STUDIES ON THE LENGTH OF THE SIDE CHAINS OF THE DEXTRAN ELABORATED BY *Leuconostoc mesenteroides* NRRL B-512*

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ABSTRACT

The length of the side chains in dextran NRRL B-512 has been studied by sequential degradation. Selective removal of the terminal D-glucosyl groups was achieved by alkaline treatment of a methylated dextran in which the hydroxymethyl groups had been replaced by *C*-*p*-tolylsulfonylmethyl groups; the 6-hydroxyl group of the new terminal units thus formed is unsubstituted. The degraded material was subjected to a second degradation by using the same procedure. The reactions were followed by methylation and/or ethylation of the products, acid hydrolysis, and analysis of the resulting mixture of ethers by g.l.c.–m.s. As a result of these studies, it is concluded that about 40% of the side chains contain only one D-glucose residue, at least 45% are two D-glucose units long, and the rest (max. 15%) are longer than two such units.

INTRODUCTION

Dextran NRRL B-512 is a (1→6)-linked α-D-glucan having side chains linked to the 3-positions of the backbone. Previous investigations by methylation analysis[1,2] and periodate oxidation studies[3,4] have demonstrated that this dextran has about 5% of branching points. Partially hydrolyzed dextran B-512 is used for the preparation of a blood-plasma substitute. The relationship between its detailed structure and its immunological properties is, therefore, of importance. The immunological properties of dextran B-512 have been extensively studied with human antibodies[5], and it has been demonstrated, by inhibition techniques, that the combining site of the anti-dextran antibodies is complementary to the series of isomaltodextrins, with an upper limit of the site corresponding to an isomaltoheptaose unit. The reactivity of the antibodies seems to be dependent on the number of side chains in the dextran and, probably, also on their length.

Previous studies[2] on the length of the side chains in dextran B-512 indicated that about 25% of these contained only one D-glucose residue. The method used was based upon catalytic oxidation of the terminal D-glucopyranosyl groups, followed by

*Dedicated to Dr. Nelson K. Richtmyer in honor of his 70th birthday.

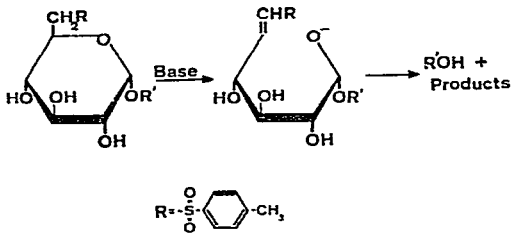
isolation and characterization of the aldobiouronic acids formed on acid hydrolysis. Only 60% of the end groups were oxidized, and the quantitative evaluation was not very accurate. The method did not discriminate between side chains containing two or more D-glucose residues.

Similar studies, by Bourne and co-workers[6], demonstrated that essentially all of the side chains in dextrans NRRL B-1375, B-1415, and B-1416 consist of single D-glucose residues.

The present investigation reports studies on the length of the side chains in dextran B-512 by use of a specific degradation method.

METHODS

The degradation method. — The glycosidic linkage in a hexopyranoside containing a 6-deoxy-6-*p*-tolylsulfonyl group is cleaved on alkaline treatment[7], by the following sequence of reactions.



The reaction proceeds *via* a β-elimination, facilitated by the electron-attracting sulfone group. This reaction, which has been investigated with simple glycosides[7], has also been used for the degradation of a polysaccharide[8]. The degradation of this polysaccharide was, however, only partially successful, as quantitative conversion of the hydroxymethyl groups into *C*-*p*-tolylsulfonylmethyl groups could not be achieved without affecting other sites in the molecule.

In dextran B-512, only the terminal D-glucosyl groups, in the backbone and in the side chains, contain a free hydroxyl group at C-6. It is, therefore, an ideal substance on which to apply this degradation method. Further, new primary hydroxyl groups are released after elimination of terminal D-glucosyl groups linked to 6-positions, and it should, therefore, be possible to subject the dextran to consecutive degradations. The unfavorable solubility properties of dextran and its derivatives created special problems which could, however, be overcome. Thus, a permethylated 6-deoxy-6-*p*-tolylsulfonyldextran in which all secondary positions were methylated was prepared by reactions 1–9 in the following reaction scheme.

Reaction scheme

1. Tritylation of dextran (**1**) to **2**; 2. methylation of **2** to **3**; 3. detritylation of **3** to **4**; 4. tritylation of **4** to **5**; 5. methylation of **5** to **6**; 6. detritylation of **6** to **7**; 7. *p*-

toluenesulfonylation of **7** to **8**; 8. replacement of *p*-tolylsulfonyloxy group in **8** by iodine, to give **9**; 9. replacement of iodine in **9** by *C*-*p*-tolylsulfonyl, to give **10**; 10. alkaline degradation of **10** to **11**; 11. *p*-toluenesulfonylation of **11** to give **12**; 12. replacement of *p*-tolylsulfonyloxy group in **12** by iodine, to give **13**; 13. replacement of iodine in **13** by *C*-*p*-tolylsulfonyl, to give **14**; and 14. alkaline degradation of **14**, with subsequent ethylation to give **15**.

