Influence of Changes in the Nurse Cell System (Syncytium) on the Development of the Cyst Nematode *Heterodera schachtii*: Single Amino Acids

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ABSTRACT

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The metabolism of seedling roots of *Brassica rapa* var. *silvestris* f. *campestris* 'Stielmus' was manipulated to investigate the influence of amino acids on the development of the beet cyst nematode, *Heterodera schachtii*. After inoculation of single J2 juveniles to germinated roots in water agar drops under aseptic conditions, 50% of the seedlings were decapitated by removing shoot and root tips. One day later, nutrient solutions containing various amounts of sucrose were flooded over the drops. In this way, a total of 10 variants was obtained, out of which seven ("+" variants) supported and three ("−" variants) inhibited female development. Using sensitive micromethods, we determined changes in the concentrations of 19 amino acids in the syncytia (nurse cell systems) and adjacent root segments, as well as in segments of uninoculated control roots. Glutamine appeared to play a beneficial role in the "+" variants, whereas methionine, phenylalanine, lysine, and tryptophan played harmful roles in the "−" variants. Changes in the concentrations of the 14 other amino acids had no influence on nematode development.

Additional keywords: autoradiography, dansyl chloride.

Knowledge of the beneficial or harmful effects of specific nutrients for sedentary root nematodes would be of great help in understanding this host-parasite relationship. For some bacteriovorous and fungivorous nematodes, essential nutritional requirements can be defined by a variation of compounds in axenic culture media, as reviewed by Vanfleteren (21) and Bolla (3).

Attempts to axenically cultivate sedentary root nematodes like *Heterodera schachtii* Schmidt will be unlikely to succeed because the development of these parasites depends on the induction and maintenance of a syncytium, a very complex nurse cell system, in host root tissue. So far, the only possible way to examine nutrient demands is to influence the nematode's development by a manipulation of host conditions (7) and to determine associated changes in available nutrients in the syncytium itself. We concentrated our investigations on amino acids, because it is known that they influence the development of nematodes. For instance, 10 amino acids are essential for the development of the bacteriovorous nematode, *Caenorhabditis briggsae* Dougherty & Nigon (20). A fungivorous *Aphelenchoides* sp. also requires specific amino acids for reproduction (1).

For the cyst nematode, *Globodera rostochiensis* Wollenw., the D- and L-forms of methionine were equally toxic when applied to potato plants grown in pots (5). In addition, DL-tyrosine changed the sex ratio in favor of males (18). Krauthausen (8) and Krauthausen and Wyss (9) examined the relative changes in the composition of amino acids in syncytia (in this case with *H. schachtii*). To determine possible "beneficial" or "harmful" effects of amino acids on the development of *H. schachtii*, a quantitative analysis in syncytia is necessary. For this reason, we used micromethods for a reliable quantification of 19 amino acids in seedling roots of a host plant grown in aseptic agar culture. Three aspects of the influence of amino acids were examined: 1) quantification of amino acids in syncytia of young females and adjacent distal (with reference to the root tip) and proximal root segments, as well as segments of uninoculated roots of identical age, 2) quantification of amino acids in uninoculated root segments, exposed to various supportive and inhibitory nutrient solutions at different intervals during the first 2 wk after germination, and 3) manipulation of nematode development by applying selected amino acids.

MATERIALS AND METHODS

The cultivation of *H. schachtii* on seedling roots of *Brassica rapa* L. var. *silvestris* Lam. f. *campestris* 'Stielmus' at a constant temperature of 25 C, the preparation of the feeding sites and control segments, the extraction processes, and the protein and amino acid determination (whole amounts) were as described by Grundler et al (7).

Quantitative determination of single amino acids. To determine amino acid amounts quantitatively on the necessary microscale, the dansyl chloride method (9,14) was modified as follows: Extracts with high amino acid contents were evaluated fluorimetrically. For some samples with low amino acid contents, a more sensitive autoradiographic technique had to be applied (10). The concentrations in the following description refer to the fluorimetric method only. For autoradiography, one-third of the concentrations were used. Depending on their whole amino acid contents, samples were diluted or concentrated to 1.67 nmol/μl of extract. Three microliters of these defined extracts (two replicates) were lyophilized and then dissolved in 6 μl of 0.1 M K_2CO_3 buffer (pH 10.5). Two microliters of amino acid standard (10 mM α-phenylglycine) and 16 μl of acetone were added. After 1 hr of protein precipitation at −20 C, followed by 15 min of centrifugation at 13,800 *g*, 20 μl of the supernatant was mixed with 4 μl of 3.3 mM dansyl chloride (or 1.1 mM ^{14}C-dansyl chloride, spec. activity 101 mCi/mmol). The dansylation reaction at 37 C was stopped after 30 min by adding 4 μl of diethylamine (1:10 in acetone).

