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CHEMICAL TRANSFORMATIONS OF LIGNIN DURING THE BIODEGRADATION AND ORGANOSOLV PULPING OF DECIDUOUS WOOD

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The lignins from the liquors in the organosolv pulping of aspen wood treated with wood-destroying fungi have been investigated using quantitative ^1H and ^{13}C NMR spectroscopy and exclusion liquid chromatography. It has been shown that the biodegradation of lignin takes place in different ways according to the complex of enzymes produced by the fungi. Phanerochaete sanguinea *causes degradation through predominant cleavage at alkyl–phenyl bonds, and* Trametes villosus *at the C–C bonds of the aliphatic chain. In addition to degradation reactions, polymerization (condensation) reactions also take place with the appearance of new $C_{ar}–O–C$ and $C_{ar}–C$ bonds. It has been established that the biological pretreatment of aspen wood ensuring partial degradation of the lignin leads to its more ready extraction in the process of organosolv pulping.*

The use of aliphatic alcohols for the delignification of wood is attracting ever greater attention by researchers as an economically pure method of obtaining cellulose. The combination of organosolv pulping (OSP) with the biological pretreatment of the wood raw material will permit an increase in the degree of delignification and a rise in the selectivity of the process, since biological action on wood leads to the freeing and degradation of the lignin component and its easier extraction [1]. The preliminary biological degradation of aspen wood by fungi permits a decrease in the hardness of the cellulose obtained by 6-9 kappa units on subsequent organosolv pulping owing to an increase in the selectivity of the process with maintenance of the yield. The fungus *Trametes villosus* increases the selectivity of OSP to the greatest degree and enables cellulose to be obtained with a yield 2% higher than a control sample at the same hardness [2].

In order to perfect the method of organosolv pulping with biological treatment of the initial raw material, it is necessary to evaluate the contribution of the stage of biological pretreatment in the delignification process. Valuable information in this respect can be given by a knowledge of the chemical reactions accompanying biodelignification and organosolv pulping.

We have studied the chemical changes taking place in lignins as the result of biological pretreatment of the wood followed by organosolv pulping.

In the first stage, we investigated liquor from organosolv pulpings containing lignin chemically changed as a result of the digestion of the lignin and the products of its degradation. Area-normalized combined gel-chromatograms of the liquors from the OSP of biologically treated wood differed from gel-chromatograms from the OSP of the initial wood and of aspen MWL itself by the fact that it had a bimodal nature, i.e., contained both high-molecular-mass and low-molecular-mass components (Fig. 1). The appearance of low-molecular-mass fractions in the liquors from the OSP of biologically treated wood shows a process of lignin degradation taking place under the action of the fungi. With an increase in the period of biological treatment up to 14 days, the proportion of low-molecular-mass fractions increased; for example, in the case of the preincubation of wood with the fungus *T. villosus*, to 24%. Similar fractions were present in only small amount in the liquor from the OSP of the initial wood.

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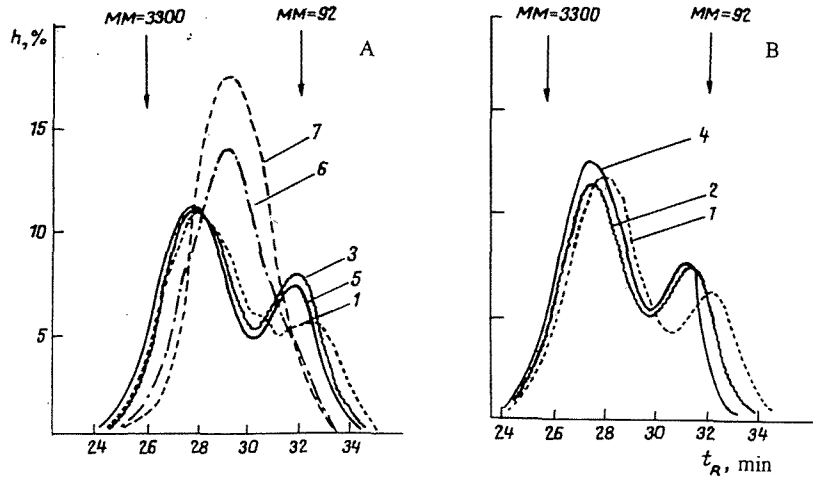

Fig. 1. Area-normalized gel chromatograms of the liquors from OSP obtained after the biodegradation of aspen by the fungi *T. villosus* (*A*) and *Ph. sanguinea* (*B*) with the following respective weight-average molecular masses: *1*) 7 days (900) and (1000); *2*) 10 days (800) and (1140); *3*) 14 days (1000); *4*) 21 days (1280); *5*) 28 days (1300); *6*) initial aspen (1200); *7*) aspen MWL (600).

