Histochemical studies on exudates of *Heterodera schachtii* (Nematoda : Heteroderidae) males

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SUMMARY

Histochemical tests on exudates, produced by males of the beet cyst nematode, *Heterodera schachtii*, showed that dyes more or less specific to proteins bound to amphidial, spicule and excretory pore exudates. For lectin binding experiments the method of exudate production was modified. Under these conditions excretory pore exudates were no longer produced, but observations of lectin binding showed that those from *Canavalia ensiformis* (Con A), *Triticum vulgare* (WGA) and *Helix pomatia* (HPA) bound specifically to the surface of amphidial and spicule exudates. The exudates of the main chemoreceptors thus consist most likely of protein backbones to which oligosaccharide chains are coupled superficially. Their possible function is discussed.

RÉSUMÉ

Études histochimiques sur les exsudats des mâles de Heterodera schachtii *(Nematoda : Heteroderidae)*

Des tests histochimiques sur les exsudats des mâles du nématode à kyste de la betterave *(Heterodera schachtii)* ont montré que des colorants plus ou moins spécifiques des protéines peuvent se fixer sur les exsudats des amphides, des spicules et du pore excréteur. Lors d'expériences sur les liaisons avec des lectines, la méthode de production des exsudats a été modifiée. De ce fait, les exsudats du pore excréteur n'ont plus été produits; mais les observations faites sur les liaisons avec les lectines ont montré que celles provenant de *Canavalia ensiformis* (Con A), *Triticum vulgare* (WGA) et *Helix pomatia* (HPA) se fixent spécifiquement à la surface des exsudats des amphides et des spicules. Les exsudats des principaux chemorécepteurs consistent donc essentiellement en un squelette principal protéique à la surface duquel sont fixées des chaînes d'oligosaccharides. Les fonctions possibles de ce dispositif sont discutées.

Nematode chemoreceptors are composed of nerve dendrites located in a cuticle-lined cavity in the head (amphids and inner labial sensilla) and tail region (phasmids) or in the spicules (spicule receptors, e.g. Wright, 1980; Coomans & De Grisse, 1981). The dendrites of these chemoreceptors (not yet proven for the spicules) are surrounded by viscous exudates produced in associated gland cells. In nematodes, at least in *Caenorhabditis elegans*, the excretory canal is also filled with an exudate synthesized in a gland cell (Nelson & Riddle, 1984). Little is yet known about the chemical composition and function of these exudates. Forrest and Robertson (1986) found specific binding sites of WGA on the amphidial exudate of second stage juveniles (J2) of *Globodera rostochiensis* and Forrest (1985) showed that lectin binding sites on this exudate can be reduced by the protease pronase E. In another electron microscopic study McClure and Stynes (1988) found UEA I binding sites on the amphidial exudate of *Meloidogyne incognita* J2. Aumann and Wyss (1987) detected specific binding sites of Con A, WGA, PNA, HPA and LFA in the region of the amphidial apertures, of Con A, WGA, PNA and HPA on the spicule tips, and of HPA in the region of the excretory pore opening of *Heterodera schachtii* males. As it was not possible to localize these binding sites definitely on the exudates, the method of Premachandran *et al.* (1988) was used and modified for inducing exudate production. Lectin binding experiments were then performed with the fluorochrome-conjugated lectins Con A, WGA, PNA, HPA and LFA from different specificity groups according to Goldstein (1981).

Materials and methods

NEMATODES

Males of *Heterodera schachtii* were obtained from aseptic root cultures of *Raphanus sativus* var. *oleiformis* cv. " Pegletta " (resistant cultivar) grown in the dark in a nutrient agar medium (Dropkin & Boone, 1966); for further details, see Wyss and Zunke (1986).