The dansyl-amino acids were separated in two dimensions on polyamide sheets (9). The autoradiographs were made according to Marx et al (10), with slight modifications (2). The polyamide sheets were evaluated by fluorescence scanning photometry with the aid of computerized digital picture analysis (15). The ab-

sorbance of the spots of the autoradiographs was measured with the same computer equipment. To quantify the amino acid values, calibration curves for each amino acid with both evaluation techniques were established (2).

Effect of selected amino acids on the development of *H. schachtii*. The five amino acids that showed a correlation to nematode development were applied at different concentrations to intact seedlings of *B. r. silvestris campestris* grown in agar drops according to the method of Grundler et al (7). Each seedling had been inoculated with a single J2 juvenile 2 days before. The "beneficial" amino acid, glutamine, was added to the nutrient solution of a "−" variant (i.e., one with few females and many juveniles stagnated at the J2/J3 stage). In this case, the medium was that of Dropkin and Boone (4), without sucrose, applied to intact seedlings. The four "harmful" amino acids (methionine, phenylalanine, lysine, and tryptophan) were added to a "+" variant, i.e., many females, few stagnated J2/J3 juveniles. In this case, intact seedlings were in distilled water. Ten days after the addition of the amino acids, the seedlings were fixed, stained, and examined for developmental stages as described by Grundler et al (7).

The effect of glutamine (800 mg/L) was tested on 80 seedlings and that of the four other amino acids at two concentrations (200 and 300 mg/L for methionine, phenylalanine, and lysine; 100 and 150 mg/L for tryptophan) on 160 seedlings. The number of controls was 80 and 160, respectively. The number of replicates per variant and the statistical evaluations were as described previously (7).

RESULTS

Quantification of amino acids 12 days after nematode inoculation. There were three relationships between amino acid concentration and nematode development. First, one amino acid, glutamine, was associated with a positive influence. Second, four amino acids (methionine, phenylalanine, lysine, and tryptophan) were associated with a negative influence (Fig. 1). Third, concentrations of 14 amino acids were not related to nematode development. These were proline, valine, alanine, glycine, glutamic acid, aspartic acid, isoleucine, leucine, ornithine, histidine, asparagine, serine, hydroxyproline, and tyrosine.

The beneficial effect of glutamine was deduced from the differences in concentrations that were found: "+" variants with high percentages of females and few stagnated J2/J3 juveniles showed high glutamine concentrations in segments of uninoculated as well as in inoculated roots. The only exception was the variant "distilled water, intact" (Fig. 1). In contrast, uninoculated root segments of "−" variants (which gave low percentages of females and many stagnated J2/J3 juveniles when inoculated) had a low glutamine content. However, in the very few syncytia of females that developed in the "−" variants, the glutamine concentration was raised compared with uninoculated control segments and approached the values of some "+" variants so closely that there was no significant difference.

The harmful effects of the four amino acids, methionine, phenylalanine, lysine, and tryptophan (Fig. 1), were deduced from a reverse situation: the seven "+" variants had, in most cases, significantly lower concentrations of these four amino acids than the "−" variants, whether inoculated or not. In the very few cases where females were able to develop in the "−" variants, the concentrations of the amino acids in the syncytia were less than in uninoculated control segments.

Quantification of amino acids in uninoculated root segments at different intervals during the first 2 wk. Of the 19 amino acids evaluated, only the beneficial amino acid, glutamine, and the harmful amino acids, methionine, phenylalanine, lysine, and tryptophan, showed significant changes that correlated with the variants that either supported or inhibited female development. Data (*not shown*) on these five amino acids can be summarized as follows. The concentration of the beneficial amino acid, glutamine, increased in the two variants, which contained 2% sucrose from the fifth day on. The differences between "+" and "−" variants were significant on the eighth and 14th days. The "+" variant (distilled water, intact) had similar low values to the three "−" variants from the seventh day on. All four harmful amino acids showed a similar pattern. Their concentrations increased in the "−" variants from the fifth day on but generally remained low in the "+" variants. In most cases, the differences between the "+" and "−" variants were significant from the eighth day on.

