Journal of Neurology, Neurosurgery, and Psychiatry, 1972, **35**, 156–162

Permeability of blood nerve barriers in the diabetic rat[1]

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SUMMARY An albumin-Evans blue conjugate has been used as a fluorescent tracer to demonstrate the increased permeability of endoneurial capillaries and perineurial sheath of the sciatic nerve of the alloxan-diabetic rat. The significance of the extravasation of protein into the endoneurial space is discussed in relation to the altered dynamics of the endoneurial microcirculation. It is suggested that tissue hypoxia produced in this way may be a cause of the segmental demyelination which occurs in these nerves.

Although the properties of the blood brain barrier have been investigated very extensively, the equivalent structures in peripheral nerve—that is, the blood nerve barrier—have attracted relatively little attention.

Shamboorov and Tchibukmakher (1938) demonstrated that several blood-borne substances which do not reach the interior of normal nerves did so when the nerve was inflamed. They recognized the existence of a blood nerve barrier made up of nerve sheaths and blood vessels and observed that the dorsal root ganglion was devoid of such a barrier. Waksmann (1961) observed that intravenously injected dyes such as Trypan blue and Niagra Sky blue stained the nerve roots and spinal ganglia of the rabbit, whereas the parenchyma of the sciatic nerve even in its fine intramuscular ramification remained unstained. Intravenously injected proteins such as egg albumen and human serum albumin labelled with fluorescein or ^{131}I showed a similar distribution and passed freely into the roots and ganglia but not into the nerve parenchyma. Intravenous diphtheria toxin produced no lesions of the central or peripheral nervous system, whereas intracisternal injection produced a rapidly fatal disease and intraneural injections regularly produced extensive lesions of the nerve parenchyma. These observations were interpreted as evidence for the existence of a blood nerve barrier located in the vascular endothelium. Olsson (1966), using intravenous injections of albumin tagged with a fluorescent dye, found that the labelled albumin appeared only within the lumen of the endoneurial blood vessels. In the external nerve sheaths, however, the albumin appeared outside the epi- and perineurial vessels, the extravasated protein extending up to the innermost laminae of the perineurial sheath but leaving the adjacent endoneurium entirely unstained.

In sectioned or crushed nerve there was a rapid and marked accumulation of extravascular labelled protein at the site of the lesion, the abnormal permeability persisting for several weeks after the nerve injury. Mellick and Cavanagh (1968) using albumin labelled with ^{131}I demonstrated the same changes in crushed chicken nerve, while Lampert, Garrow, and Pentschew (1970) have adduced electron microscopic evidence of increased vascular permeability of endoneurial capillaries in tellurium induced neuropathy. Krücke (1941) drew attention to the importance of increased capillary permeability as a cause of nerve oedema and believed that a serous infiltration of nerve led to a derangement of nerve fibre metabolism and a proliferation of intercellular connective tissue within the nerve sheath. Nerve oedema resulting from an increased permeability of endoneurial capillaries has also been incriminated as the causative factor of nerve damage in Bell's palsy (Jain and Sharma, 1964).

Fagerberg (1959) argued that occlusive lesions of the vasa nervorum produced by the deposition

[1] A preliminary communication of these results was made at the annual general meeting of the Association of British Neurologists—Dublin, April 1971.

