SYNTHESIS OF THE OCTADEOXYRIBONUCLEOTIDE COMPLEMENTARY TO A SECTION OF THE 3'-TERMINAL FRAGMENT OF THE 16 S RNA of *E. coli* RIBOSOMES

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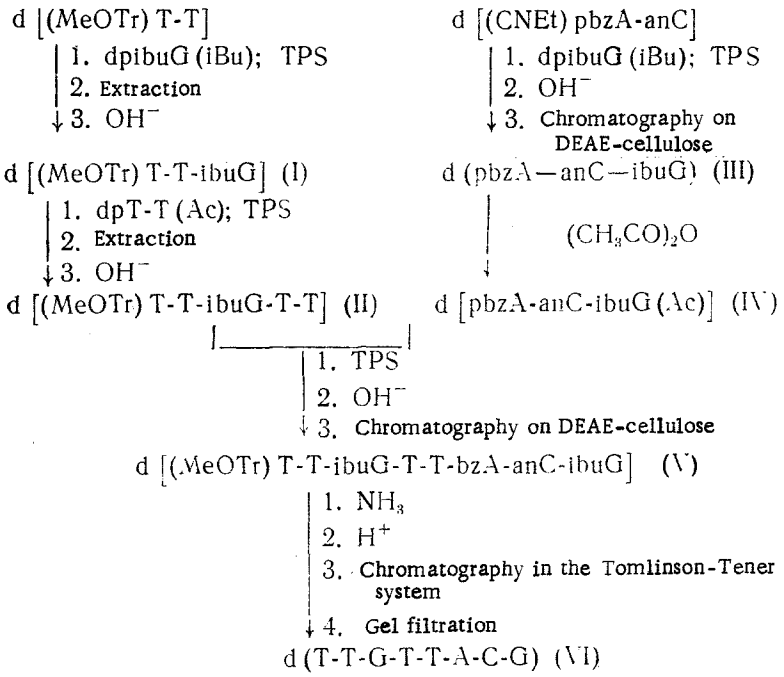
In recent years, information has appeared on the functional importance of the 3'-terminal fragment of the 16 S rRNA of *E. coli* for the process of initiating protein synthesis. Thus, it is known that it is precisely with the 3'-terminal fragment of the 16 S rRNA that mRNA binds [1]. A number of proteins necessary for the initiation of protein synthesis (S1, S3, S12, IF2, IF3) [2-5] and some antibiotics (colicin E3 and cloacin DF13) [6] interact precisely with this region of rRNA. Finally, there are facts which indicate an interaction of the 30S and 50S ribosomal particles just in this region [7]. However, there is no information on the mutual spatial arrangement of the ribonucleotide chain and the ribosomal proteins in this region nor of a change in their spatial arrangement on interaction with initiating factors, antibiotics, and mRNA.

For subsequent use in the study of the structure and functions of the 3'-terminal fragment of the 16S of *E. coli* rRNA we have synthesized the octadeoxyribonucleotide d(T-T-G-T-T-A-C-G), which is complementary to a section of the 16S rRNA close to the position of its cleavage by the antibiotic colicin E3 or cloacin DF13. It was proposed to study, with the aid of the octanucleotide synthesized, the accessibility of the 3'-terminal fragments in native RNA and in the composition of the 30S ribosomal particle, and also the possible change in the interaction of this fragment with the octanucleotide with a change in the composition of the 30S subparticle.

The synthesis was carried out by the methods developed by H. Khorana et al. [8] according to the following scheme:



In all stages of the synthesis we used traditional protective groups [9]. As the condensing agent we used triisopropylbenzenesulfonyl chloride (TPS) in an amount of one molar

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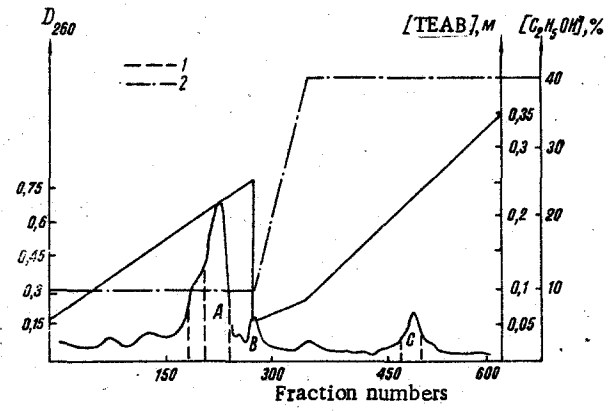