Manipulation of nematode development by applying selected amino acids. The individual influences of the five amino acids with deduced effects on the development of *H. schachtii* are shown in Table 1. Glutamine added to the selected "−" variant (i.e., intact seedlings without sucrose) clearly supported female development. In the control of this variant, two-thirds of the invaded J2 juveniles stagnated at an early developmental stage. The four harmful amino acids, methionine, phenylalanine, lysine, and tryptophan, added to a selected "+" variant (distilled water, intact), inhibited female development at both concentrations tested with no significant difference between the concentrations.

DISCUSSION

As an obligate parasite, the sedentary cyst nematode *H. schachtii* induces and maintains a highly specialized nurse cell system (syncytium), which serves as a continuous nutrient supply throughout development. Müller et al (11) calculated that females require about 40 times more food than males, which stop feeding after termination of the J3 developmental stage. Quality and quantity of nutrients in the syncytium may influence nematode development in two ways: male formation may be favored or juveniles may fail to develop beyond the early stages. Both are possible strategies by means of which plants may exercise resistance reactions.

The agar drop fluid culture with singly inoculated J2 juveniles, described by Grundler et al (7), where stagnation in female development predominated in some variants, proved to be a suitable system for examination of the effects of nutrient components on the development of *H. schachtii*. Although 14 out of 19 amino acids evaluated could not be associated with either beneficial or harmful effects, it would be premature to declare them unimportant in nematode development. Only under the specific experimental conditions described was no correlation evident. However, it cannot be excluded that some of these amino acids may become a limiting factor in lower or higher concentrations.

The divergent results in one "+" variant (distilled water, intact) in which the concentration of the beneficial amino acid, glutamine, was similar to that in the "−" variants, and in which the harmful amino acids methionine and phenylalanine were increased, underlines the possible importance of the ratio of the amino acid concentrations to each other. If the ratios of all four harmful amino acids (methionine, phenylalanine, lysine, and tryptophan) to glutamine is calculated, it becomes evident that the abovementioned "+" variant has a position between the other six "+" and the three "−" variants. In two of the "−" variants (distilled water, decapitated and nutrient solution without sucrose, intact) the ratio values in the uninoculated root segments were significantly higher than in all other variants. However, in root segments in which some nematodes occasionally were able to establish a syncytium supporting female development, the ratios were much reduced. The combined influence of the harmful amino acids thus appears to be more decisive in inhibiting female development than low glutamine values.

Figure 2 shows the changes in the sum of these four amino acids in uninoculated root segments. In the "−" variants, a marked increase occurred from the fifth day on, whereas the level in the "+" variants remained more or less at the same level or was reduced. For glutamine, a significant difference between the "+" and "−" variants becomes discernible from the eighth day on. The nematodes were always inoculated 2 days after germination and would have encountered the first changes in nutrient concentrations after decapitation (1 day after inoculation) and the subsequent addition (1 day after decapitation) of the nutrient

solutions. After invading roots, J2 juveniles of *H. schachtii* have to feed from the developing syncytium for at least 3 days at a constant temperature of 25 C before they molt to the J3 stage, which reinitiates feeding 1 day later (U. Wyss, *unpublished*). This means that the marked increase in the total of the harmful amino acids on the fifth day after germination will have occurred at a time during which the J2 juveniles were still feeding (corresponding to 3 days after nematode inoculation in experiment I). Grundler's investigations (6) have shown that the future sex of the developing J2 juveniles is already irreversibly determined before they molt; thus, they are confronted with the drastic change in amino acid concentrations at a stage when sex has already been determined as female, as can be concluded from experiment I. Favorable conditions for sex determination toward the female apparently predominate during the first 2 or 3 days after nematode invasion in all 10 variants (7). Also, the results from experiment 3 can be considered as a confirmation that the female sex of the juveniles is in most cases already determined before the