of a hyaline, PAS staining material in the vascular endothelium were responsible for the nerve ischaemia that caused segmental demyelination in diabetic nerve, but Thomas and Lascelles (1966) and Dolman (1967) have drawn attention to the poor correlation that exists between the severity of the vascular lesion and the extent and distribution of the demyelinated fibres. There is, however, good evidence to suggest that microangiopathy is a cardinal feature of diabetes and that it is a generalized process involving vessels in many tissues. The characteristic morphological change seen in the diabetic capillary is a gross thickening of the basement membrane and such changes have been seen in diabetic capillaries in the human dermis (Aagenaes and Moe, 1961), retina (Toussaint and Dustin, 1963), glomerulus (Bergstrand and Bucht, 1957), and placenta (Burstein, Soule, and Blumenthal, 1957). Similar changes have been identified in the capillaries of some animal species where spontaneous diabetes is known to occur; in the spiny mouse (Orci, Stauffacher, Amherdt, Pictet, Renold, and Rouiller, 1970), the Japanese KK mouse (Camerini-Davalos, Offermann, Mittl, and Ehrenreich, 1970), and in the Chinese hamster (Federmann and Gerritsen, 1970). Capillary basement membrane changes are also seen when experimental diabetes is induced—whether by alloxan (Bloodworth, Engermann, and Powers, 1969), somatotrophin (Bloodworth and Molitor, 1969), or by pancreatectomy (Levene, Lazzarini-Robertson, Foglia, and Singer, 1963). Although the structural changes in diabetic vessels have been well documented, the functional effects of these changes have attracted less attention. Ditzel (1967) made direct *in vivo* observations of the reactions of small blood vessels in the bulbar conjunctiva, skin, and retina in diabetic subjects and reported severe functional changes in the microvasculature. These changes occurred early during the course of the disease and before the onset of clinical signs indicative of a microangiopathy. The common features observed were a loss of normal vascular tone, an increase in the permeability of the smaller vessels, and a decreased rate of blood flow through the microvasculature. The continuous seepage of plasma components through the endothelium led to an oedema of the perivascular tissues, and Ditzel considered this to be the precursor of the hyaline mucoid changes seen in the later stages of the disease. Churg, Mautner, Grishman, and Eisner (1962) have described the increased permeability of the diabetic glomerular capillary to plasma protein. Kohner, Dollery, Paterson, Oakley, and Frazer (1967) used fluorescein angiography to demonstrate the leakage of dye from diabetic retinal vessels, and more recently Trap-Jensen (1970) has used double indicator diffusion techniques to measure the increased diffusion capacity of capillaries in the skeletal muscle of longterm juvenile diabetics.

The experiments described below were undertaken to investigate the integrity of the blood nerve barriers in the alloxan diabetic rat nerve, since recent studies (Seneviratne and Peiris, 1968, 1969, 1970) have shown that the alloxan diabetic rat nerve exhibits many of the structural and functional changes that occur in human disease. The extent and distribution of the demyelination of peripheral nerve, the decreased conduction velocity of the nerve impulse, and the resistance of the nerve to inactivation by hypoxia are so comparable that the alloxan diabetic rat provides a satisfactory experimental animal model for the study of the human disease.

METHODS

Experimental diabetes was induced in 6 month old rats, weighing between 150 to 200 g, by a modification of the method described by Klebanoff and Greenbaum (1954). A single dose of alloxan monohydrate (B.D.H.) of 150 mg/kg body weight in freshly prepared 0·125 M citrate-phosphate buffer at pH 4·0 was injected intraperitoneally at a concentration of 10 mg/ml., and the rats maintained in separate metabolism cages with free access to food and water. The criteria used to establish diabetes in these animals were a blood glucose level of more than 200 mg/100 ml. and a glycosuria of over 1%. These animals were maintained for at least four weeks in this condition before being used for study. Litter mates of these rats and the alloxanized non-diabetic rats were maintained under the same condition and served as controls.

