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Mineralization of Detrital Lignocelluloses by Salt Marsh Sediment Microflora†

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Specifically radiolabeled ^{14}C-(cellulose)-lignocellulose and ^{14}C-(lignin)-lignocellulose were isolated from labeled cuttings of *Spartina alterniflora* (cordgrass) and *Pinus elliottii* (slash pine). These were used to estimate the rates of mineralization to CO_2 of lignocelluloses of estuarine and terrestrial origin in salt marsh estuarine sediments. The lignin moiety of pine lignocellulose was mineralized 10 to 14 times more slowly than that of *Spartina* lignocellulose, depending on the source of inoculum. Average values for percent mineralization after 835 h of incubation were 1.4 and 13.9%, respectively. For *Spartina* lignocellulose, mineralization of the cellulose moiety was three times faster than that of the lignin moiety. Average values for percent mineralization after 720 h of incubation were 32.1 and 10.6%, respectively. Lignocellulose and lignin contents of live pine and *Spartina* plants were analyzed and found to be 60.7 and 20.9%, respectively, for pine and 75.6 and 15.1%, respectively, for *Spartina*.

Microbial degradation of plant material serves as the principal link between primary and secondary productions in salt marsh estuaries (25). Only small percentages of the dominant marsh grasses, such as *Spartina alterniflora*, are eaten while living; after death and physical disintegration, the plant material serves as a carbon and energy source for the microflora of the marsh sediments (3, 14, 27). The resultant microbial biomass, in turn, enhances the nutritive value of the detritus as food for a variety of grazers (18, 28). Plant material of terrestrial origin may also add significantly to the detrital carbon in salt marsh estuaries (17).

Rates of degradation of natural plant material in salt marsh estuaries have been examined by using both field and laboratory incubations. Such experiments have been conducted in submerged litter bags or aerated laboratory microcosms and, therefore, estimate degradation under well-oxygenated conditions. In general, water-soluble (leachable) components of *Spartina* decomposed rapidly, leaving a residue of fibrous material more resistant to microbial degradation (2, 13, 15). Although the chemical composition of the more refractory component of the *Spartina* detritus has not been reported previously, it has been presumed to consist primarily of lignin and cellulose (2, 11). In support of this hypothesis are field observations indicating that soft, flexible leaves of *S. alterniflora* and *Zostera marina* degrade more rapidly than rigid, "lignified" stems (2, 20).

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Lignin and cellulose in plants are almost always associated by virtue of both intimate physical contact and probably some degree of covalent bonding, resulting in a complex referred to as "lignocellulose" (8, 19, 26). Various plant lignins differ in the ratios of three possible phenylpropane subunits and in the relative abundances of different intermonomeric linkages, resulting in species-specific variations in the vulnerability of lignocelluloses to microbial attack (12, 21). Generally, cellulose is considered the more readily degradable component of lignocellulose (7), although, under certain conditions, the lignin moiety can be degraded at similar or higher rates than the cellulose (21, 22).

Although lignocellulose is presumed to be a major component of salt marsh detritus, there have been no reports of either the lignin or lignocellulose content of *S. alterniflora* or the rates at which lignin and cellulose moieties of the lignocellulose are microbially degraded in salt marsh and adjacent estuarine sediments. In this study, the rates of degradation of specifically radiolabeled, extractive-free lignocelluloses from *S. alterniflora* and *Pinus elliotii* (slash pine) were determined in incubations approximating conditions in the aerobic surface layers of various marsh and estuarine habitats. The pine lignocellulose was included as a representative lignocellulose of terrestrial origin which is likely to be transported into the estuarine environment via stream runoff. The lignocellulose and lignin contents of these plants are also reported.