On a gel chromatogram, the peak of the high-molecular-mass fraction of the lignins from the liquors of the OSP of biologically treated wood was shifted into the region of higher molecular masses as compared with the peak of the liquor from the OSP of the initial wood. The latter, in contrast to a gel chromatogram of aspen MWL were also located in the region of higher molecular masses (see Fig. 1). It follows from this that in the process of OSP the lignin was not only extracted from the wood and degraded but also underwent polymerization (condensation) transformations. After the biological pretreatment of the wood, these transformations became more pronounced: on the one hand, a peak of the relatively low-molecular-mass compounds appeared, and on the other hand the peak of high-molecular-mass compounds shifted into the region of higher molecular masses than for the MWL and for the lignin from the liquor of the OSP of the initial wood (see Fig. 1).

Polymerization reactions taking place with lignin in the OSP process have also been reported previously [3]. The intensification of this effect on biological pretreatment can be explained, on the one hand, as the consequence of the capacity of the fungal hyphae for readily penetrating into the depths of the cell walls of the wood and, with the aid of secretory enzymes of ligninolytic (ligninase, laccase, N-peroxidase) and hemicellulasic (β-glucosidase and xylanase) natures [4, 5] rupturing lignin-carbohydrate and C–C bonds, facilitating in the subsequent digestion the passage into the liquor of the higher-molecular-mass lignin located in the primary layer of the cell wall and the middle lammella [6]. On the other hand, it is known that the enzymatic oxidation of lignin is accompanied by the formation of phenoxy radicals which are capable of participating in polymerization processes. The latter hypothesis requires additional experimental proofs in our case.

With the aim of studying the chemical reactions taking place in lignin during the OSP process we isolated the OSP lignins from the liquors and investigated the changes in their chemical composition, using ^1H and ^{13}C NMR. The ^1H spectra were used to determine the number of phenolic hydroxy groups [8].

As an example, Fig. 2 gives the ^{13}C NMR spectrum of an OSP lignin. The distribution of the carbon atoms over the main fragments of the lignins is shown in Tables 1 and 2. In analyzing the results obtained, we first evaluated the changes taking place in lignins solely as the result of an aqueous ethanolic digestion process.

The OSP lignin of the initial wood differed from aspen WML above all by a lower content of ether bonds (see Table 2) because of the fact that in the OSP process they undergo catalytic hydrolysis in the presence of HCl [9]. This lignin contained a larger amount of carbonyl groups and phenolic hydroxy groups the appearance of which was due to oxidative and hydrolytic processes [9]. In the OSP lignin, the amounts of CHO, CH_2O, and CH fragments had fallen (see Table 1), which may be connected with the disruption of pinoresinol and phenylcoumaran structures in the acid medium. In addition, the aliphatic chain had undergone degradation (see Table 2), and, probably because of this, the degree of aromaticity (f_a) in the OSP lignin had increased.

TABLE 1. Distribution of the Carbon Atom Over the Structural Fragments of Organic-Solvent Lignins (number (*N*) per 100 ARs)