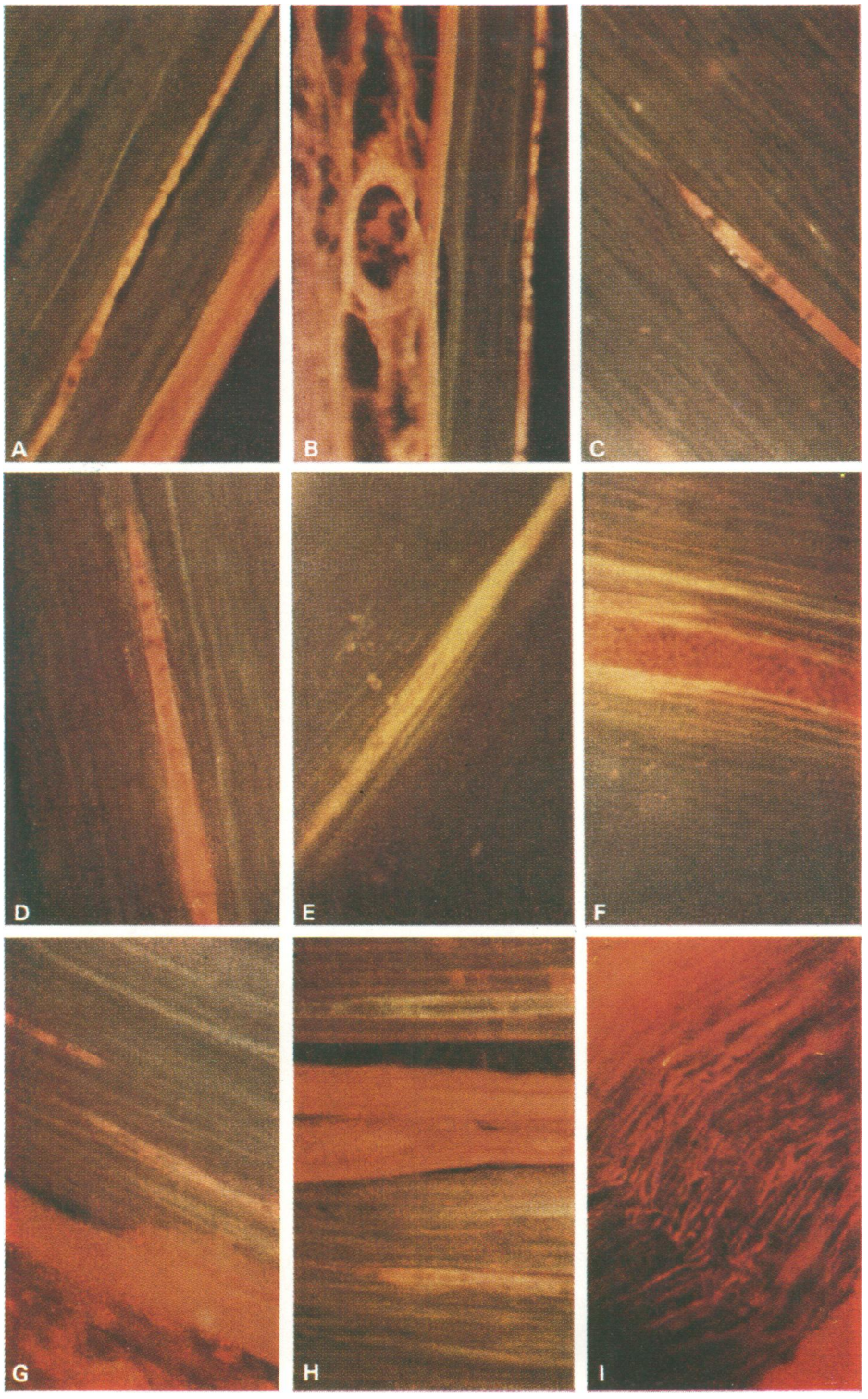
The technique for demonstrating vascular permeability change was based on the method used by Steinwell and Klatzo (1966). Each rat was injected intravenously with 1·0 ml./100 g body weight of a solution containing the freshly prepared and filtered Evans blue—albumin complex. Evans blue (T 1824, B.D.H., mol. wt. 982) 100 ml. in 0·01 M solution in physiological saline was conjugated with 8 g bovine crystalline albumin (B.D.H.) according to the method of Levick and Michel (1970) to ensure that the complex contained no free dye in solution. The sciatic nerves were removed after varying intervals of time and fixed in 5% buffered formalin for 24 hours.

Frozen longitudinal sections, 15 μ thick, were mounted in 50% aqueous glycerine and the fresh preparations examined in a Leitz fluorescent microscope in light from an Osram HBO 200 W, high pressure mercury lamp. The incident light was filtered through a Schott BG 12/3 mm filter and the emitted light through a combination of Schott OG 4 and GG 4 barrier filters. Selected specimens were photographed on 35 mm high speed Ektachrome colour film. In the control and diabetic rats the sciatic nerves were dissected out at time intervals varying from 15 minutes to six hours after the injection of the dye complex. In some experiments a further 2 ml. of the dye-albumin complex was injected into the space around the sciatic nerve in the thigh, and the nerves dissected out two hours after this second injection.