CHEMICALS

Organic solvents : Ethanol was purchased from a local dealer, toluene from Fluka (89681) and n-butyl acetate from Merck (9652). *Dyes :* Coomassie Brilliant Blue R-250 was from Serva (Serva Blue R, 35051) and aniline blue from Aldrich (methyl blue, 86,102-2). The cover-

slip sealer Glyceel (Hooper, 1986) was a gift from Dr. N. v. Mende, Department of Biological Sciences, University of Missouri. *Lectins :* The tetramethylrhodamine isothiocyanate (TRITC) labelled lectins Con A (L-3636) and WGA (L-5266) and the fluoresceine isothiocyanate (FITC) labelled PNA (L-7381) and HPA (L-1511) were from Sigma. TRITC-LFA (R-5101) was from E. Y. *Carbohydrates :* α-methylmannoside (α-ManMe, M-6882) and N-acetylgalactosamine (GalNAc, A-2795) were purchased from Sigma, and D-galactose (D-Gal, 4058) and N-acetylneuraminic acid (Neu5Ac, 24800) from Merck. The oligomers of N-acetylglucosamine (GlcNAc) were kindly provided by Dr. J. M. S. Forrest from the Scottish Crop Research Institute.

Induction of exudate production for lectin binding experiments

Freshly emerged aseptic males were washed twice for 10 min each in 0.1 $mol \cdot l^{-1}$ phosphate buffered saline, pH 6.8, with 1 $mmol \cdot l^{-1}$ $CaCl_2$ and $MnCl_2$ (PBS). Then they were transferred into the well of a glass microscope slide, filled with 80 µl of a solution of 0.05 % Coomassie Brilliant Blue R-250 in distilled water. The slides were placed on the lid of a Petri dish (90 mm diam.) within a second dish (145 mm diam.). The lid was surrounded by a mixture of ethanol, toluene and n-butyl acetate (1:2:2). The nematodes were kept in the saturated atmosphere for 20-26 h during which time they produced amphidial and spicule exudates that became visible as blue stained strands emanating from the apertures. Nematodes that produced visible exudates were removed from the staining solution with a needle and immediately washed twice in PBS (for Con A, WGA, PNA and HPA binding experiments) or in 0.1 $mol \cdot l^{-1}$ tris buffered saline, pH 8.0, with 10 $mmol \cdot l^{-1}$ $CaCl_2$ (TBS, for LFA binding experiments). The exudate strands broke off during handling. All procedures were performed at room temperature.

Lectin binding

The nematodes were transferred from the washing buffer into a drop (30 µl) of lectin solution (100 $\mu g \cdot ml^{-1}$) in PBS (Con A, WGA, PNA and HPA) or TBS (LFA) on a glass microscope slide and incubated for 19-24 h in a moisture chamber at room temperature. During incubation in lectin solutions the males continued to produce exudates, but to a lesser extent than during the organic solvent treatment. They became visible as drop-like emanations from the amphidial apertures and spicule tips.

Controls of specificity of lectin binding

Control experiments were performed by incubating the lectins (50 $\mu g \cdot ml^{-1}$) first at 37° (see Aumann and Wyss, 1987) for 90 min with the appropriate inhibitory carbohydrates (Tab. 1). The following solutions were tested : TRITC-Con A with 200 $mmol \cdot l^{-1}$ α-methylmannoside, TRITC-WGA with 15 $mg \cdot ml^{-1}$ N-acetylglucosamine oligomers, FITC-PNA with 200, 300, 400 and 500 $mmol \cdot l^{-1}$ D-galactose, FITC-HPA with 200 $mmol \cdot l^{-1}$ N-acetylgalactosamine and TRITC-LFA with 200, 300 and 400 $mmol \cdot l^{-1}$ N-acetylneuraminic acid. The nematodes were then incubated with these solutions as described under Lectin binding.

Table 1

Sugar specificities of the lectins and inhibitory sugars tested in control experiments

Lectin	*Sugar specificity**	*Inhibitory sugars tested*
Con A	Man-α-1, 2-Man-α-1, 2-Man > Man-α-1, 2-Man > α-Man > α-Glc ⩾ αGlcNAc	α-ManMe
WGA	GlcNAc (β-1, 4-GlcNAc)$_{1-2}$ ⩾ β-GlcNAc > Neu5Ac	oligomers of GlcNAc
PNA	D-Gal-β-1, 3-GalNAc > α- and β-Gal	D-Gal
HPA	GalNAc-α-1, 3-GalNAc > α-GalNAc > GalNac α-GlcNAc ⩾ α-Gal	GalNAc
LFA	α-Neu5Ac > α-N-glycolylneuraminic acid	Neu5Ac

* According to Goldstein and Poretz (1986).

