# The Effects of Myoinositol on the Autonomic Neuropathy in the Streptozotocin Diabetic Rat - A Freeze Fracture Study

G. Monckton and H. Marusyk

**ABSTRACT:** This report describes a freeze fracture study of rat dorsal sympathetic chain in normal, streptozotocin diabetic on normal diet, and a group fed a 1% myoinositol normal diet. In nonmyelinated fibres, the diabetic group had significant loss of particles on the P face of juxta-axonal Schwann cell membranes, whilst myelinated nerves showed a profound loss of particles on P and E faces. In the group with adjuvant myoinositol the particle numbers were normal in both types of nerve. Axonal plasma membranes showed increased numbers of particles in the P face of the nonmyelinated membranes in the diabetic, but were normal in the myoinositol group. Myelinated axonal membranes showed no significant difference between the diabetic and the normal. Rats on a myoinositol diet showed a significant decrease in the E face particles. These results are consistent with those described in the sciatic myelinated nerves by earlier authors and reinforce the view that myoinositol is an important membrane constituent as phosphatidylinositol and is essential for the expression of normal protein particle numbers.

**RÉSUMÉ: Les effets du myoinositol sur la neuropathie du système nerveux autonome chez le rat diabétique traité à la streptozotocine - une étude par technique "freeze fracture".** Nous décrivons une étude, utilisant la technique "freeze fracture", de la chaîne sympathique dorsale chez le rat normal, chez le rat diabétique traité à la streptozotocine et maintenu à une diète normale et chez un groupe sous diète normale à 1% de myoinositol. Le groupe diabétique avait une perte significative de particules sur la face P de la membrane des cellules de Schwann juxta-axonales dans les fibres non-myélinisées, tandis que les nerfs myélinisés présentaient une perte importante de particules sur les faces P et E. Dans le groupe recevant le myoinositol comme adjuvant à la diète, le nombre des particules était normal dans les deux types de nerfs. Les membranes plasmatiques des axones présentaient un nombre accru de particules sur la face P des membranes non-myélinisées chez le rat diabétique, mais étaient normales dans le groupe traité au myoinositol. Les membranes axonales myélinisées n'étaient pas significativement différentes chez le diabétique et le normal. Les rats sous diète avec myoinositol présentaient une diminution significative des particules de la face E. Ces résultats sont compatibles avec ceux déjà décrits au niveau des nerfs sciatiques myélinisés et appuient l'hypothèse que le myoinositol est un constituant important de la membrane sous forme de phosphatidylinositol et est essentiel à l'expression d'un nombre normal de particules protéiques.

*Can. J. Neurol. Sci. 1988; 15:147-151*

It has been previously shown that severe pathological changes occur in the dorsal sympathetic chain in the acute streptozotocin diabetic rat.[1] Fukuma et al[2] have demonstrated changes in the numbers and distribution of protein particles in myelin membranes of sciatic nerve in acute streptozotocin diabetic rats. At fourteen days following the induction of diabetes these authors also demonstrated that these particle changes were prevented by supplementation of the diet with 1% myoinositol during the course of acute diabetes. The present report describes the results obtained by freeze fracture studies of the interganglionic segments of the dorsal sympathetic chain fourteen days following the induction of diabetes and the effects of a 1% myoinositol adjuvant in the diet given during the course of the diabetes.

## METHODS

Thirty-one male Wistar rats weighing 200-250 g were used in this study, and following an initial blood glucose assay, 21 were rendered diabetic by the injection of streptozotocin into the penile vein (70 mg/kg of streptozotocin in 0.1 M citrate buffer, pH 4.5). Ten normal and 10 diabetic rats were fed a standard rat diet *ad libitum* for 14 days. The remaining diabetic rats were fed similarly with a standard rat diet supplemented with 1% (w/w) free myoinositol (ICN Canada Ltd., Montreal, P.Q.). The plasma glucose assays were obtained on all animals on day 7 and day 14 of the study. On day 14 the rats were anaesthetized with pentobarbitol and perfused with 3.5% glutaraldehyde in phosphate buffer, pH 7.4. The sympathetic chain was dissected out and placed in the glutaraldehyde phosphate buffer solution as above for 4 hours. The nerves were then placed in 30% glycerol in phosphate buffer, pH 7.4, for 30 minutes, oriented longitudinally onto gold discs and frozen in the liquid phase of Freon 22, cooled with liquid nitrogen. The nerves were fractured in a Balzers BAE 080 apparatus (Balzers Instruments, Lichtenstein).