RESULTS

A representative selection of the results obtained in this study is illustrated in the colour plate. In A—a longitudinal section from a healthy rat nerve taken four hours after the intravenous injection of the albumin—the fluorescence of the dye complex is seen confined within an endoneurial capillary and no fluorescence is seen in or outside the vascular wall. The epi- and perineurial nerve sheath shows the presence of extravascular albumin extending to the innermost limit of the perineurium which it clearly demarcates from the adjacent part of the endoneurium. B is a section taken from a healthy rat where in addition to the intravenous injection the dye complex was also introduced into the space around the sciatic nerve and left *in situ* for two hours. This section, too, shows the fluorescence confined entirely within the endoneurial capillary, together with extensive staining of the connective tissue sheath of the nerve. The innermost layer of the perineurium remains clearly demarcated from the adjacent endoneurium. In C—a section from a diabetic rat, taken 15 minutes after intravenous injection of the dye complex—a small quantity of the fluorescing albumin can be seen immediately outside the endoneurial capillary. In D and E—sections taken from diabetic rats 30 minutes after intravenous injection of the dye—the albumin can be seen diffusing out through the endoneurial vessels. F—a section from a diabetic rat one hour after injection of the dye—shows a more extensive spread of the extravascular albumin tracking down longitudinally along the endoneurial spaces. A segment of the vessel seems markedly affected while the adjacent part remains relatively normal. G is a section taken from a diabetic rat two hours after the protein-dye complex had been injected intravenously and introduced into the space around the sciatic nerve in the thigh. The shorter segment of endoneurial capillary shows no evident abnormality; the longer stretch of vessel, however, shows faint traces of fluorescence within and immediately outside the vessel wall. The most evident change, however, is in the nerve sheath which fluoresces brightly with the extravascular protein. At its peripheral end, the innermost layers of the perineurium remain, as in the healthy nerve, clearly demarcated from the subjacent endoneurium. The central zone of the sheath shows an evident breach in the effectiveness of the perineurium as a diffusion barrier, for a high concentration of albumin can be seen within the endoneurial spaces, immediately adjacent to the external nerve sheath. H is a section from a diabetic rat taken two hours after the albumin dye complex had been injected intravenously and around the sciatic nerve. One leaking endoneurial capillary still contains some intravascular labelled protein while the other two are nearly empty, their walls being faintly outlined by traces of fluorescent material. The perineurium fluoresces brightly with the extravascular protein, but the integrity of the perineurial diffusion barrier remains intact, for its innermost laminae clearly demarcate it from the adjacent endoneurium. I—a section from a diabetic rat taken two hours after the introduction of albumin around the nerve in the thigh—shows massive infiltration of albumin across the perineurial barrier. Within the nerve parenchyma the albumin can be seen in high concentration tracking down along the longitudinal endoneurial spaces between the axons. This section, too, illustrates the patchy distribution of the perineurial permeability changes. The perineurium at the lower border shows no gross change, for the discrete junction between perineurium and endoneurium can be readily identified. At the upper border, however, the gross change of perineurial permeability has led to a massive influx of albumin across it, com-

PLATE *Longitudinal sections (15 μ) of sciatic nerve of control (A and B) and alloxan-diabetic (C–I) rats after intravenous injection of albumin-Evans blue conjugate. In animals B, G, H, and I the albumin dye conjugate was also introduced into the space around the sciatic nerve.*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pletely obscuring the junction between the sheath and endoneurium.

DISCUSSION

The results obtained in this study show that the healthy perineurium prevents the passage of albumin across its innermost laminae into the adjacent endoneurium. These findings confirm the work of numerous earlier investigators who have used a number of techniques to demonstrate that the external sheath of the nerve trunk acts as an effective diffusion barrier to a wide variety of substances applied to its surface. (Feng and Gerard, 1930; Bishop, 1932; Lundberg, 1951; Crescitelli, 1951; Krnjević, 1954; Shanes, 1954; Weiss and Rohlich, 1954; Emiroğlu, 1955; Martin, 1964; Waggener, Bunn, and Beggs, 1965; Olsson and Reese, 1971).