Binding of histochemical dyes

Aseptic freshly emerged males were washed twice for 10 min each in PBS and transferred into a drop (40 µl) of 0.05 % Coomassie Brilliant Blue R-250 or aniline blue in distilled water on a glass microscope slide. The dye solution was surrounded by a circle of Glyceel (see Premachandran *et al.*, 1988) onto which a coverslip was placed. The slides were then incubated for 20-23 h at room temperature before light microscopic examination. The dyes were used to visualize the nematode exudates produced during incubation.

Microscopy

The dye-stained nematodes were examined under a Reichert-Jung Polyvar photomicroscope with Nomarski interference microscopy at 100 to 1 000 fold magnification. Binding of fluorochrome-conjugated lectins was visualized with an epifluorescence system at 400 to 1 000 fold magnification. TRITC-conjugated lectins were examined with a green filter IG2 (exciter filter BP 520-560 nm, splitting filter DS 580 nm and barrier filter LP 590 nm) and FITC-conjugated lectins with a blue filter IB1 (BP 450-495 nm, DS 510 nm and LP 520 nm).

The fluorescence of FITC-lectins appeared green, whereas the autofluorescence was yellowish. Secondary fluorescence of TRITC-conjugated lectins was visible as a strong red colour and the autofluorescence appeared as a pale red. Photomicrographs were taken on 50 ASA Kodak daylight colour films (lectins and dyes) or on Agfa PAN 100 black-white films (Nomarski interference microscopy). The lectin and dye binding experiments were repeated at least three times, each with ten nematodes, whereas the specificity controls of lectin binding were performed with at least ten males per treatment.

Results

Table 2 shows the binding of different lectins to the amphidial and spicule exudates and to the excretory pore opening of *Heterodera schachtii* males, together with the results of the specificity controls of lectin binding. All lectins tested bound to the amphidial and spicule exudates. In specimens where the excretory pore was visible only PNA bound to the region of the opening. Apart from LFA that bound only to 25 % of the nematodes tested, nearly all specimens were labelled at the amphidial exudates. The majority of the nematodes, with the exception of LFA, were also labelled at the spicule exudates. The binding of Con A, HPA and WGA was carbohydrate-specific, because the lectin binding was completely prevented with the usual concentration of the appropriate inhibitory sugars (Tab. 1). The binding of PNA and LFA, visualized as the intensity of fluorescence, was reduced in the presence of increasing concentrations of D-galactose and N-acetylneuraminic acid respectively (data not shown).

The exudate production of nematodes decreased markedly in the presence of inhibitory carbohydrates

Table 2

Binding of lectins (capital) to exudates of *Heterodera schachtii* males and controls of binding specificity

Treatment	*n*	*Labelling*		
		amphidial exudates	*spicule exudates*	*excretory pore opening*
TRITC-Con A	30	30	17	0
TRITC-Con A + 200 mmol·l^{-1} α-ManMe	27	2	0	0
TRITC-WGA	30	25	30	0
TRITC-WGA + 15 mg·ml^{-1} GlcNAc-oligomers	16	0	0	0
FITC-PNA	30	30	28	21
FITC-PNA + 200 mmol·l^{-1} D-Gal	20	17	9	9
FITC-PNA + 300 mmol·l^{-1} D-Gal	10	10	6	9
FITC-PNA + 400 mmol·l^{-1} D-Gal	10	8	2	3
FITC-PNA + 500 mmol·l^{-1} D-Gal	10	10	3	4
FITC-HPA	30	30	25	1
FITC-HPA + 200 mmol·l^{-1} GalNAc	10	0	0	0
TRITC-LFA	60	15	5	0
TRITC-LFA + 200 mmol·l^{-1} Neu5Ac	10	3	0	0
TRITC-LFA + 300 mmol·l^{-1} Neu5Ac	20	15	2	0
TRITC-LFA + 400 mmol·l^{-1} Neu5Ac	20	9	4	0