MATERIALS AND METHODS

Preparation of labeled lignocelluloses. The pro-

cedures used to prepare radiolabeled lignocelluloses from *S. alterniflora* and *P. elliottii* were adapted from methodology described by Crawford and Crawford (5). Plant cuttings for radiolabeling included young pine branches (stem diameter of ≤1 cm) and the entire above-ground portions of short-form *Spartina* plants (approximately 20 cm in height). Each plant cutting was placed in 0.5 ml of 0.1 M phosphate buffer solution (pH 7.4) (pine) or 5‰ sterile seawater (*Spartina*), to which was added 5 μCi of L-[*U*-^{14}C]phenylalanine (500 mCi/mmol). The cuttings were placed under constant illumination and incubated until 80 to 90% of the liquid volume had been taken up. Additional liquid was then added intermittently to maintain the volume at about 1.0 ml, and the cuttings were allowed to metabolize the phenylalanine for 72 h. Some *Spartina* cuttings were labeled preferentially in the cellulose moiety by substituting D-[*U*-^{14}C]glucose (247 mCi/mmol) for the L-[*U*-^{14}C]phenylalanine (7).

Entire *Spartina* shoots and the inner cambial wood of the pine twigs were dried at 60°C for 24 h, ground in a Wiley mill to pass a 40-mesh (425-μm) wire screen, and weighed. An extractive-free lignocellulose fraction was separated from nonincorporated label and other plant components by extracting the plant material with boiling ethanol, ethanol-benzene (1:2 [vol/vol]), and water (1). The extracted lignocellulose was collected on tared Reeve Angel 984H ultrafine glass fiber filters, dried, and weighed to determine the percent lignocellulose of the original plant material.

The percent lignin in the original plant material was calculated after determining the lignin content of the extractive-free lignocellulose by using a Klason extraction as described by Browning (1). One-gram portions of extractive-free lignocellulose were placed in 125-ml Erlenmeyer flasks and digested with 20 ml of 72% H_2SO_4 at 20°C for 2 h with continuous shaking. Contents of the flasks were quantitatively transferred to 1-liter flasks and diluted with 560 ml of water, yielding a final acid concentration of about 3%. Samples were then placed in an autoclave and digested at 100°C for 3 h. The residual acid-insoluble Klason lignin was collected on tared glass fiber filters, dried, and weighed. The percent nitrogen in extractive-free lignocelluloses and Klason lignins was determined by combusting 0.6- to 0.8-mg samples in a Hewlett-Packard model 185 carbon-hydrogen-nitrogen analyzer.

Distribution of radiolabel in lignocellulose. Specific activity of ^{14}C in extractive-free lignocelluloses and Klason lignins was determined by combusting samples (0.4 to 0.7 mg) in a Hewlett-Packard model 185 carbon-hydrogen-nitrogen analyzer, and trapping the evolved $^{14}CO_2$ in liquid scintillation medium (5). Radioactivity in the cellulose moiety of lignocellulose (solubilized during Klason extraction) was determined by assaying 1 ml of neutralized Klason filtrate in 10 ml of Scintiverse liquid scintillation counting medium. The filtrates from the Klason procedure contain sugars which are liberated during the acid hydrolysis of the carbohydrate fraction of the lignocellulose. Distribution of radioactivity among sugars was determined after separation via paper chromatography (1, 9).

It has been reported previously (4) that protein is sometimes present as a contaminant in the extractive-free lignocellulose fraction of plant material. In such cases, part of the radiolabel can be incorporated into protein rather than lignocellulose and could lead to overestimations of lignocellulose degradation rates (4). The percent protein in our lignocellulose fractions and the percentage of label associated with that protein were determined by comparing the weights and specific activities of samples with and without pepsin digestion to remove protein (1, 9).

Degradation experiments. Sediment samples were collected from various marsh sites on Sapelo Island, Georgia, including two short-form *Spartina* stands, a tall-form *Spartina* stand, and a tidal creek bottom. One of the short *Spartina* stands, located in a high marsh area, contained sediment which was a loosely packed "mud," whereas the other stand, located on the edge of a salt panne, contained sediment which was sandy and tightly packed with roots. The tall-form *Spartina* stand was located on the bank of a tidal creek. Sediment samples from the creek bottom were collected during exposure at low tide. In all cases, only the upper 1 to 5 mm of the aerobic zone of the sediment was collected.