Fragment	Lignin		*Phanerochaete chrysosporium*		*Phanerochaete sanguinea*		*Trametes villosus*		Range of the spectrum (^{13}C), ppm from TMS, assignment [16-18]
	MWL	init. OSP	10d	28d	10d	84d	10d	56d	
C=O	13.0	20.1	22.9	18.6	45.0	27.2	18.9	8.6	220—185 ketonic, aldehydic
COO	40.3	34.6	27.2	21.7	39.4	22.1	27.4	29.8	185—164 acidic, ester
C_{ar}—O tot	233.6	229.7	218.9	209.6	222.9	198.3	201.0	210.2	163—151 C,H, H′ 161—154 $C_{3,4}$G with α-CO 154—151 $C_{3,5}$ S 151—141 $C_{3,4}$ G G′; C_4S with α-CO; 136—134 C_4 S, S′
C_{ar}—C tot	118.5	124.9	178.6	185.9	154.2	127.9	184.2	145.9	140—125 C_1H, H′; G, G′; S, S′; C_5—C_5; $C_\beta - C_1$; C_β—C_5
C_{ar}—H	235.6	194.0	161.1	158.0	182.3	179.2	188.4	185.3	132—117 C_6 G, G′; $C_{2,6}$ H, H′
CHO	261.6	85.9	71.9	77.5	72.0	60.4	64.2	82.6	92—65 C_α, C_β
CH_2O	137.9	66.9	53.3	35.1	55.2	32.2	34.7	51.8	65—58 C_γ
CH_3O	144.5	147.2	149.1	145.6	144.1	147.0	148.4	136.3	61—55 methoxyls
CH_2O	65.1	20.1	16.3	13.4	11.3	62.4	13.9	10.6	72.2 C_γ pinoresinol
CH	65.1	23.4	18.5	26.9	14.6	31.2	18.9	22.1	54—52 C_β—C_β; C_β—C_5 phenylcoumaran
CH, CH_2, CH_3	32.5	59.0	46.0	35.0	108.0	57.0	74.0	52.0	31—12 CH, CH_2, CH_3 not bound to an oxygen atom
CH_2—CH_3	0	13.7	17.4	16.5	18.4	15.5	19.1	13.1	28.8; 15.3—14.2 CH_2 and CH_3 of an ethyl group
f_a	0.461	0.538	0.551	0.581	0.533	0.596	0.570	0.625	

TABLE 2. Distribution of Carbon Atom Over the Structural Fragments of the Lignins from Organosolv Pulping, Calculated per 100 ARs

Fragment	Lignin		*Phanerochaete chrysosporium*		*Phanerochaete sanguinea*		*Trametes villosus*	
	MWL	init. OSP	10 d	28 d	10 d	84 d	10 d	56 d
S	58±9.0	55±8.8	55±8.8	58±9.2	56±8.9	54±8.6	51±8.1	57±9.1
G	28±6.0	36±7.9	35±7.7	31±6.8	31±6.8	38±8.3	39±8.5	24±5.2
H	14±0.7	12±1.0	4±1.0	2±0.1	12±1.0	7±1.0	4±1.0	8±1.0
S_{α} -CO*	21±1.0	13±0.7	13±0.7	13±0.7	18±1.0	19±1.0	14±0.7	18±1.0
S**	5±0.6	6±3.6	34±3.4	35±3.5	35±3.5	37±3.7	28±2.8	31±3.1
$C_{ap}-C$	18±3.0	25±4 4	79±12	86±14	53±9.5	28±5.0	84±14	46±8.3
$C_{ap}-O-C$	60±12	5`±11	43±10	49±11	65±15	30±7.2	35±7.0	31±7.7
$C_{side\ chain}$	53`±74	340±50	321±50	270±40	370±50	240±40	270±30	200±20
OH_{phe}	29±2.0	79±9.0	50±6.0	61±6.0	70±8.9	65±7.8	51±6.0	46±6.0

*Number of syringyl rings with an α-CO substitutent.