Fig. 1. Chromatographic separation of the mixture after the synthesis of the octanucleotide d[(MeOTr) T-T-ibuG-T-T-bzA-anC-ibuG] on DEAE-cellulose (HCO_3^-; 25 × 600 mm). Fractions of 14 ml/9 min: 1) concentration of TEAB; 2) concentration of ethanol. Zone C contained 500 OU_{260} of the octanucleotide contaminated with pyrophosphate.

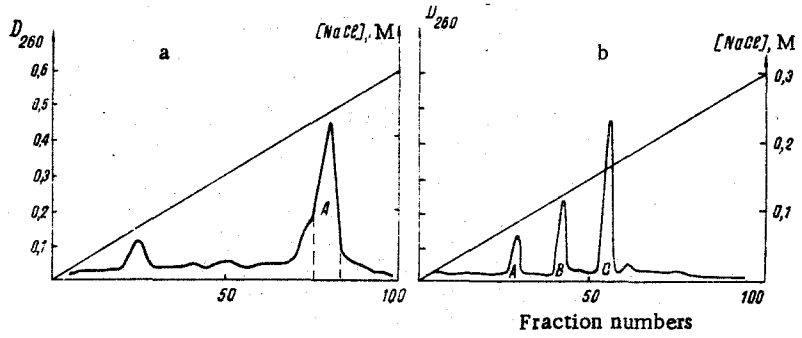

Fig. 2. Rechromatography on a column of DEAE-cellulose (Cl^-, 6 × 300 mm) in a linear concentration gradient of NaCl in 8 M urea: a) 53 OU_{260} of the octanucleotide d[(MeOTr)T-T-ibu-G-T-T-bzA-anC-ibuG] (0.02 M Tris-HCl buffer, pH 7.0, fractions of 4.5 ml/7 min; zone A contained 40 OU_{260} of the octanucleotide contaminated with pyrophosphate); b) 21 OU_{260} of the octanucleotide d(T-T-G-T-T-A-C-G) (pH 3.5, fractions of 1.0 ml/1 min; zone C contained 11 OU_{260} of octanucleotide).

equivalent per one molar equivalent of dissociated phosphate groups in the reaction components. At the stage of the isolation of the trinucleotide (III) we used ion-exchange chromatography in 8 M urea in a concentration gradient of NaCl in place of the chromatography in a concentration gradient of triethylammonium bicarbonate (TEAB) that is usually used in this case. This enabled us to achieve purity of the preparation isolated and to shorten the time of its isolation from the eluate, since the evaporation of a large amount of triethylammonium bicarbonate buffer is replaced by a desalting stage.

The pentanucleotide tetraphosphate (II) was isolated by extraction with organic solvents, and the purity of the preparation so obtained was not inferior to that of compound (III) isolated chromatographically. The octanucleotide (V) was isolated by ion-exchange chromatography on DEAE-cellulose in a gradient of TEAB in ethanol (Fig. 1). However, the substance could not be freed completely from the symmetrical trinucleotide pyrophosphate (III), apparently because of the high lipophilicity of the latter. Ion-exchange chromatography in 8 M urea (pH 7.5) enabled the contaminating pyrophosphate impurity to be eliminated only partially (after separation, the amount of pyrophosphate impurity was about 15%) because