The replicas were floated and cleaned in bleach, then distilled water, mounted on copper grids and examined in a Siemens Elmiskop 102. P and E faces of both juxta-axonal and axonal internodal plasma membranes of nonmyelinated and myelinated nerves were examined. These were photographed at a magnification of 60,000x. Because of the potential error caused by curvature of the surfaces to be examined, we counted particles only on the surface of membranes to which the beam was perpendicular. A 0.25 micrometer square grid was superimposed on the centre of each face and the number of particles within this area was counted. The resulting numbers were expressed as the number of particles per micrometer square plus or minus the standard deviation. Effective enlargement of these areas by 120,000x was carried out and the length of the shadows of the protein particles were carefully measured to determine particle size. These observations were plotted in histogram form.

## RESULTS

The graph in Figure 1 shows the mean plasma glucose levels for the normal, streptozotocin diabetic and streptozotocin diabetic rats on the myoinositol diet, which does not significantly alter their diabetic status. The general appearance of the freeze fracture replicas of portions of the dorsal sympathetic chain are illustrated in Figure 2. There is an even distribution of particles in normal, diabetic and myoinositol diabetic axons, showing a less dense distribution on the E than the P faces.

### Juxta Axonal Plasma Membranes

#### A. Nonmyelinated Axons

The histogram in Figure 3 indicates the range of numbers of particles counted expressed as a percentage of the total in all three groups of animals on the P and E face. In the normal animals there are significantly more particles on the P face than the E face (p < 0.01). In the diabetic animals the P and E faces have almost the same numbers of particles, but when compared with the normal P face, the diabetic animals had significantly fewer particles (p < 0.02). The myoinositol diabetic animals P and E face numbers are normal. Referring to the mean number of particles per micrometer squared it is seen that both in

the normal and myoinositol group the P faces have significantly more particles than the E faces, whereas in the diabetic animals the P and E faces have similar numbers. The histogram in Figure 4 displays the sizes of particles expressed as a percentage of the total. In normal animals, the ranges of sizes are similar and the P face shows a greater percentage of larger particles than the E face, but the latter have greater numbers of smaller particles. In the diabetic animals there is a significant reduction in larger particles and an increase in smaller particles as compared to the normal and myoinositol diabetic groups. The diabetic E face particle size distribution closely matches the P face (p > 0.1).

#### B. Myelinated Axons

The changes in juxta-axonal internodal Schwann cell membranes are shown in figure 5A. In the normal there is a clear difference between the P and E faces (p < 0.01). In the diabetic group there is a profound reduction in the P face numbers of particles per micrometer squared with a significant drop in the numbers of particles in the E face (p < 0.01). In the group of animals on adjuvant myoinositol the P and E faces showed normal numbers of particles. The comparison of the number of particles per micrometer squared on the myelinated and nonmyelinated juxta-axonal membranes is shown in this histogram and the changes seen in the myelinated group are more severe than those in the nonmyelinated.

### Axonal Plasma Membranes

#### A. Nonmyelinated Axons

In the diabetic animals (Figure 5B) there are significantly increased numbers of particles noted on the P faces as compared to normal (p < 0.02). In the myoinositol fed animals this increase is reduced to normal. The E faces are similar in all three groups of animals and are unaffected by diabetes or myoinositol.

#### B. Myelinated Axons

The number of particles on the P face in normal rats is significantly greater than the E-face. In the diabetic and myoinositol diabetic animals there is a significant increase in the P-face particle numbers as compared to normal. The E-faces
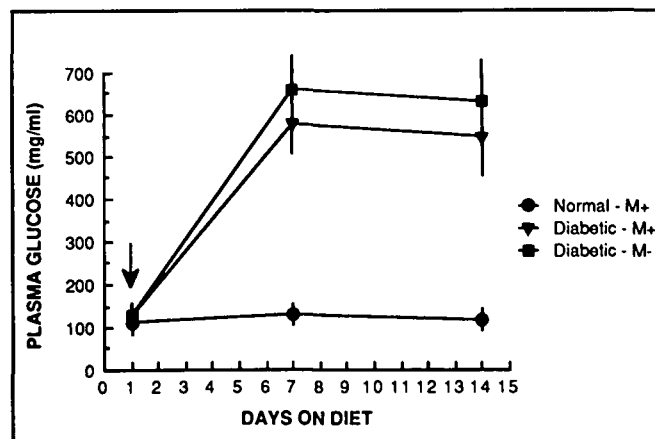