**Unsterified syringyl structures.

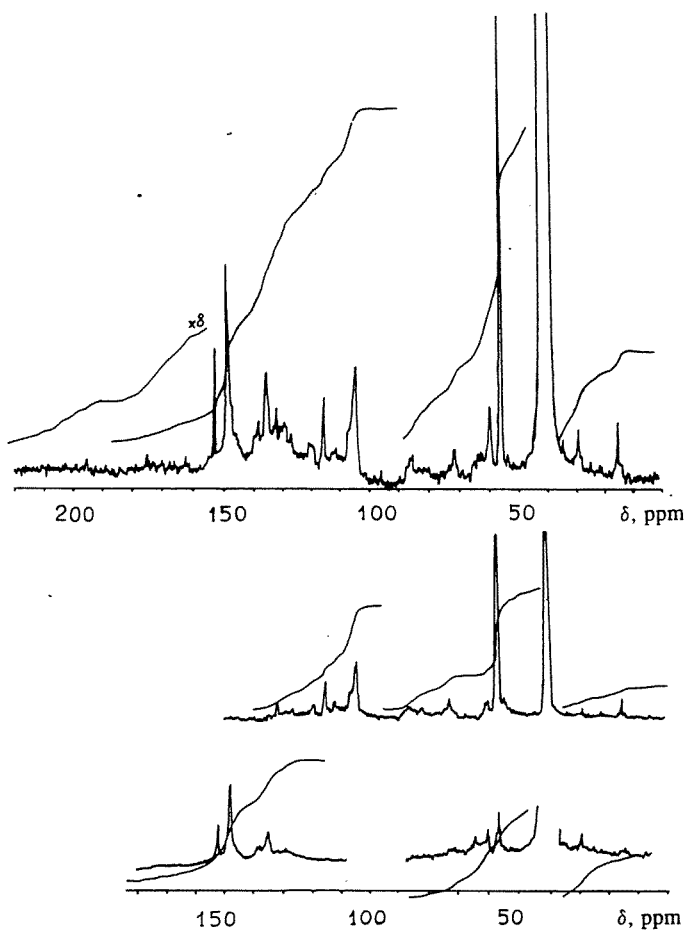

Fig. 2. ^{13}C spectrum and subspectra of the lignin from the organosolv pulping of aspen wood that had been biologically degraded by the fungus *Ph. sanguinea* for 10 days, recorded in (dimethyl sulfoxide)-d_6 solution.

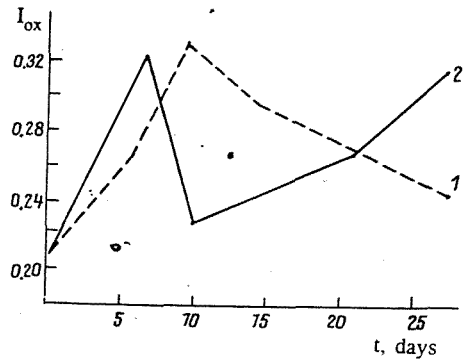

Fig. 3. Dependence of the integral degree of oxidation of the lignins from the OSP liquors of biologically degraded aspen wood on the time of incubation with the fungi *T. villosus* (*1*) and *Ph. chrysosporium* (*2*).

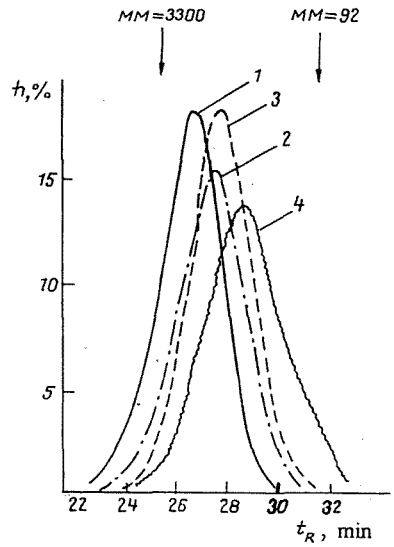

Fig. 4. Normalized gel-chromatograms of the OSP lignins obtained after biodegradation of aspen wood by various fungi at long times of incubation with their weight-averaged molecular masses: *1*) *T. villosus* (2890); *2*) *Ph. chrysosporium* (1900); *3*) *Ph. sanguinea* (1330); *4*) initial aspen (1200).

The OSP lignin contained a greater number of $C_{ar}-C$ bonds than the WML lignin. It is obvious that the polymerization processes revealed by gel chromatography and referred to above take place thanks to the appearance of new $C_{ar}-C$ bonds. In the OSP lignin, the proportion of carbon atoms not bound to oxygen had increased, and aliphatic (ethyl) groups, absent from aspen MWL had appeared (see Table 1), which indicates the alkylation of aromatic rings taking place during aqueous ethanolic digestion and recorded previously for the lignins of the ALCELL process [10].

The biological pretreatment of the wood before OSP must have a substantial influence on the structure of the lignins isolated. This assumption is based on previous investigations performed with the fungi *H. sanguinea* [117] and *T. villosus* [12], which showed that the biolignins differ considerably from the lignin of the initial wood in functional composition and amounts of individual fragments.