A 10-ml amount of sediment was homogenized with 90 ml of autoclaved creek water (25‰), and 25 ml of the resulting slurry was added to each of a series of 125-ml milk dilution bottles. Each bottle received 10 mg of ^{14}C-(lignin)-lignocellulose from *Spartina* or pine or 10 mg of ^{14}C-(cellulose)-lignocellulose from *Spartina*. Samples were incubated in the dark at 25°C, maintaining aerobiosis by constant aeration (12 ml/min) of the slurry with CO_2-free, humidified air. Mineralization of the radiolabeled lignocelluloses was monitored by continuously trapping the evolved $^{14}CO_2$ in a series of two scintillation vials containing 10 ml of liquid scintillation counting medium (5). The trapping efficiency was determined by adding 0.1 μCi of $NaH^{14}CO_3$ to incubation vessels with sterile sediment slurries. After acidification, 100% of the released $^{14}CO_2$ was recovered in the two traps, with greater than 90% being trapped in the first scintillation vial. Traps were changed periodically, and the $^{14}CO_2$ was assayed in a liquid scintillation counter. Controls, killed with 5% Formalin, evolved no $^{14}CO_2$ during the incubation period.

Upon termination of each mineralization experiment, slurries were examined for the presence of radiolabeled soluble organic material. This was done by acidifying to pH 1 with 6 N H_2SO_4 and sparging with N_2 for 20 min to remove $^{14}CO_2$, followed by filtration of the supernatant through Nuclepore filters (0.2-μm pore size) to remove the remaining particulate ^{14}C-lignocellulose. A 1-ml amount of each filtrate was added to 10 ml of Scintiverse, and the radioactivity present was quantitated by using liquid scintillation spectrometry.

Chemicals, radiochemicals, and supplies. D-Glucose, D-mannose, and D-xylose were obtained from Sigma Chemical Co.; Scintiverse liquid scintillation counting medium was obtained from Fisher Scientific Co.; Reeve Angel glass fiber filters were obtained from Whatman Filter Co.; Nuclepore membrane filters were obtained from Nuclepore Corp.; and D-[*U*-^{14}C]glucose, L-[*U*-^{14}C]phenylalanine, and sodium [^{14}C]bicarbonate were obtained from New England Nuclear Corp. All other chemicals were of analytical reagent grade.

RESULTS

Distribution and specific activities of lignin and lignocellulose. The percentages of lignocellulose and Klason lignin in young twigs of *P. elliottii* and above-ground portions of short-form *S. alterniflora* are shown in Table 1. Values are means of nine replicate samples. The lignocellulose content of pine and *Spartina* plants accounted for 60.7 ± 2.6 and 75.6 ± 1.6%, respectively, of the ash-free dry weights; Klason lignin concentrations averaged 20.9 ± 0.9 and 15.1 ± 1.3%, respectively. Detailed analyses of the lignocellulose and Klason lignin contents of various parts of *Spartina* plants indicated that the highest concentrations of both substances occur in the stems, whereas the lowest concentrations occur in young leaves (R. E. Hodson, A. E. Maccubbin, and R. R. Christian, manuscript in preparation).

Table 2 shows typical results of radiolabeling of pine and *Spartina* lignocelluloses. Specific activities of lignocelluloses extracted from plants labeled in the lignin moiety were consistently higher than those of lignocelluloses labeled in the cellulose moiety, averaging 5×10^3 to 7×10^3 and 0.8×10^3 dpm/mg (dry weight), respectively. This difference is probably due to the extraction of some ^{14}C-labeled, water-soluble carbohydrates and other compounds which become labeled by metabolism of the D-[^{14}C]glucose.

Extraction of the labeled lignocelluloses with 72% sulfuric acid revealed that the bulk of incorporated label (87.1% for pine; 65.5% for *Spartina*) was localized in the insoluble Klason lignin fraction in plants that had been labeled with L-[^{14}C]phenylalanine. In lignocellulose from *Spartina* plants labeled with D-[^{14}C]glucose, most of the label (59.9%) was present in the acid-soluble "cellulosic" fraction. Theoretically, the radioactivity in the acid-soluble Klason filtrates could be due to either glucose derived from acid-hydrolyzed cellulose or acid-soluble lignin. Previous studies have indicated that, depending on plant species, various amounts of lignins are solubilized by the Klason extraction procedure (10, 23, 24, 26). To test which of the possibilities was correct for these lignocelluloses, the acid-soluble filtrates from Klason extraction fractions were subjected to paper chromatographic analysis (9) to separate sugars from solubilized lignin. Extracts were cochromatographed with D-glucose, D-mannose, and D-xylose. In filtrates from ^{14}C-(cellulose)-lignocelluloses, most of the applied radioactivity was accounted for in the spot corresponding to D-glucose, the acid hydrolysis product of cellulose. In contrast, Klason filtrates from ^{14}C-(lignin)-lignocelluloses contained virtually no radioactivity in spots corresponding to sugars (data not shown). Therefore, the radioactivity in the filtrates of Klason-extracted lignocelluloses from [^{14}C]phenylalanine-labeled pine and *Spartina* plants can be attributed to acid-soluble lignin. A similar distribution of label was reported by Crawford and Crawford (9) for specifically labeled lignocelluloses from hemlock. Labeling of *Spartina* with D-[^{14}C]glucose apparently resulted in some synthesis of labeled lignin, as evidenced by the 34% label retained in the Klason lignin fraction (Table 2).