*Figure 1 — Graph showing mean (± SEM) plasma glucose levels in normal (M⁺) and streptozotocin diabetic with (M⁺) and without (M⁻) a myoinositol diet. The blood glucose levels of diabetic animals on the myoinositol diet showed no significant difference to those diabetics not on diet. Both were significantly higher than the normal (p > 0.005).*

in normal and diabetic animals have similar numbers of particles, but the myoinositol treated rats show significant decrease ($p < 0.01$).

### C. Internodal Myelin Membranes Distant to the Axon

Examination of particles in Schwann cell membranes (myelin) at a distance of at least 10 laminae from the axon, show that

particle numbers in normal animals are significantly greater than in the juxta-axonal plasma membrane ($p < 0.02$). The numbers of particles in the diabetic and myoinositol diabetic animals are similar to normal. On the E-faces of the three groups, the normal and diabetic rats were similar but the myoinositol group showed a significant reduction as compared to the other two ($p < 0.05$).

### Discussion

In our study we have been interested particularly in the numbers and size of particles in the juxta-axonal and axonal membranes in autonomic nerves. Since the introduction of freeze-etching techniques[3] and their application to cell membranes, there has been a great deal of interest as to the nature of intramembranous particles demonstrated. The consensus of opinion is that the majority of these particles are protein in nature, but it is recognized that occasional individual particles may represent lipids in hexagonal phase.[4] It is accepted that protein particles exposed in replicas are heterogeneous and do not necessarily represent a single protein type. Rosenbluth[5] in an excellent discussion has pointed out some of the problems associated with assessing particle sizes, but states that there is an acceptable amount of agreement from different laboratories on particle size and distribution by simple observation and that
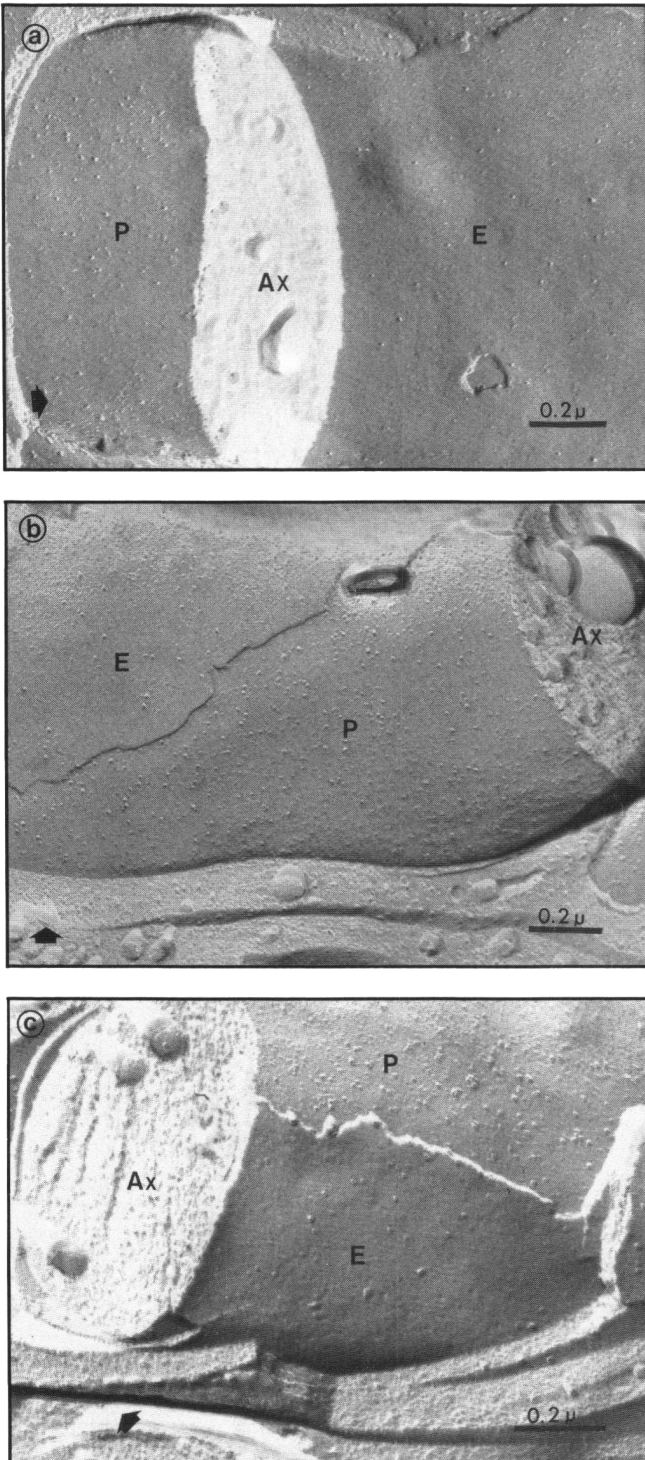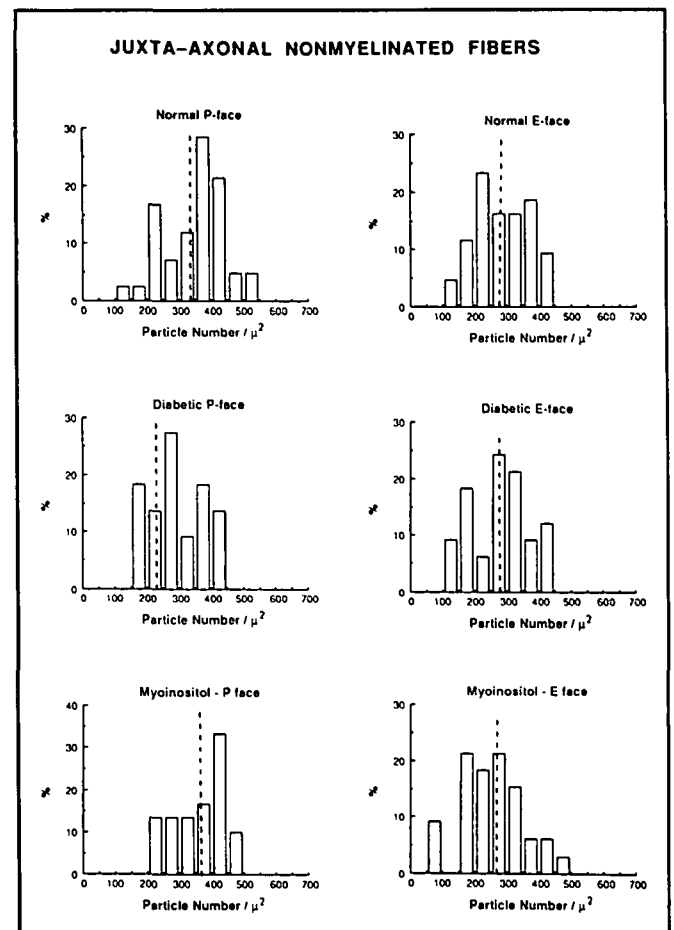


Figure 2 — Freeze-fracture replicas of (a) normal, (b) diabetic and (c) diabetic-myoinositol treated nonmyelinated nerves demonstrating particles on the P and E faces. Ax represents a cross-fractured axon. Arrow represents the angle of shadowing.



Figure 3 — Histogram shows the number of particles/$\mu m^2$ expressed as a percentage of the total. A minimum of 95 unit areas ($\mu^2$) were counted from each category. The means are represented by broken lines.

comparisons of such data are valid. Counting particles on curved surfaces can also give rise to error,[6] but our methodology has attempted to minimize this.