The OSP lignins isolated from the liquors after the digestion of wood incubated with the fungi (*Ph. chrysosporium*, *Ph. sanguinea*, and *T. villosus* for a short period (10 days), just like the OSP lignin from the initial wood, contained a larger amount of carbonyl and phenolic hydroxy groups than the MWL. With an increase in the time of incubation, the amounts of carbonyl and, to a smaller degree, phenolic hydroxy groups in the OSP lignins from the biologically treated wood decreased,

as was confirmed by the results of gel chromatography – the degree of oxidation of the OSP lignins isolated after the digestion of wood with prolonged periods of biological pretreatment became smaller (Fig. 3). Such relationships are probably a consequence of the fact that the oxidized lignin fragments were split out and passed into the liquor in the form of lower-molecular-mass products.

The biological treatment of the wood led to a deeper degradation of the aliphatic chains in the lignin than organosolv pulping. With a prolonged time of biotreatment, less than three aliphatic carbon atoms per one aromatic ring remained in the OSP lignins (see Table 2), and the degree of aromaticity f_a became higher than in the OSP lignins of the initial wood.

It is interesting to note that neither OSP nor biological pretreatment affected the methoxy groups. Their amounts changed little in all the OSP lignins, just like the total amount of guaiacyl and syringyl structures. The conversion of the syringyl and guaiacyl structures is connected with the cleavage of O–C bonds in C_{ar}–O–C (but not C_{ar}–OCH_3) fragments (see Table 2). This leads to the appearance of syringyl and guaiacyl units with free phenolic hydroxyls: as compared with the MWL, their amount increased approximately 7-fold and simultaneously, through this, the proportion of hydroxy groups rose. The calculated semiempirical formulas of the lignins – $C_9H_{10.70}O_{3.36}(OCH_3)_{1.11}$ for the MWL, $C_9H_{8.34}O_{3.19}(OCH_3)_{1.36}$ for the OSP lignin, and $C_9H_{7.62}O_{2.96}(OCH_3)_{1.53}$ for the OSP lignin from aspen treated with *Ph. sanguinea* for 84 days – indicated changes in its phenylpropane units taking place during the process of digestion and biological degradation. Analysis of the structural changes was therefore calculated per 100 aromatic rings (ARs), since the ^{13}C NMR method permits an estimate of the change in lengths of the side chains in lignins under biological and chemical actions.

For biological pretreatment we used three cultures of basidial fungi. It proved to be difficult to reveal any difference in their actions on the lignin after the performance of OSP: the OSP lignins from the biologically treated woods were fairly close (see Table 2). The OSP process probably levels out these differences. Nevertheless it is possible to note distinguishing features of the action of *Ph. sanguinea* and *T. villosus* on lignin. As preceding investigations [5] have shown, thanks to the presence of a Mn-dependent peroxidase, *Ph. sanguinea* brings about the degradation of the lignin by cleaving alkyl–phenyl bonds. A parallel polymerization process takes place with the formation of new C_{ar}–O–C bonds.

This feature of the action of the fungus was reflected on the structure of the lignin: among the OSP lignins it was just the lignin isolated from the liquor after the action of *Ph. sanguinea* on the wood (incubation for 10 days) followed by organosolv pulping that had the smallest content of C_{ar}–C bonds and the largest content of C_{ar}–O–C bonds (see Table 2). In contrast to *Ph. sanguinea*, because of the action of a ligninase *T. villosus* degraded lignin at C–C bonds of the aliphatic chain [4]. As a result, the amount of $C_{side\ chain}$ in the OSP lignin after the action of *T. villosus* was the lowest, and the aromaticity of the lignin the highest (see Table 2).

A feature of *T. villosus* is its capacity for a more active polymerization of the lignin: the OSP lignin after the action of *T. villosus* on the wood contained more C_{ar}–C bonds (at short incubation times) than the other OSP lignins after biological treatment of the wood. This characteristic was also confirmed by gel-chromatograms of the lignins (Fig. 4): the OSP lignin after the action of *T. villosus* had fragments with the highest molecular mass. This may be connected with the specific enzyme system of the fungus – *T. villosus*, unlike *Ph. chrysosporium* and *Ph. sanguinea* is capable of producing laccase [4], which, it is known, actively catalyzes polymerization processes.