Some of the radioactivity in the labeled lignocellulose could be associated with small amounts of contaminating protein rather than with lignin or cellulose. Low concentrations of protein have been detected in Klason lignin fractions of several plant species (26). However, nitrogen determinations of these lignocellulose preparations indicated that they contained no more than 6.5 and 1.0% protein for *Spartina* and pine, respectively, assuming that protein equals 6.25 × N. The amount of labeled protein was evaluated by comparing levels of radioactivity in the lignocelluloses before and after digestion with 1.0% pepsin to remove protein (1). In the case of ^{14}C-(lignin)-lignocellulose from pine, only a 3% loss of label resulted from pepsin digestion,

TABLE 1. *Lignocellulose and Klason lignin contents of S. alterniflora and P. elliottii*

Plant	% Lignocellulose[a]	% Klason lignin[a]
S. alterniflora	75.6 ± 1.6	15.1 ± 1.3
P. elliottii	60.7 ± 2.6	20.9 ± 0.9

[a] Mean values of nine replicates ± one standard deviation.

TABLE 2. *Distribution of label in ^{14}C-lignocellulose and Klason lignin*

Source	Labeled precursor	sp act (dpm/mg)	% Activity in Klason lignin	% Activity in acid-soluble fraction	% Activity recovered	% Activity in lignocellulose after pepsin hydrolysis
P. elliottii	L-[*U*-^{14}C]Phenylalanine	7,289	87.1	6.2	93.3	97.0
S. alterniflora	L-[*U*-^{14}C]Phenylalanine	5,480	65.5	37.3	102.8	65.8
S. alterniflora	D-[*U*-^{14}C]Glucose	868	34.3	59.9	94.2	77.1

indicating that only a small percentage of the label was associated with protein. Pepsin-mediated losses of radioactivity from labeled *Spartina* lignocelluloses were higher, averaging 34.2 and 22.5% for ^{14}C-(lignin)-lignocellulose and ^{14}C-(cellulose)-lignocellulose, respectively. Thus, compared with pine lignocellulose, a greater percentage of label in the *Spartina* lignocellulose could be associated with protein. This, however, does not interfere with measurement of lignocellulose mineralization (see below). Subsequent work in our laboratory (unpublished data) indicates that incubation of *Spartina* cuttings, with a deaminated precursor such as cinnamic acid (as suggested by Don L. Crawford, personal communication), yields a labeled lignin preparation which contains relatively less label as protein.

Microbial degradation of lignocellulose. Rates of microbial mineralization of ^{14}C-(lignin)-lignocellulose from *P. elliottii* and *S. alterniflora* by the microflora of various salt marsh sediments are shown in Fig. 1. For all inocula tested, rates of mineralization were 10 to 14 times higher for the *Spartina* lignin component than for the pine lignin component during most of the incubation period. For example, after 732 h of incubation, depending on the type of sediment, only 0.5 to 2.05% of the added label from pine lignocellulose had been recovered as $^{14}CO_2$, compared with 8.6 to 17.9% from the *Spartina* lignocellulose. These observed differences in the lignocellulose degradation rates could have been due to actual structural differences between the lignins of pine and *Spartina* or to mineralization of labeled protein in the *Spartina* lignocellulose preparations. These alternatives were investigated by comparing rates of mineralization of pepsin-digested and non-pepsin-digested ^{14}C-(lignin)-lignocelluloses from *Spartina*. The results of two separate 500-h incubations in slurries of seawater and short *Spartina* sediments indicated that the two lignocellulose preparations were mineralized at identical rates (data not shown). Thus, under our laboratory conditions, formation of $^{14}CO_2$ from labeled protein is negligible.

