°; pH 7.7.

Fig. 11. Antagonism of the action of ACh on the action potential of lobster nerve by atropine. A, control in sea water. B shows the effect of 5 mM ACh after 9 min. There is no apparent change in the membrane potential but the descending phase of the spike is elevated and prolonged. The effect was reversed in sea water and then exposed to 1 mM atropine for 9 min (C). The nerve was then rinsed in sea water containing 1 mM atropine plus 5 mM ACh; there was no significant effect after 9 min (D). Even after rinsing the nerve for 9 min in sea water the reapplication of 5 mM ACh only produces a small effect after 9 min exposure (E). F, reversibility in sea water. Temperature, 23°; pH 7.7.



DISCUSSION

This investigation has revealed that ACh has a definite action on the membrane and action potential of the walking-leg nerve of lobster. ACh depolarizes the membrane potential, the rate and magnitude depending on concentration. The action potential is reduced in height after an initial increase and with adequate exposure the excitation process is finally blocked. Accompanying the onset of the depolarization the descending phase of the action potential is prolonged and appears oscillatory. With increasing depolarization and decreasing spike height the prolongation becomes less

marked. All these effects are reversible and are effects of ACh, since neither choline nor acetate in similar concentration have any effect. Only in 10-fold high concentrations can small effects be seen with choline.

According to NACHMANSOHN's theory ACh should, by its action on the receptor protein, depolarize the axonal membrane in a way similar to that observed on synaptic membranes[2]. The failure to affect axons has been attributed to structural barriers preventing lipid-insoluble quaternary ammonium derivatives from reaching the conducting membrane. Chemical pretreatment which apparently reduces the barrier has recently been shown to block reversibly axonal electrical activity[7,8]. An action of ACh was also observed on the axons of the rabbit vagus; however, the effect was only obtained after removal of the sheath[6].

Two aspects of the ACh action may be briefly discussed: the time course of the effect and the concentration required. The depolarization takes place within seconds, the return to normal is even slower. Thus, the time course seems to be very slow when compared with the physiological events during activity. But obviously, it cannot be expected that a biological process taking place in μseconds in a membrane of 80 Å thickness is reproducible with such crude techniques as the application of a compound from the outside: under these experimental conditions the compound applied has to diffuse through several layers of tissue surrounding conducting membranes. It can *a priori* not be expected that such a slow diffusion process parallels the speed of the biological event in which the active ester, when dissociated from the storage form, may be assumed to act on the receptor at a distance of a few Å. Structural organization is probably the basis of the efficiency and speed of many biological processes.

The high concentration of ACh required is also hardly surprising. Previous observations indicate that only a very small fraction of externally applied compounds enter the axon. Even a lipid-soluble compound, such as DFP, was found inside the axon in a concentration less than one thousandth of that on the outside at the time when electrical activity was blocked[16]. The outside concentration is, therefore, not an important aspect, considered by itself. It is true that many compounds in sufficiently high concentration may block activity. However, such an effect considered by itself would not permit one to attribute a physiological function to the chemical used. The effect observed with ACh is only meaningful in association with the large amount of data accumulated in favor of the postulated role of the ACh system: its presence in all axons, its many remarkable properties which are a prerequisite for the proposed role, *i.e.* the control of ion movements underlying electrical activity, etc. The most essential evidence is, however, the functional interdependence between electrical activity and the two active protein members of the system, the inseparable association of electrical activity with either ACh-esterase or the receptor protein demonstrated by powerful and specific inhibitors[2,8,17-19].

Another difficulty must be kept in mind in evaluating the effects of ACh and the other compounds tested. The preparation used is a bundle of probably several hundred fibers. In such a preparation the electrical records represent the sum of the responses of individual fibers. It is difficult to determine how the individual fibers are affected. In some fibers conduction may be blocked while in others electrical activity may be either still unaffected or even potentiated. With the techniques used the analysis of the electrical response would not reveal such modifications of the

response of single fibers. Moreover, neither the degree nor the rate of penetration are known. The complexity of the response is still further increased by the fact that many of the compounds react with both receptor and enzyme depending on the concentration. All these findings become even more disturbing for a proper evaluation in the case of two compounds applied to the same preparation, since it is known that the rate of penetration varies greatly with small modifications of structure and differs greatly according to the types of fibers used. The analysis of the data obtained so far must keep in mind these factors which limit the interpretation of the complex responses of the multifiber preparation[20].

Physostigmine, in low concentrations, inhibits ACh-esterase. At concentrations where the enzyme is completely blocked, a block of conduction would be expected, as has been demonstrated to be the case[17]. At lower concentrations it may exhibit ACh-like effects by slowing the hydrolysis of ACh released. Such effects have indeed been observed in the present studies. Physostigmine is, however, also a receptor inhibitor and may compete with ACh for the receptor site and have a blocking action on that basis[6,21–23]. At a certain level of inhibition of ACh-esterase a potentiation of added ACh would be expected. But in view of the many competing factors outlined above, it is not surprising that this particular effect has not been observed thus far. Electrical activity in some more peripheral fibers may have been completely blocked and potentiation in the more centrally located axons may escape detection[20].

The classical view of curare as a competitive inhibitor of the ACh receptor is considered to be one of the fundamental criteria in the characterization of vertebrate neuromuscular transmission. Our experiments indicate that in the axonal membrane tested a similar system is present. This is not the first time that this kind of evidence has been found. Curare in even lower concentrations than used here, reversibly and rapidly blocked conduction in single nerve fibers of the frog sciatic nerve[18]. WALSH AND DEAL[7] showed that after pretreatment with CTMC the sciatic nerve bundle reacted with curare. ROSENBERG *et al.*[8] showed that after pretreatment of the squid giant axon with snake venoms, which apparently effectively reduced the permeability barriers surrounding the nerve, curare and ACh reversibly blocked conduction. In the present experiments curare inhibits the action of ACh, but by itself even in high concentrations does not block conduction; this was also observed by ARMETT AND RITCHIE[6] on the rabbit vagus. This may be due to poor penetration by the compound, affecting perhaps only peripheral fibers, an effect which might escape detection. The action of atropine on the muscarinic action of ACh is well known. In the present experiments it blocked conduction and, depending on the concentration, inhibited the action of ACh. This also supports the view that the ACh receptors present in the axonal membrane have similar properties as those at the parasympathetic postganglionic effector sites.

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