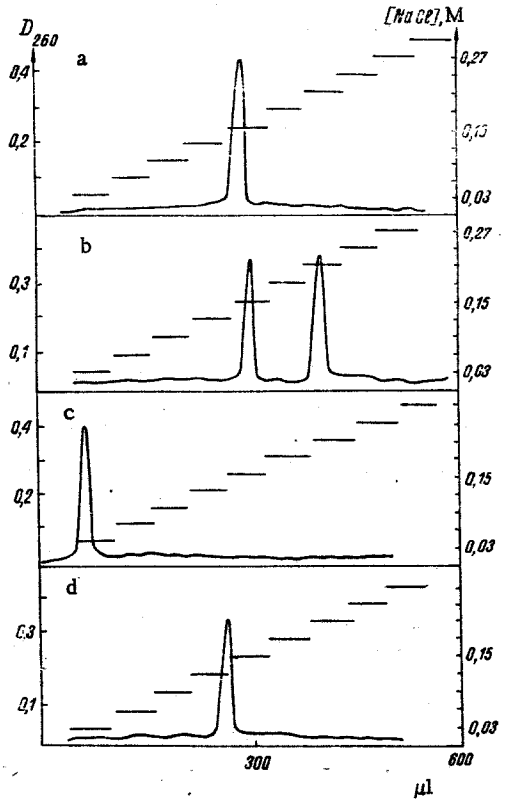

Fig. 3. Chromatographic separation on DEAE-cellulose (Cl^-, 1 × 55 mm) in a linear gradient of NaCl in 7 M urea, pH 3.0: a) the octanucleotide d(T-T-G-T-T-A-C-G); b) the octanucleotide modified with a mixture of sodium bisulfite and O-methylhydroxylamine; c) hydrolyzate of the unmodified octanucleotide by snake venom phosphodiesterase; d) hydrolyzate of the modified octanucleotide d(T-T-G-T-T-A-C*-G) by snake venom phosphodiesterase.

of the small difference in the number of charges in the compounds to be separated (see Fig. 2a). The final purification of the octanucleotide (VI) was achieved (after the elimination of all the protective groups) by chromatography in the Tomlinson-Tener system [10] at pH 3.5 (see Fig. 2b).

The spectral and chromatographic characteristics of the oligonucleotides synthesized are given in Table 1. The composition of the oligonucleotides were confirmed by determining the ratio of the products of their hydrolysis by snake venom phosphodiesterase or a mixture of snake venom phosphodiesterase and *E. coli* alkaline phosphatase (see Table 1).

In order to confirm the primary structure of the octanucleotide synthesized, we used specific chemical modification of the cytidine and guanosine units followed by hydrolysis of the modified oligonucleotides with snake venom phosphodiesterases. E. D. Sverdlov et al. [11] have shown that snake venom phosphodiesterase stops its action at that point of the polynucleotide chain where the cytidine units modified with a mixture of sodium bisulfite and O-methylhydroxylamine are located. Since, after the modification of the cytosine, the total charge of the oligonucleotide changes, chromatographic separation permits the determination of the number of cytidine units in the oligonucleotide under investigation. As can be seen from a comparison of Figs. 3a and 3b, the octanucleotide (V) contained only one cytidine unit. The position of the unit in the oligonucleotide chain was determined in the products of the hydrolysis of the modified oligonucleotide by snake venom phosphodiesterase.

TABLE 1. Some Characteristics of the Oligodeoxyribonucleotides Synthesized

Compound	Spectral characteristics				R_f in systems*		Ratio of the products of enzymatic hydrolysis
	λ_{max} / λ_{min}	$E\frac{240}{260}$	$E\frac{280}{260}$	$E\frac{300}{260}$	1	2	
d [(MeOTr)T-T-ibuG-T-T] II	260 / 240	0,37	0,71	0,11	0,49	0,55	
d [T-T-G-T-T) IIa	266 / 235	0,50	0,74	0,11	0,08	0,11	dpT : dpG : dT=2,93 : 1,0 : 1,05†
d (pbzA-anC-ibuG)III	280 / 233	0,67	1,20	0,81	0,40	0,48	
d (pA-C-G) IIIa	257 / 232	0,67	0,61	0,22	0,09	0,11	dpA : dpC : dpG=1,04 : 1,02 : 1,0†
d (T-T-G-T-T-A-C-G) VI	260 / 232	0,59	0,69	0,07	0,00	0,05	dC : dT : dG : dA=1,01 : 4,0 : 2,1 : 1,0‡

*For the symbols of the chromatographic systems, see Experimental part.
†The results of hydrolysis by snake venom phosphodiesterase are given.
‡The results of hydrolysis by a mixture of snake venom phosphodiesterase and of *E. coli* alkaline phosphatase are given.