At any given time during the incubation period, the means of the percent mineralization of both the pine and *Spartina* lignin components were highest in incubations with sandy sediment from a short-form *Spartina* stand, intermediate in incubations with sediment from a tall-form *Spartina* stand, and lowest in incubations with sediment from a tidal creek bottom (Fig. 1). Since the values reported here are the means of only two replicates which often had overlapping ranges, the significance of their apparent order requires verification via further experimentation involving higher numbers of replicates.

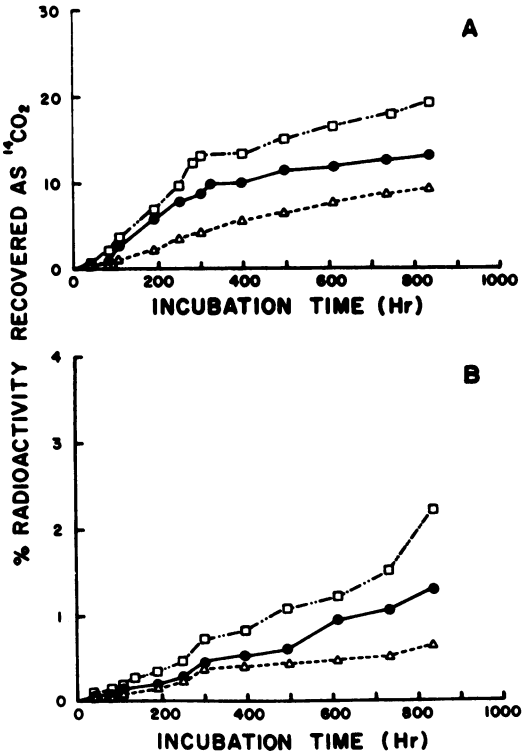

FIG. 1. *Mineralization of ^{14}C-(lignin)-lignocelluloses from S. alterniflora (A) and P. elliottii (B) by salt marsh sediment microflora. Symbols: □, short-form Spartina zone; ●, tall-form Spartina zone; △, creek bottom. Each point represents the mean of two replicates.*

Figure 2 shows the results of an experiment in which the rates of degradation of ^{14}C-(lignin)-lignocellulose and ^{14}C-(cellulose)-lignocellulose from *Spartina* were determined by using sediments from tall-form and short-form *Spartina* marsh zones as inocula. With both inocula, rates of $^{14}CO_2$ production from ^{14}C-(cellulose)-lignocellulose were much higher than those of ^{14}C-(lignin)-lignocellulose. After 720 h of incubation, only 9.9 to 11.6% of label from ^{14}C-(lignin)-lignocellulose had been recovered as $^{14}CO_2$, compared with 30.0 to 34.1% of the label from ^{14}C-(cellulose)-lignocellulose.

After each experiment had been terminated, slurries were examined for the presence of radiolabeled dissolved organic material. In no case was the radioactivity in the soluble organic fraction significantly higher than the background level.

DISCUSSION

The 10- to 14-fold difference in the rates of mineralization of the lignin moieties of lignocelluloses from *S. alterniflora* and *P. elliottii* is