Thus, it has been found that the biodegradation of lignin is accompanied by oxidative reactions that are particularly clearly expressed in the case of *Ph. sanguinea*: the amounts of carbonyl and phenolic hydroxy groups in the lignin increased considerably. Depending on the species of microorganism or, more precisely, on the complex of enzymes produced by them, biodegradation of lignin takes place by various routes: through the predominant cleavage at alkyl–phenyl bonds in the presence of *Ph. sanguinea* and of C–C bonds of the aliphatic chain in the case of *T. villosus*. In addition to the predominating destructive reactions, polymerization (condensation) reactions also take place with the appearance of new C_{ar}–C and C_{ar}–O–C bonds. That is, the biological pretreatment of wood leads to the partial degradation of the lignin and thereby promotes its easier extraction. As the result of a subsequent aqueous ethanolic digestion in the presence of HCl, further degradation takes place through the cleavage of ether bonds and C–C bonds of the aliphatic chain. The combination of these methods ensures a more profound delignification of the wood.

EXPERIMENTAL

The fungi of white rot were used: *Phanerochaete chrysosporium* ATCC 24725 (Biological Institute), *Phanerochaete sanguinea* (Fr) Pouz BKMF 2487D (Leningrad Academy of Wood Technology), *Trametes (Coriolus) villosus* (Lloyd) Kreisel

0276 (All-Union Production Combine of the Hydrolysis Industry). The fungi were maintained on 2% wort-agar. The mycelium was grown in a liquid nutrient medium the composition of which has been described elsewhere [13]. Sterile aspen splints (18.5% lignin, 55.1% cellulose) were inoculated with the mycelium from the cultures of *Ph. chrysosporium*, *Ph. sanguinea*, and *T. villosus*, and were incubated at 38, 27, and 33°C, respectively, for 10-84 days.

Samples with similar losses of mass were investigated. Organosolv pulping was carried out in steel autoclaves in the ethanol–water (55:45 vol. %) system at 165°C for 210 min with the catalyst HCl (0.2% on the weight of the absolutely dry wood) at a liquid ratio of 5:1. The pulpings were performed by M. I. Buryachenkov. After pulping, the liquor was filtered and was analyzed by gel chromatography. The lignin from the liquors was precipitated with distilled water, filtered off, and dried in vacuum. The milled wood lignin (MWL) of the initial aspen was isolated by the procedure of [14].

The molecular mass distributions both of the lignins isolated and of the lignins in the liquors without preliminary isolation were determined by exclusion liquid chromatography on a Milikhrom-1 instrument as described in [6]. Molecular masses were determined by a procedure presupposing the calibration of the column on fractions of dioxane lignin the molecular masses of which were determined by vapor-phase osmometry [6].

^{13}C and ^{1}H NMR spectra were recorded on a Bruker WP-200SY spectrometer by the procedure described in [15]. The assignment of the signals was made in the light of [16-18]. The numbers (N) of carbon atoms in the structural fragments of the lignin were calculated from the formula [19]: $N = q \cdot 6 \cdot 100/f_a$ where q is the proportion of carbon atoms in the structural fragment and f_a is the degree of aromaticity.

The lignin preparations were characterized by their elementary compositions: C (mass %) and H (mass %) and their developed empirical formulas: MWL – 58.67; 6.80; $C_9H_{10.70}O_{3.36}(OCH_3)_{1.11}$; initial OSP lignin – 59.34; 5.95; $C_9H_{8.34}O_{3.19}(OCH_3)_{1.36}$; *Ph. chrysosporium* 10d – 59.25; 5.65; $C_9H_{7.61}O_{3.21}(OCH_3)_{1.43}$ and 28d – 61.79; 6.34; $C_9H_{8.86}O_{2.71}(OCH_3)_{1.55}$; *Ph. sanguinea* 10d – 62.40; 6.79; $C_9H_{9.48}O_{2.21}(OCH_3)_{1.32}$ and 84d – 60.06; 5.82; $C_9H_{7.62}O_{2.96}(OCH_3)_{1.53}$; *T. villosus* 10d – 62.00; 6.01; $C_9H_{7.72}O_{2.58}(OCH_3)_{1.48}$ and 56d – 57.83; 5.98; $C_9H_{8.51}O_{3.44}$-$(OCH_3)_{1.49}$, respectively.

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