As can be seen from Fig. 3c, hydrolysis led to the appearance of a pentanucleoside hexaphosphate (the charge was determined from the position of the peak on the chromatogram of the hydrolyzate), which confirmed the assumed position of the cytidine in it in compound (V).

The positions of the guanosine residues in the octanucleotide were determined similarly after modification of the guanine heterocycles with glyoxal. The oligonucleotides modified in this way were subjected to complete hydrolysis by snake venom phosphodiesterase, but hydrolysis slowed down considerably in the modified units. Because of this it is possible to expect that of the hydrolyzate of d(T-T-G*-T-T-A-C-G*) (the modified guanosine residues are indicated by an asterisk) octa- and trinucleotide derivatives will be detected. A chromatographic investigation ofthe hydrolyzate (see Fig. 4) completely confirmed this assumption.

Thus, the use of the specified modification of the cytosine and guanosine units has enabled us to confirm the primary structure of the octanucleotide synthesized.

EXPERIMENTAL

Chromatography on paper (FN-1, GDR) was performed in the following systems: 1) ethanol—1 M ammonium acetate (7:3; pH 3.5) and 2) n-propanol—concentrated aqueous ammonia—water (55:10:35). Thin-layer chromatography was performed on silica gel plates (Eastman Kodak, USA) in the following systems: 3) acetonitrile—water (85:15) and 4) acetonitrile—water (8:2). Ion-exchange chromatography was carried out on DEAE-cellulose DE-23 (Whatman, UK) and DEAE-Sephadex A-25 (Pharmacia, Sweden). Gel filtration was carried out on Sephadexes G-25 and G-10 (Pharmacia, Sweden).

The work was carried out with the mononucleotides of dpT and dpibuG(iBu) and the dinucleotides d(pbzA-anC) and d(pT-T). The preparation of the cyanoethyl and acetyl derivatives of the oligonucleotides, and also the elimination of the protective groups was performed by known methods [8]. The dinucleotides d[(MeOTr)T-T] were obtained by the method of Khorana et al. [9]. The compositions of the oligonucleotides after the elimination of the protective groups were determined by hydrolysis with snake venom phosphodiesterase (E.C. 3.1.4.1.) or with a mixture of snake venom phosphodiesterase and *E. coli* alkaline phosphatase (E.C. 3.1.3.1.) (Worthington, UK). The ratio of the products in the hydrolyzate was established with the aid of a Varian (USA) high-pressure liquid chromatograph on a column containing Aminex A-6 (2.4 × 250 mm) with 0.4 M ammonium formate, pH 4.55 as the eluent at a rate of elution of 15 ml/h.

Analytical microcolumn chromatography was performed with the aid of a MKSFP-4 instrument [12] on DEAE-cellulose (Cl^- 1 × 55 mm) in a linear concentration gradient of NaCl in Tris-HCl buffer, 0.005 M, pH 7.5, and 7 M urea acidified with HCl to pH 3.5; rate of elution 360 µl/h.

d[(MeOTr)T-T-ibuG] (I) was obtained by analogy with the general method of synthesis described by Khorana [8] from 1.93 g (2 mmole) of d(MeOTr)T-T and 3 g (5 mmole) of dpibuG(iBu) using 3.7 g (12 mmole) of TPS (5 h, 20°C). After the decomposition of the TPS and the evaporation of the solution, the residue was dissolved in 100 ml of 0.12 M TEAB (pH 8.2) and was extracted with ether (10 × 60 ml), ethyl acetate (4 × 120 ml) and mixtures of ethyl acetate and butanol (9:1, 2 × 100 ml; and 85:15, 4 × 50 ml) to separate the impurities. The 3'-acetate of compound (I) was isolated from the buffer solution by extraction with a mixture of methylene chloride and n-butanol (7:3, 2 × 100 ml), the course of extraction being monitored by TLC in system 3 (R_f of compound (I), 0.25). The solution was treated with 1 liter of pyridine and evaporated, and the residue was dissolved in 40 ml of 2 N NaOH; the solution was kept at 20°C for 30 min and then 120 ml of Dowex-50 in the pyridinium form was added. The suspension was transferred to a 30 × 400 mm column containing 50 ml of pyridine Dowex-50 and the compound (I) was eluted with 1 liter of 50% pyridine. The eluate was evaporated and the trinucleoside diphosphate was isolated by precipitation in ether (2 liters) from solution in anhydrous pyridine (45 ml).