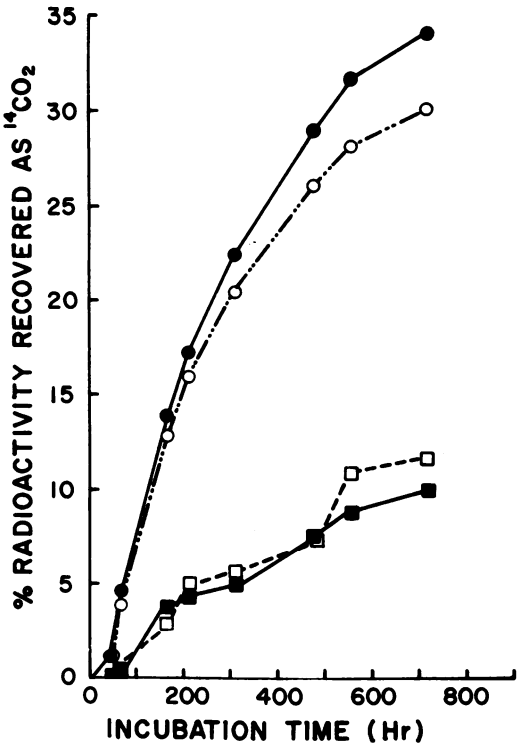

FIG. 2. *Mineralization of ^{14}C-(lignin)-lignocellulose and ^{14}C-(cellulose)-lignocellulose from S. alterniflora by salt marsh sediment microflora. Symbols: ■, ^{14}C-(lignin)-lignocellulose, tall-form Spartina zone; □, short-form Spartina zone; ●, ^{14}C-(cellulose)-lignocellulose, tall-form Spartina zone; ○, short-form Spartina zone. Each point represents the mean of two replicates.*

probably indicative of basic structural differences between lignins of grasses and gymnosperms. Basically, all lignins are three-dimensional polymers of phenylpropane units derived from the oxidative polymerization of *p*-coumaryl, coniferyl, and sinapyl alcohols. The relative proportions of the individual alcohols and the types of bonds forming the polymers vary with plant species and can affect the relative resistance to microbial degradation of the resultant lignin. In grass lignins, sinapyl alcohol is the major monomer, whereas in conifer lignins, coniferyl alcohol predominates (21).

Rates of degradation of the cellulose moiety of *Spartina* lignocellulose were consistently higher than those of the lignin moiety. Extrapolation of these results to the marsh environment would suggest, that, with time, detritus derived from *Spartina* would become progressively enriched in lignin relative to cellulose. Extensive chemical analyses of the lignin content of naturally aged *Spartina* detritus from the salt marsh at Sapelo Island, Georgia, supports this hypothesis (R. E. Hodson, A. E. Maccubbin, and R. R. Christian, manuscript in preparation). Similar differences in the rates of mineralization of lignin and cellulose in lignocelluloses from terrestrial plants have been reported by Crawford et al. (7).

Our estimates of degradation rates probably overestimate the in situ values. Experiments to date have been run exclusively at 25°C; lower winter temperatures may result in different mineralization rates. In addition, during degradation, the more easily utilizable constituents of lignocellulose molecules would be removed first, rendering the remaining material progressively more refractory. Incubations which lasted a maximum of 900 h might not accurately reflect the lower rates of mineralization of partially degraded lignocellulose. The observed decreases in degradation rates after only 300 h of incubation may, in fact, reflect the exhaustion of the most readily utilizable components of the lignocellulose.

To date, all of our experiments have been conducted with aerobic incubations to simulate conditions at water-sediment interfaces or in deeper sediments which may be oxygenated via diffusion of O_2 from *Spartina* roots (29). Rates of lignin degradation in the anaerobic horizons of salt marsh sediments have not yet been determined. However, they can be assumed to be much lower than those under aerobic conditions. In a study of synthetic lignin degradation in freshwater sediments and terrestrial soils, Hackett et al. (16) found that production of $^{14}CO_2$ or other degradation products was negligible in incubations conducted under anoxic conditions.

Only a limited range of microorganisms have been shown to be capable of lignin degradation. Certain obligately aerobic fungi have been identified as the principal mediators of lignin degradation in forest soils (e.g., see reference 21); bacterial strains of the genera *Nocardia*, *Streptomyces*, and *Bacillus* have been shown to degrade lignin to CO_2 (6). Microscopic examination of both standing dead and detrital *Spartina* material invariably reveals extensive invasion by fungal hyphae and colonization by epiphytic bacteria. However, the microorganisms responsible for lignin degradation in estuarine environments have not been identified.

Our data confirm the widely held assumption that lignocellulose accounts for a large percentage of the standing stock of plant material in salt marsh estuaries (3, 14, 27). This organic material is converted to the microbial biomass before it can be assimilated by higher organisms. Therefore, knowledge of the factors controlling rates of lignin and cellulose degradations in marsh and coastal sediments will enhance our under-

standing of carbon flow through such detritus-based ecosystems. Our preliminary data reported here suggest that specifically radiolabeling *Spartina* lignocellulose and following its mineralization should have wide applicability in studies of microbial processes in marsh-dominated coastal marine environments.

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