There is a difference in the effects of diabetes on the juxta-axonal membranes in the two types of nerve fiber. In the nonmyelinated group, the diabetes appears to affect the P faces mainly, whereas in the myelinated group both faces are severely affected. Our results show that there is a significant reduction in numbers of particles in the juxta-axonal membranes of the autonomic nerve fibres in the diabetic rats. This reduction is associated with a relative increase in the percentage representation of smaller particles, and indicates a reduction in the larger particle population. The addition of myoinositol to the diet returns the particle count and size distribution to normal. These findings are similar, but less prominent than those noted in the internodal juxta-axonal Schwann cell membranes of myelinated nerve fibers.[2]

The changes in the numbers of particles per micrometer squared in nonmyelinated and myelinated axolemma show no correlation. In nonmyelinated axolemma there is a significant increase in the numbers of particles on the P face with no change on the E face. The addition of myoinositol to the diet reduces the increased numbers in the diabetic to the normal range. In the myelinated nerve axolemma P face, particles appear to be significantly increased by the addition of myoinositol with a significant reduction in the E face particles as compared to the normal or untreated diabetic rat.

In our study of myelinated membranes at a distance from the axon in myelinated nerve fibres, the number of protein particles on the P and E faces are essentially unchanged by diabetes or myoinositol. This is in contrast to the juxta-axonal membranes which are noted to have marked changes and normally to have a larger number of particles than elsewhere in the cell. This seems to provide anatomical correlation with the accepted intercellular communication which occurs between Schwann cells and axons. It would seem possible that the reduction in particle numbers in the juxta-axonal myelinated membranes to levels which are not dissimilar from that seen in myelinated
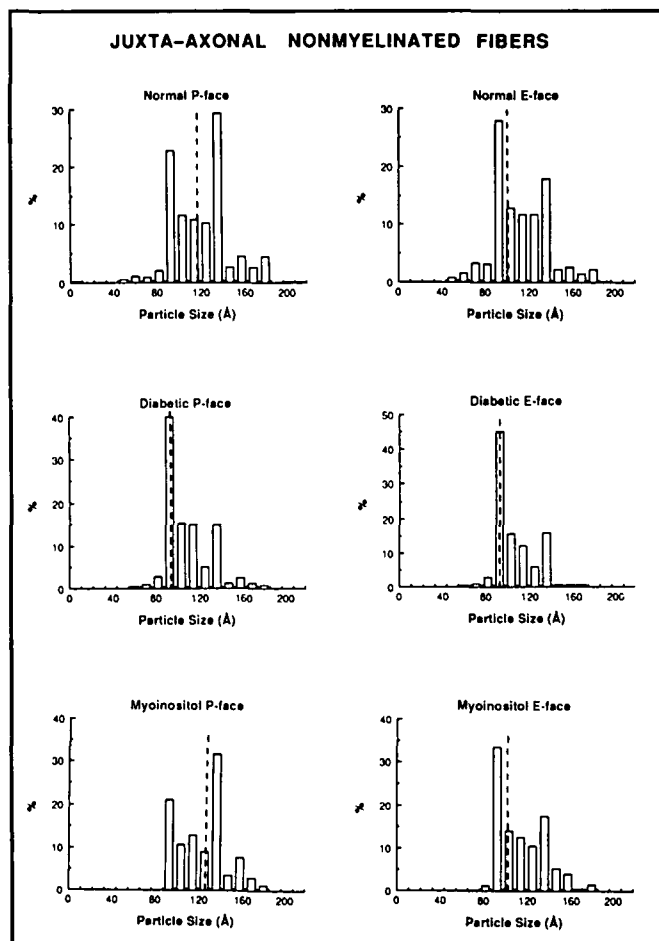