The yield of d[(MeOTr)T-T-ibuG] was 1.6 g (1.2 mmole; 60%).

d[(MeOTr)T-T-ibuG-T-T] (II) was obtained in a similar manner to compound (I) from 0.97 g (0.7 mmole) of d[(MeOTr)T-T-ibuG] and 2.12 g (2.7 mmole) of d[pT-T(Ac)] using 3.0 g (10 mmole) of TPS. After the decomposition of TPS and evaporation of the solution, the residue was dissolved in 50 ml of 0.2 M TEAB (pH 7.0) and extracted with ether (2 × 30 ml), methylene

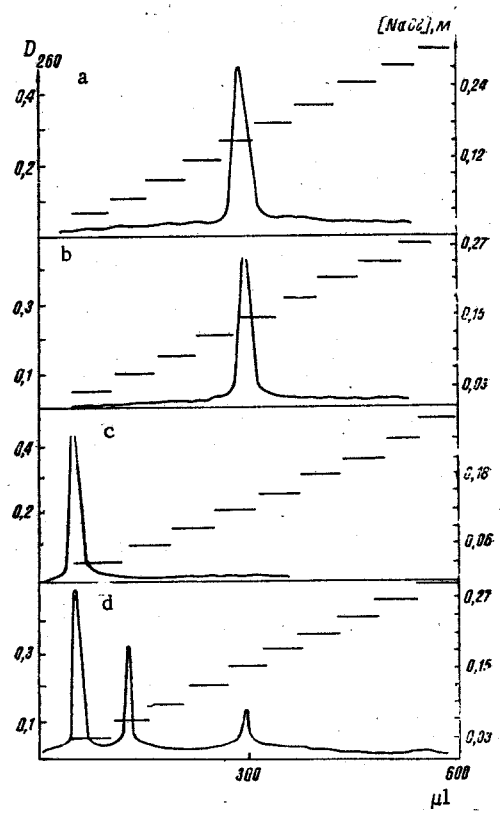

Fig. 4. Chromatographic separation on DEAE-cellulose (Cl^-, 1 × 55 mm) in a linear gradient of NaCl in 7 M urea, pH 3.0: a) initial octanucleotide d(T-T-G-T-T-A-C-G), b) octanucleotide modified with glyoxal; c) hydrolyzate of d(T-T-G-T-T-A-C-G) produced by snake venom phosphodiesterase; d) hydrolyzate of the modified octanucleotide d(T-T-G*-T-T-A-C-G*) produced by snake venom phosphodiesterase.

chloride (3 × 30 ml), butan-1-ol—ethyl acetate (1:9, 2 × 25 ml; 2:8, 3 × 25 ml; 3:7, 5 × 40 ml) and butan-1-ol—methylene chloride (3:7, 6 × 50 ml; 4:6, 4 × 100 ml). The extraction process was monitored by GLC on silica gel in system 4. After precipitation in absolute ether, the pentanucleotide [(MeOTr)T-T-ibuG-T-T(Ac)] was dissolved in 5 ml of 50% aqueous pyridine and the solution was treated in 5 ml of 2 N NaOH (0°C, 20 min) and was then neutralized with Dowex-50 (pyridinium form) to pH 8, and the resin was washed with 30% aqueous pyridine (200 ml). The filtrate was evaporated, and the residue was dissolved in 10 ml of absolute pyridine and precipitated in two liters of absolute ether.