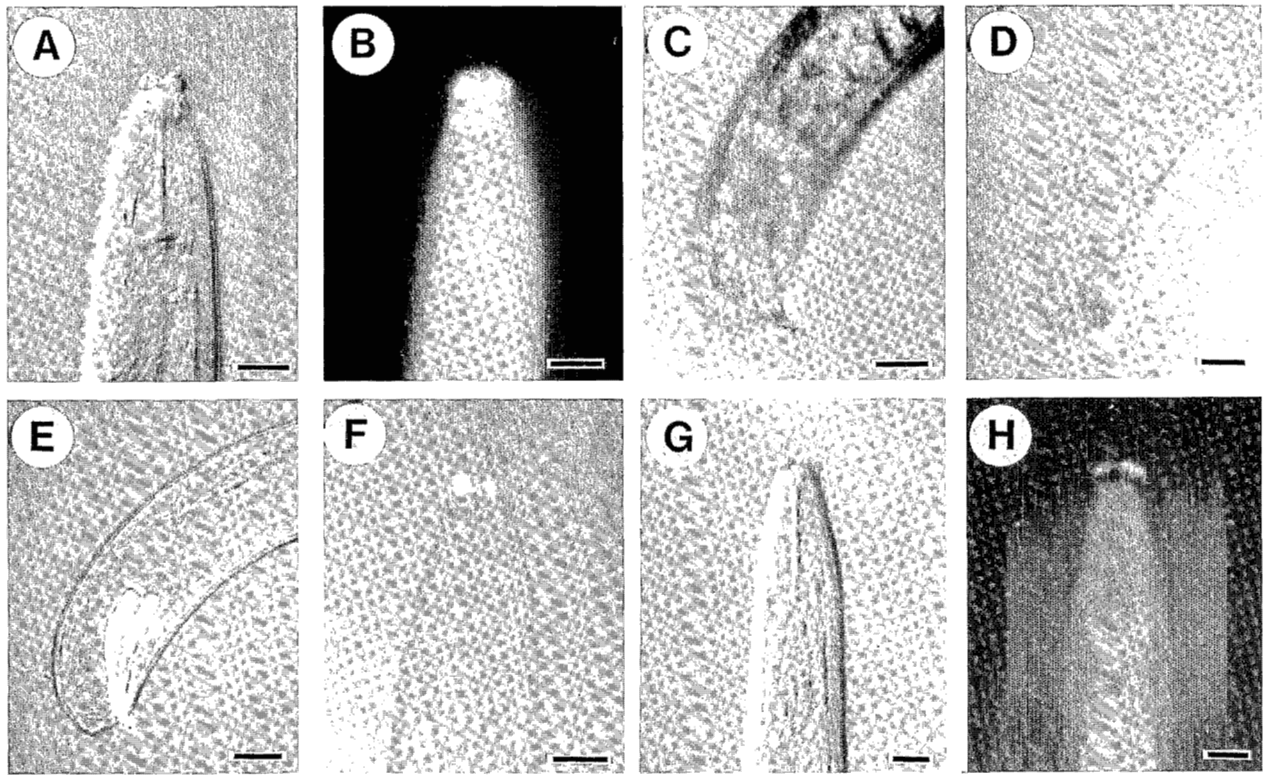

Fig. 1. Binding of fluorochrome-conjugated lectins to exudates of *Heterodera schachtii* males. A, B : Binding of TRITC-Con A to amphidial exudates (A : Nomarski interference microscopy; B : fluorescence microscopy). C, D : Binding of TRITC-WGA to spicule exudates (C : Nomarski interference microscopy; D : fluorescence microscopy). E : Binding of FITC-PNA to spicule exudates; F : Binding of FITC-HPA to amphidial exudates; G, H : Binding of TRITC-LFA to amphidial exudates (G : Nomarski interference microscopy; H : fluorescence microscopy). Bars = 10 µm.

and also when the males were treated with LFA. Figure 1, A-H shows some selected typical binding patterns of FITC- and TRITC-conjugated lectins at the amphidial and spicule exudates of *H. schachtii* males.

Table 3 presents the binding of the dyes aniline blue and Coomassie Brilliant Blue R-250 to exudates of *H. schachtii* males under the influence of Glyceel solvents. In contrast to the lectin binding experiments, where the nematodes produced only amphidial and spicule exudates, the production of excretory pore exudates was now also induced. Fig. 2 shows binding to the amphidial exudates (Fig. 2, A), excretory pore exudate (Fig. 2, B) and spicule exudates (Fig. 2, C & D). Most of the nematodes examined produced abundant exudates and all exudates were stained with the dyes.