*Figure 4 — Histogram showing size distribution of particles on P and E faces of the three groups of animals. Note the similarity of distribution of normal and myoinositol treated animals. The number (N) of particles measured in each category was 500. The means are represented by broken lines.*
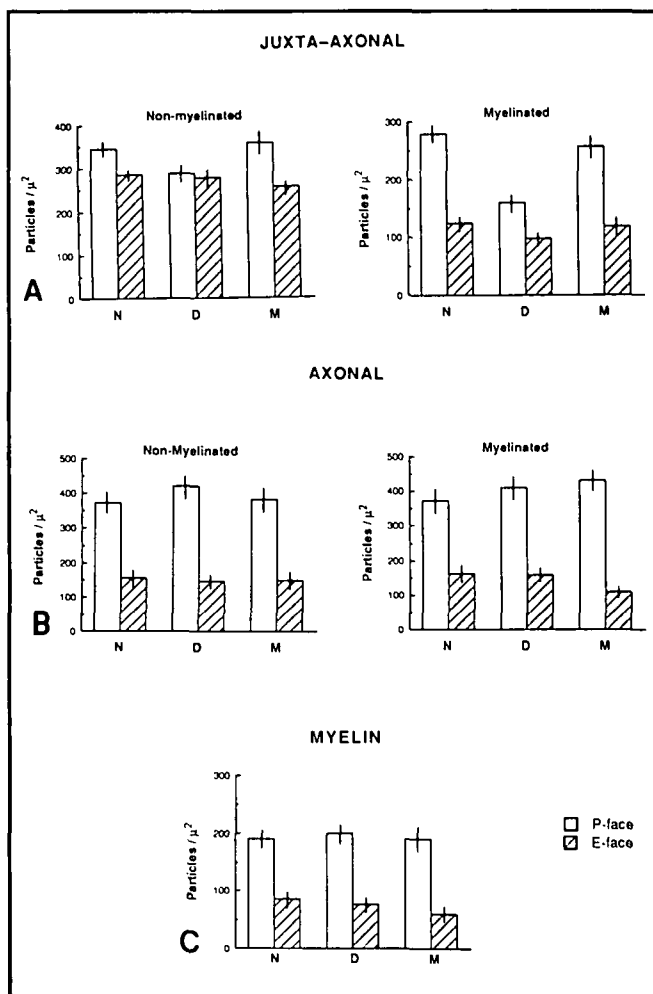


*Figure 5a — Comparison of juxta-axonal membrane P and E face particle numbers/μm² in nonmyelinated and myelinated membranes in three groups of animals demonstrating the more severe effect of diabetes on myelinated juxta-axonal membranes (p < 0.02).*

*Figure 5b — Histogram showing numbers of particles/μm² on axolemma P and E faces of nonmyelinated and myelinated axons. Note in diabetic animals the apparent significant increase in particles on the P face of nonmyelinated axons and the normal number of particles on the P face of myoinositol treated axons and reduction of E face particles.*

*Figure 5c — Myelin membrane particle number/μm² at a distance from the axon. Note reduced numbers of particles and relatively slight effect of diabetes or myoinositol treatment. Bars represent the mean ± SEM.*

membranes at a distance from the axon, may be a further factor modifying axonal function by reducing intercellular communication. Studies[7] have demonstrated marked slowing of nerve conduction velocity in diabetic rats and that this conduction defect could be remedied by the addition of myoinositol to the diet. Fukuma et al[2] demonstrated that there was close correlation between the loss of protein particle numbers in the diabetic rat and the slowing of conduction velocity in myelinated nerve fibres. It is not clear what this correlation indicates. An excellent review of background biochemical changes in experimental diabetes and in particular the effects of myoinositol depletion in its relation to reduced nerve conduction velocity has been provided by Greene et al.[8] These authors propose a self-reinforcing metabolic defect based upon hyperglycemia and an increased polyol pathway activity causing decreased sodium dependent myoinositol uptake. As a result of interdependent steps, sodium is accumulated at the node of Ranvier and blocks nodal depolarization in large rapidly conducting nerve fibres effectively diminishing composite nerve conduction velocity. The biochemical cascade described is prevented by myoinositol adjuvant in the diet and also maintains normal numbers of protein particles in the juxta-axonal membranes both of myelinated and nonmyelinated nerve fibres. The fact that diabetic rats on the myoinositol diet are still hyperglycaemic and insulin deficient would seem to exclude these two factors as being responsible for protein particle depletion. It might be considered that the reduced myoinositol associated with diabetes may have some impact upon the structure of plasma membranes and although it is interesting to speculate on what the effect of reduced myoinositol might be in membrane function and composition, it is unlikely that this would directly affect numbers of protein particles embedded in it. It is difficult to arrive at a conclusion as to the cause of the changes in numbers and sizes of protein particles that we have observed, but it is reasonable to assume, since myoinositol rectifies these changes, that the same cycle of events is occurring in Schwann cell membranes of autonomic nerves as in the myelinated nerve fibres.

Whatever the basis for the reduction in protein particles may be, the observation of a number of authors,[2,7,8,9] as well as those reported here, that a myoinositol adjuvant diet reverses chemical, structural and functional changes in juxta-axonal and axonal membranes in acutely diabetic rats and that aldose reductase inhibition can do the same,[10] urges further evaluation of both myoinositol supplements and aldose reductase inhibitors in the management of diabetic neuropathy.

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