Histological studies on the perineurium have shown it to consist of several laminae of closely apposed flattened cells lined by basement membrane (Röhlich and Knoop, 1961; Shanthaverappa and Bourne, 1962; Cravioto, 1966; Burkel, 1967; Lieberman, 1968). The barrier properties of the perineurium have been attributed to the 'closed contacts' or 'tight junctions' which the adjacent epithelial cells make with one another. Usually the epithelial cells are 'dovetailed' one into another; on other occasions lateral processes from the perineurial cells make contact between neighbouring laminae fixing them together in tight apposition. At these sites the basement membranes are lost and the plasma membranes of the two cells are separated by a gap of 150 to 170 Å with a thin intervening line making up a 'three lined system' (Thomas, 1963). Near the endoneurium the perineurial lamellae completely encircle the nerve and the tight junctions generally form extensive and perhaps complete belts around the cells (Olsson and Reese, 1971).

The difference in permeability between the endoneurial vessels and those of the sheath raises the question whether these vessels display corresponding differences in structure. Key and Retzius (1876) drew attention to the fact that blood vessels passing through the perineurium to enter the endoneurium were covered by a sleeve of perineurial cells, but the electron micrographs of Burkel (1967) show clearly that this perineural sheath may often be a discontinuous one. Permeability studies of the blood brain barrier have led Lassen, Alexander, and Trap-Jensen (1970) to conclude that this barrier is likely to be sited at the luminal wall of the capillary endothelium, and that the blood brain barrier is much tighter than is the case for capillary membrane junctions in most other tissues. Pappenheimer (1970) has argued that cerebral capillaries behave as if diffusion and exchange occurred through effective interendothelial cell slits 10 to 15 Å wide instead of 50 to 60 Å as in other tissues. Olsson and Reese (1971) have used horse-radish peroxidase as a tracer to demonstrate the route by which substances escape from the lumen of sheath and endoneurial vessels of nerve and conclude that the interendothelial cell clefts of epineurial and perineurial vessels are wider than those found in the endoneurial vessels, the sheath vessels being of the 'open' variety similar to those found in cardiac and skeletal muscle. The presence of an effective blood-nerve diffusion barrier in the healthy rat would, no doubt, serve to maintain a special endoneurial micro-environment within the 'milieu intérieur', buffering the axons against the adverse effects which would result from any changes that might occur in the electrolyte constitution of the extracellular fluid pool of the body. Olsson (1968) has, however, shown that the presence of an effective barrier is not common to all species, and that the endoneurial capillaries of the guinea-pig, rabbit, and hen are permeable to protein, while Kristensson (1970) reports that the perineurium of the newborn mouse is permeable to protein for up to 12 days after birth. There is also good evidence (Waksmann, 1961; Olsson, 1968; Liebermann, 1968) that the dorsal root ganglia are unprotected by such a barrier, and that the ganglion has a much denser capillary plexus than peripheral nerve (Adams, 1942; Bergmann and Alexander, 1941). It is conceivable that these morphological differences between ganglia and nerve are related to the greater metabolic requirements of the ganglion; being the site of protein synthesis, it is likely that the presence of a diffusion barrier might impair the rate of transport of substrates to it.

The results obtained in this study show that there is a gross impairment of the integrity of the blood nerve barrier in the alloxan diabetic rat. Since the technique used allows of only a limited sampling of the peripheral nerves, no attempt has been made to correlate the extent of this impairment with the severity or duration of the diabetic state. All of 20 diabetic rats studied