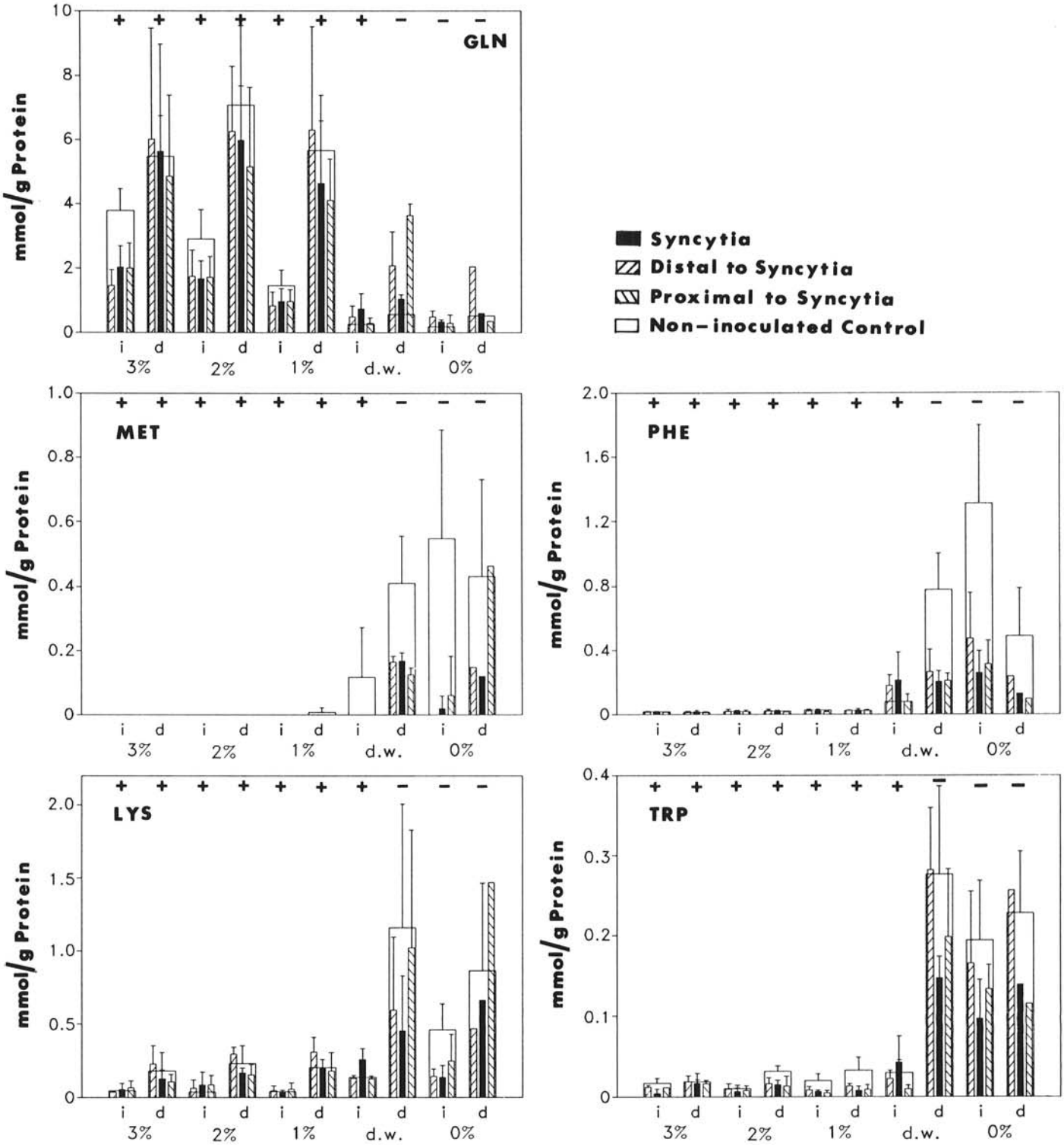

Fig. 1. Concentrations of amino acids in seedling roots of *Brassica rapa* var. *silvestris* f. *campestris* 'Stielmus' inoculated with *Heterodera schachtii*, and in uninoculated roots 12 days after inoculation. Seedlings were cultured in the aseptic agar drop fluid culture system at 25 C in the dark. The values are means ± standard deviation. Abbreviations are as follows: i = intact seedlings; d = decapitated seedlings; + = "+" variants; − = "−" variants; 0, 1, 2, and 3% = sucrose concentration in the nutrient solution; d.w. = distilled water.

addition of the selected amino acids.

The means by which the five amino acids affect nematode development remains unknown. The necessity of glutamine as an important compound in syncytium and nematode metabolism is conceivable (for instance, as an amino donor in some reactions). Krauthausen and Wyss (9) have shown that the relative amount of glutamine in the total pool of amino acids remains at a markedly low level in the syncytia of *H. schachtii* in two cruciferous host plants throughout female development. In our "+" variant "3% sucrose, intact," which more or less corresponded to the culture conditions of Krauthausen and Wyss (9), the concentration pattern of this amino acid (8) in syncytia and uninoculated root segments was similar. This indicates that glutamine can indeed be considered as one of the key amino acids.