The yield of d[(MeOTr)T-T-ibuG-T-T] was 0.71 g (0.26 mmole, 32%). The characteristics of the compound (II) are given in Table 1. The homogeneity of the preparation was confirmed by ion-exchange chromatography on a microcolumn containing DEAE-cellulose.

d(pbzA-anC-ibuG) (III) was obtained similarly from 1.75 g (1.5 mmole) of d[(CNEt)pbzA-anC] and 2.83 g (5.0 mmole) of dpibuG(iBu) using 5.8 g (19 mmole) of TPS (20°C, 6 h). After decomposition of the TPS and evaporation of the solution, the residue was dissolved in 100 ml of 0.15 M TEAB, pH 7.5, and extracted in an extractor with ether for 5 h. The aqueous layer was evaporated with pyridine, the residue was dissolved in 10 ml of 50% aqueous pyridine, the solution was treated with 10 ml of 2 N NaOH (0°C, 20 min) and neutralized by the addition of Dowex-50 (pyridinium form), and the resin was filtered off and washed with 30% aqueous pyridine (500 ml). The filtrate was evaporated and the residue was dissolved in 1000 ml of 8 M urea containing 0.02 M Tris-HCl, pH 7.0, and was deposited on a column of DEAE-Sephadex (Cl^-, 25 × 400 mm) previously equilibrated with 8 M urea containing 0.02 M

Tris-HCl buffer (pH 7.0) and it was chromatographed in 8 M urea and 0.02 M Tris-HCl buffer (3 liters of 0.1 M NaCl – 3 liters of 0.3 M NaCl), 16-ml fractions being collected every 5 min. Fractions 275-325 were desalted on DEAE-cellulose. The trinucleotide (III) was isolated by precipitation in 2 liters of absolute ether from 80 ml of absolute pyridine.

The yield of d(pbzA-anC-ibuG) was 0.75 g (0.51 mmole, 35%).

d[(MeOTr)T-T-ibuG-T-T-bzA-anC-ibuG] (V) was obtained from 0.113 g (0.005 mmole) of d[(MeOTr)T-T-ibuG-T-T] and 0.36 g (0.25 mmole) of d[(pbzA-anC-ibuG(Ac)] using 0.43 g (1.4 mmole) of TPS. The reaction mixture after the decomposition of the TPS was evaporated with pyridine, and the residue was dissolved in 15 ml of 0.2 M TEAB (pH 7.3). The solution was extracted with ether (5 × 60 ml) and with methylene chloride (4 × 50 ml), the course of extraction being monitored by TLC. The aqueous layer was evaporated with pyridine and the residue was dissolved in 10 ml of 50% aqueous pyridine and treated with 10 ml of 2 N NaOH (0°C, 20 min). After neutralization of the solution and conversion of the nucleotide material into the pyridinium salt, it was dissolved into 50 ml of 0.05 M TEAB in 10% ethanol (pH 7.15) and deposited on a column of DEAE-cellulose. The chromatographic conditions are shown in the caption to Fig. 1.

d(T-T-G-T-T-A-C-G) (VI). The octanucleotide (V) (53 OU_{260}) was treated with 25% aqueous ammonia (50°C, 16 h) and with 80% acetic acid (20°C, 45 min) to eliminate the protective groups, and was chromatographed on DEAE-cellulose in 8 M urea at pH 7.0 and 3.5 (see Fig. 2, a, b), and it was then desalted by gel filtration on a column of Sephadex G-25.

Determination of the Positions of the Cytidylic Acid Residues in the Octanucleotide (VI). To 0.25 OU_{260} of the octanucleotide (VI) was added 11 µl of a 2 M solution of O-methylhydroxylamine and 11 µl of a 2 M solution of sodium metabisulfite (pH 6.0). The solution was carefully stirred and was kept at 20°C for 40 min, and then the nucleotide material was departed from the modifying agents by means of gel filtration on a column (0.8 × 140 mm) of Sephadex G-10 and was subjected to ion-exchange chromatography on a microcolumn of DEAE-cellulose (Cl^-, 1 × 55 mm) at pH 3.0. The fractions containing the unmodified and modified oligonucleotides were collected separately and were hydrolyzed with snake venom phosphodiesterase. The hydrolyzate was deposited on a microcolumn and was subjected to ion-exchange chromatography on DEAE-cellulose at pH 3.0. The results of the experiment are shown in Fig. 3.