Discussion

Following the first report of a lectin binding site on a nematode (Nordbring-Hertz & Mattiasson, 1979),

Table 3

Binding of histochemical dyes to exudates of *Heterodera schachtii* males

Dye	*n*	*Exudate staining*		
		Amphids	*Spicules*	*Excretory pore*
Aniline blue	32	30	32	28
Coomassie Brilliant Blue R-250	46	43	46	42

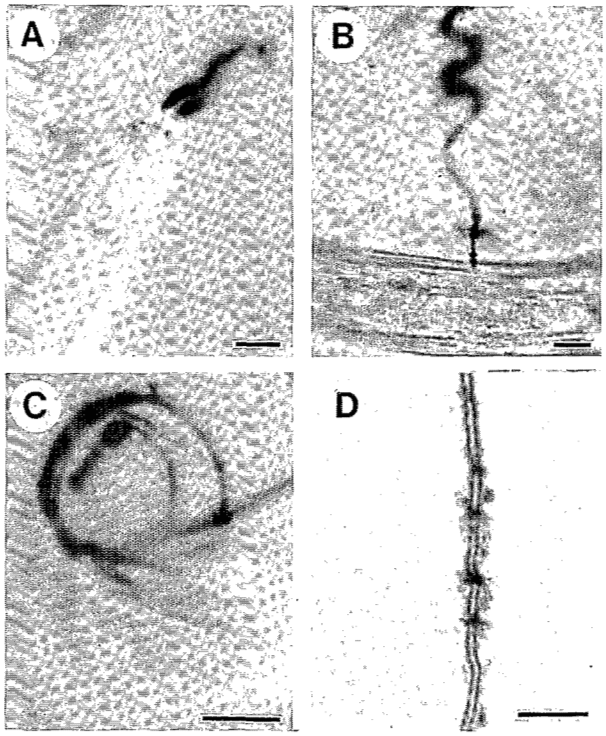

Fig. 2. Binding of histochemical dyes to exudates of *Heterodera schachtii* males. A : Binding of Coomassie Blue to the amphidial exudates; B : Binding of Coomassie Blue to the excretory pore exudate; C, D : Binding of aniline blue to spicule exudates; the two strands, one per spicule pore, are clearly visible. Bars = 10 µm.

similar reports were published, especially in recent years (for a review see Jansson, 1987). Among these Forrest and Robertson (1986) were the first to demonstrate the binding of a lectin, WGA, on the amphidial exudate of freshly hatched juveniles of the potato cyst nematode *Globodera rostochiensis*. McClure and Stynes (1988) found binding sites of different lectins in the region of the amphidial apertures of several races of *Meloidogyne* species (J2) and of the lectin UEA I on the amphid exudate of *M. incognita*. Spiegel, Cohn and Spiegel (1982) showed the binding of a sialic acid-specific reagent in the region of the amphidial apertures of the dagger nematode *Xiphinema index*. We detected binding sites of Con A, WGA, PNA, HPA and LFA in the region of the amphidial apertures of juveniles and males of the beet cyst nematode, *Heterodera schachtii* (Aumann & Wyss, 1987). Furthermore we found binding sites of Con A, WGA, PNA and HPA at the spicule tips of males and binding sites of Con A (only in J2) and HPA in the region of the excretory pore opening. It seemed that these binding sites were located on the corresponding exudates, but unequivocal proof was not possible by light microscopy.

Premachandran *et al.* (1988) described a method for inducing exudate production of nematodes under sealed coverslips. As the exudates break off immediately even with slight turbulence when the coverslip is lifted and the removal of nematodes with intact exudates is virtually impossible, we modified this method as described. The stimulus for producing exudates persists for several days so that lectin binding experiments can be performed with carefully washed nematodes.

The results of the present study show that the lectin binding sites found earlier around the openings of the amphidial pore and spicules (Aumann & Wyss, 1987) are actually located on the exudates. However, some differences in lectin binding patterns occurred. The most striking was that lectin binding in the region of the excretory pore opening in our previous study was restricted to HPA, whereas here only PNA (with the exception of one male labelled by HPA) bound to this area. We cannot explain this change at the present time; perhaps the organic solvent mixture may have enhanced production of a special component of the excretory gland cell. It is still a matter of speculation how dead nematodes, killed during exposure to the solvents, continue producing exudates.