showed an increase in the permeability of the endoneurial vessels and of the perineural barrier, and a characteristic feature was the patchy and discontinuous involvement of the vessels and nerve sheath. In a given segment of nerve, lengths of leaking capillary or perineurium lay interspersed among lengths whose function seemed unimpaired. In most segments the vessels were more defective than the sheath, and traces of extravasated protein could be detected as early as 15 minutes after injection. Since there are no lymphatics within the nerve trunk (Shdanow 1931), it is expected that any protein escaping from leaking vessels into the endoneurial space would tend to accumulate within it. There is, however, evidence for a steady proximodistal movement of endoneurial fluid within the nerve trunk (Weiss, Wang, Taylor, and Edds, 1945) and this would transport the protein extravasated within the proximal part of the nerve into the more distal and peripheral reaches of the nerve. The osmotic effects of extravasated protein would cause an endoneurial oedema and, where the perineurium is intact and inextensible, lead to a rise of interstitial cell pressure which would effectively inhibit capillary filtration and formation of endoneurial tissue fluid and result in tissue hypoxia and cellular damage. Since the metabolism of the Schwann cell is solely dependent on the integrity of this capillary microcirculation, a cessation of capillary filtration would reproduce the effects of maintained ischaemia. Mayer and Denny-Brown (1964) and Lundborg (1970) have shown that nerve ischaemia of six to 10 hours produces an irreversible conduction block and local loss of myelin sheaths, the axons themselves being preserved unless the ischaemia is much more extensive. This evidence suggests that the segmental demyelination of the alloxan diabetic rat (Preston, 1967; Hildebrand, Joffroy, Graff, and Coërs, 1968; Seneviratne and Peiris, 1970) is due to tissue hypoxia which occurs within a nerve trunk where an increase in the permeability of the endoneurial capillary to protein occurs in the presence of an intact perineurial sheath. There is evidence that segmental demyelination of nerve occurs in several disorders such as periarteritis nodosa (Lovshin and Kernohan, 1948), where there is widespread vascular damage, and in conditions such as experimental allergic neuritis and the Guillain-Barré syndrome where the activation of a specific complement (Vulpe, Hawkins, and Rozdilsky, 1960; Ratnoff and Lepow, 1963; Pette, Mannweiter, Palacios, and Mütze, 1965) causes an increase of vascular permeability which precedes the phase of cellular infiltration. The hypothesis outlined above would also explain the predominantly peripheral distribution, and the patchy, discontinuous, and 'segmental' nature of the demyelinating lesion. The special vulnerability of peripheral nerve at the common sites of nerve entrapment, at the carpal and ulnar tunnels and at the lower end of the bony facial canal are also likely to be due to the presence of a rigid and unyielding support around the nerve, which by restricting the extensibility of the oedematous nerve trunk, contributes to a further increase of intra-endoneurial pressure which hastens the onset of tissue hypoxia.

There is increasing evidence (Garner, 1970) that the microangiopathy of diabetes is associated with the production of a basement membrane, the physicochemical properties of which are significantly different from those which occur in the healthy state. The basement membrane mucopolysaccharides have the property of polymerizing in molecular groups of varying size linking up with proteins and giving rise to aggregates of varying complexity. The greater or lesser degree of polymerization and protein linkage directly determines the properties of permeability and strength. An increase in polymerization increases the viscosity of the fundamental substance while a reduction makes it more permeable and fluid (Businco, 1959).

Lazarow and Spiedel (1964) measured the rate of formation and replacement of basement membrane in glomerular capillaries of control and alloxan diabetic rats and found a significant reduction in the rate of basement membrane removal in the diabetic rat. They have argued that a slower rate of removal would provide a greater opportunity for the cross linkages between the protein constituents of the basement membrane which could account for the paradoxical observation of increased basement membrane thickness associated with increased permeability of the glomerular capillary. Bischoff (1968) has observed that a significant thickening of the Schwann cell basement membrane was the earliest recognizable change in diabetic nerve. Seneviratne, Peiris, and Weerasuriya (1971) suggest that the resistance of diabetic nerve to inactivation by ischaemia may be due to an increase in the permeability of the muco-

polysaccharide gap substance which occurs at the node of Ranvier, while the results reported in this study demonstrate an increased permeability of the 'tight junctions' between perineurial cells. These changes in the structure and properties of basement membranes of non-vascular structures draw attention to the likelihood that the diabetic process produces a more universal change in basement membrane function than has been hitherto recognized.

A part of the experimental work reported here was carried out at the University Laboratory of Physiology, Oxford, during the tenure of a Commonwealth Medical Fellowship. The author is indebted to Professor David Whitteridge, Wayneflete Professor of Physiology, University of Oxford, for facilities provided for this study, and to the Wellcome Trust for a research grant.

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