Determination of the Position of the Guanylic Acid Residues in the Octanucleotide (VI). To 0.3 OU_{260} of the octanucleotide (VI) was added 22 µl of a 1% solution of glyoxal in 0.1 M phosphate buffer (pH 8.3), and the solution was carefully stirred and was left at 50°C for 50 min. Then the nucleotide material was separated from the modifying reagent by gel filtration on a column (0.8 × 140 mm) of Sephadex G-10 in borate buffer (0.05 M borate buffer, pH 8.5; $5 \cdot 10^{-3}$ M $MgCl_2$). Snake venom phosphodiesterase was added to the nucleotide material in the borate buffer and the mixture was kept at 37°C for 20 min. From this solution, 48 µl was diluted to 150 ml with water and was heated on the water bath at 100°C for 5 min. Then the hydrolyzate was chromatographed on a microcolumn of DEAE-cellulose (1 × 55 mm; pH 3.0). The results of the separation are given in Fig. 4.

E. D. Sverdlov (Institute of Bioorganic Chemistry of the Academy of Sciences of the USSR) kindly supplied the enzyme preparations and took part in the discussion of the results on the determination of the positions of the cytidine and guanosine residues in the octanucleotide.

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AN INVESTIGATION OF THE LIGNINS OF HEALTHY AND WILT-AFFECTED COTTON PLANTS OF VARIETY TASHKENT-1 ACCORDING TO THE VEGETATION PERIODS

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Wilt — one of the most dangerous diseases of the cotton plant — causes great losses to cotton growers. In recent years, breeders have isolated new wilt-resistant varieties of the cotton plant Tashkent-1, -2, -3, and others [1], but with time they have also become subject to the action of wilt.

We have performed a comparative study of the dioxane lignins of healthy cotton plants and plants affected by the fungus *Verticillium dahliae* Kleb. of variety Tashkent-1 according to the vegetation period. The samples were collected on the territory of the F. Engels kolkhoz in the Srednechirchikskii region, Tashkent oblast. The characteristics of the samples — healthy stems of the early period (I), healthy and wilt-affected stems of the flowering period (II, III), and healthy and wilt-affected bolls and stems after the harvesting of the crop (IV-VII) — are given below (%):

Chemical component	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*
Ethanol—benzene extract	8,15	5,10	6,80	12,73	7,25	6,60	4,22
Aqueous extract	19,41	19,33	25,26	7,77	9,56	3,14	12,82
Komarov lignin	23,20	23,20	23,25	22,80	24,50	22,94	24,49
Cellulose	31,00	38,00	34,60	28,95	32,25	38,07	31,30
Ash substances	9,60	4,15	3,60	10,20	7,10	5,40	5,22

Thus, the amount of substances extractable by hot water from the welt-damaged samples of the cotton plant was greater than from healthy specimens, but the amount of substances extractable by ethanol—benzene (1:1) from the healthy specimens was greater. Apparently, under the action of the wilt fungi the cellulose and polysaccharides are broken down into lower-molecular-weight fractions and become water-soluble. This can explain the marked increase in water-extractive substances in wilt-affected specimens and the decrease in the amount of cellulose in them.

In wilt-damaged specimens of the cotton plant, the amount of Komarov lignin and ash substances increases. The increase in the amount of Komarov lignin can be explained, on the one hand, by the protective reaction of the plants to the fungus. On the other hand, investigations of recent years [2] have shown that the parisitic fungus is to be less sensitive to the toxic action of an excess of phenolic substances than the cotton plant: the phenolic substances cause necrotization and withering of the affected tissues of the host plant, which aggravates the growth and development of the fungus in the conducting vessels adjacent to these tissues.

The dioxane lignins of the cottonplant of variety Tashkent-1 (DLCT) from stems of the early period finely comminuted (0.25 mm) and pre-extracted with ethanol—benzene (1:1) and hot water (DLCT-I), from healthy and wilt-affected plants in the flowering period (DLCT-II and -III), from healthy and affected mature plants (DLCT-VI, and -VII), and from healthy and wilt-affected bolls (DLCT-IV and -V) were isolated by a modification of Pepper's method [3]

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