In the present study the specificity controls of lectin binding gave more conclusive results. Previously (Aumann & Wyss, 1987), only a reduction of fluorescence intensity of fluorochrome-conjugated lectins was observed when exposed to increasing concentrations of the appropriate inhibitory sugars (up to 800 mmol·l^{-1}). A total inhibition of binding could in most cases only be achieved at a high concentration (1 mol·l^{-1}). Now, however, a total inhibition of binding of Con A and HPA was already possible in the presence of each 200 mmol ·l^{-1} α-methylmannoside and N-acetylgalactosamine and a more or less equivalent concentration of GlcNAc oligomers. An obvious reduction of fluorescence intensity of PNA and LFA was visible in the presence of at least 200 mmol·l^{-1} D-galactose or N-acetylneuraminic acid. A possible explanation for this reduction of the minimal concentration necessary to block lectin binding totally may be the extended incubation period of nematodes with the lectin-carbohydrate solutions. The binding of the dyes aniline blue and Coomassie Brilliant Blue R-250 on the exudates of the amphids, spicules and excretory pore suggests that the exudates consist partially of proteins. Coomassie Blue has a high protein specificity (Bradford, 1976; Wolf & Fric, 1981), whereas aniline blue binds to a broader range of tissue compounds (Mallory, 1900; Crossomon, 1937). The two strands emanating from the spicule tips in Fig. 2, D show to our knowledge for the first time that dendrites in spicules are also surrounded by an exudate like that in the amphids, inner labial sensilla and phasmids. The reason for the differences in exudate production between the nematodes sealed with Glyceel and those treated with the organic solvents are not clear. The males

sealed with Glyceel produced excretory pore exudates, whereas those treated with the organic solvents failed to do so. Possibly, the Glyceel compound methanol may play a role in inducing the production of excretory pore exudates.

The results with the lectin and dye binding sites on the exudates indicate, that these are composed of glycoproteins. Possibly, they consist of a protein backbone to which oligosaccharide chains are coupled superficially. The partial protein and carbohydrate nature of the amphidial exudate of J2 of potato cyst nematodes was shown by Forrest (1985). We observed a reduction of lectin binding sites in the regions of exudate contact after *H. schachtii* males had been treated with chymotrypsin, trypsin and pronase E (unpubl.). Veech, Starr and Nordgren (1987) analyzed the amino acid composition of the stylet exudate of *M. incognita* females and found a relatively high level of serine. This indicates that not the N-glycosidic linkeage between an N-acetylhexosamine and asparagine, but rather the O-glycosidic linkeage of N-acetylgalatosamine and serine or threonine prevails in proteincarbohydrate bonds (Kornfeld & Kornfeld, 1985). The carbohydrate composition of amphidial and spicule exudates, as tested by lecpn binding patterns, is apparently identical, but it differs markedly from that of the excretory pore exudate. As lectins are ,t specific for one sugar only, sophisticated experiments (e.g. destruction of the sugars with specific enzymes) are necessary xr an exact characterization of the carbohydrates on the exudate surface.

Any discussion of the function of these exudates is still speculative. Wright (1980) described parallels in the chemoreceptor morphology between arthropods and nematodes, and assumed that the exudates may protect the dendrites of the chemosensory nerves. The pores of insect chemosensilla in direct contact with the environment also contain partially viscous exudates (Zacharuk, 1980) which, according to Altner (1977), may serve both for stimulus transmission to the dendrite membranes and as a protectant against dehydration. In nematodes the exudates surrounding chemoreceptive dendrites probably function primarily as protectants against microbial metabolites to which, in the soil, they are continuously exposed. At the moment we stillhesitate to assign them a major function in chemoreception as preliminary biotests of sugar blockings with lectins did not affect the orientation of *H. schachtii* males to the female pheromone. Out of the five lectins that bound to the exudates in the present study, only HPA showed a slight effect (unpublished results). The function of the excretory pore exudate, like the excretory system itself, is completely unknown. Nelson and Riddle (1984), who ablated single cells in the excretory system in *Caenorhabditis elegans* by laser microbeam concluded that one function may be osmoregulation. So the pore exudate may act as a carrier of osmotically significant substances. Bird, Bonig and Bacic (1988) hypothesized that one function of the excretory system in secernentean nematodes may be the secretion of the glycocalyx covering the cuticle.

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