Methionine, lysine, phenylalanine, and tryptophan, classified as harmful, may either have a negative influence on syncytium metabolism or may be toxic to the nematodes. In particular, methionine has an inhibitory effect on the development of different nematode species, as shown by Prasad and Webster (16) for *Heterodera avenae* Wollenweber and *Aphelenchoides ritzemabosi* (Schwartz) Steiner & Behrer; Evans and Trudgill (5) and Trudgill (18) for *Globodera rostochiensis*, and Tsai and van Gundy (19) for *Meloidogyne incognita* (Kofoid & White) Chitwood.

All of these investigations were based on an indirect manipulation of the nematode's development by applying the amino acids to the host plants, while experiments I and II in our paper can be considered as a combination of indirect and direct intervention: indirect because the amino acid concentrations could only be varied by a manipulation of the host-metabolism; direct because the quantification of the amino acids in the syncytium allowed a correlation with nematode development similar to that which would be possible in axenic culture media. Under such conditions, Myers and Balasubramanian (12) reported a stagnation in the development of the fungivorous nematode *Aphelenchoides rutgersi* Hooper & Myers with high histidine and tryptophan concentrations as well as a slight decline in nematode numbers with a α-phenylalanine concentration exceeding 150 mg/L. Methionine, however, supported reproduction of this nematode even at high concentrations, which indicates obvious differences between different plant parasitic nematode species in their sensitivity to amino acids.

Besides a trophic and also possibly a toxic function, amino acids may be involved in excretory processes in nematode metabolism. *Caenorhabditis briggsae* eliminates, for instance, a high percentage of its waste nitrogen bound in amino acids (17). This was also shown by Myers and Krusberg (13) for *Meloidogyne* spp. which have a similar host relationship as cyst nematodes. Therefore, deficiencies in the amounts of some amino acids may cause problems in the detoxification of nitrogen.

TABLE 1. Influence of methionine, phenylalanine, lysine, and tryptophan on the development of *Heterodera schachtii* in intact seedlings of *Brassica rapa* var. *silvestris* f. *campestris* 'Stielmus'

Amino acid	Concentration of amino acid (mg/L)	Females (no.)	Stagnated J2/J3 juveniles (no.)	Males (no.)
Control[a]		32	6	1
		46	3	6
Methionine	200	0	32	0
	300	2	41	6
Phenylalanine	200	0	38	2
	300	4	47	4
Lysine	200	0	20	1
	300	1	21	10
Tryptophan	100	3	29	1
	150	4	35	4
Control[b]		6	18	3
Glutamine	800	29	3	1

[a] This control corresponds to the "+" variant "distilled water, intact."
[b] This control corresponds to the "−" variant "0% sucrose, intact."

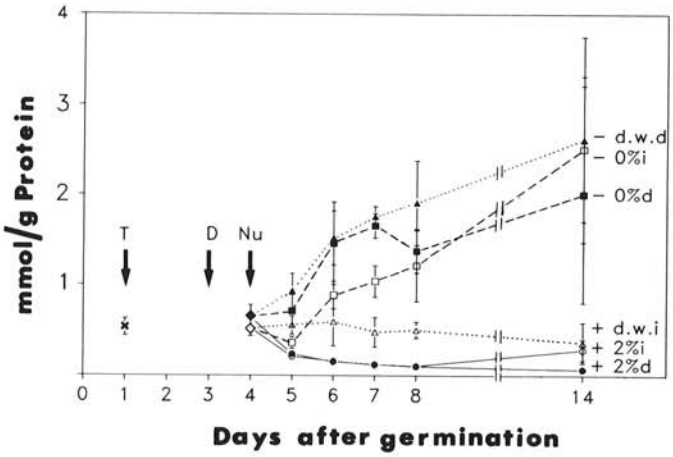

Fig. 2. Changes in the total concentration of the four harmful amino acids, methionine, phenylalanine, lysine, and tryptophan, in uninoculated root segments of *Brassica rapa* var. *silvestris* f. *campestris* 'Stielmus' seedling roots within the first 2 wk after germination. Fifty percent of all seedlings were decapitated on the third day (D), hence two values on the fourth day. The nutrient solutions were added on the fourth day (Nu), hence six values from the fifth day on. T = transfer of 1-day-old seedlings into distilled water agar drops (hence one value only). N = 5 except for the 14th day, where n = 4 for 2%i, 2%d, and 0%d variants. Abbreviations are as follows: i = intact seedlings; d = decapitated seedlings; + = "+" variants; − = "−" variants; 0, 1, 2, and 3% = sucrose concentration in the nutrient solution; d.w. = distilled water.

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