This material was then subjected to alkaline degradation (Reaction 10), the product was converted into the 6-deoxy-6-*p*-tolylsulfonyl derivative (Reactions 11–13), and this was subjected to a second alkaline degradation (Reaction 14). The reactions were monitored by hydrolysis of the modified dextrans (or their fully methylated or ethylated derivatives) and analysis by g.l.c.[9]–m.s.[10] of the alditol acetates prepared from the D-glucose ethers obtained.

*Preparation of permethylated 6-deoxy-6-*p*-tolylsulfonyldextran* (**10**). — Dextran (**1**) was tritylated, in methyl sulfoxide–pyridine[11], with an excess of chlorotriphenylmethane (trityl chloride). The trityl derivative (**2**) was then methylated by treatment[12] with methylsulfinylsodium and methyl iodide in Me_2SO, giving **3**. Part of this material was hydrolyzed, and the resulting mixture of methylated D-glucoses was analyzed (see Table I). The analysis showed that all of the primary positions in **3** were tritylated (absence of 2,3,4,6-tetra-*O*-methyl-D-glucose) and that some of the secondary positions were also tritylated (high percentage of di-*O*-methyl-D-glucoses).

TABLE I

METHYL ETHERS FROM THE HYDROLYZATES OF MODIFIED DEXTRANS

Modified dextran	*2,3,4,6-Tetra*-O-*methyl*-D-*glucose* (%) (T = 1.00[a])	*2,3,4-Tri*-O-*methyl*-D-*glucose* (%) (T = 2.50)	*2,4-Di*-O-*methyl*-D-*glucose* (%) (T = 5.10)
1, methylated	5.5	89.0	5.5
4		81.0	19.0[b]
4, methylated	5.4	89.2	5.4
7	0.1	94.8	5.1
7, methylated	5.4	89.2	5.4
11, methylated	3.8	92.8	3.4
11, methylated[c]	3.6	87.7	3.2

[a]*T* is the retention time of the corresponding alditol acetate on an ECNSS-M column, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as unity. [b]Also contains other di-*O*-methyl-D-glucoses. [c]Recalculated values, assuming that 5.5% of the D-glucose residues have been eliminated.

The material (**3**) was therefore detritylated by treatment with hydrogen chloride in methanol, affording the methylated product **4**. Part of this material was methylated, the product hydrolyzed, and the mixture of D-glucose ethers analyzed as before (see Table I). The good agreement between this methylation analysis and that of the original dextran (**1**) showed that no appreciable degradation of the material had occurred during the detritylation. In **3**, the ratio of secondary to primary hydroxyl groups was only 2:1, compared to 60:1 in the original dextran. On tritylation of **4**, a

more selective reaction of the primary hydroxyl groups could, therefore, be expected. The tritylated dextran (**5**) was methylated; part of the product (**6**) was hydrolyzed, and the resulting mixture of methylated D-glucoses analyzed (see Table I). The analysis confirmed that tritylation had occurred essentially at the primary positions of **4**, with negligible substitution at the secondary positions.

Detritylation of **6** yielded **7**, and, again, methylation analysis revealed that no significant degradation had occurred.

Partially methylated dextran (**7**) was *p*-toluenesulfonylated by reaction with *p*-toluenesulfonyl chloride in chloroform–pyridine, and the product (**8**) was treated with sodium iodide in chloroform–*N*,*N*-dimethylformamide (DMF). The resulting 6-deoxy-6-iodo derivative (**9**), on treatment with sodium *p*-toluenesulfinate in chloroform–DMF, yielded the fully methylated 6-deoxy-6-*p*-tolylsulfonyldextran (**10**).

First degradation. — The terminal sugar residues in **10** were eliminated by treatment with methylsulfinylsodium in Me_2SO, yielding **11**. The reagent was not rigorously dried and, therefore, contained some sodium hydroxide. Methyl iodide or ethyl iodide was added after the reaction, thereby effecting complete methylation or ethylation of the hydroxyl groups released during the elimination. The alkylated products were hydrolyzed, and the hydrolyzates were analyzed as before. The methylation analysis (see Table I) revealed that (*a*) the degradation was complete, as equal quantities of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,4-di-*O*-methyl-D-glucose were obtained, and (*b*) about 40% of the branches had been lost during the degradation.

The ethylation analysis (see Table II) confirmed these results, and established, in addition, that all of the terminal units had, in fact, been formed during the degrada-

TABLE II

METHYL AND ETHYL ETHERS FROM THE HYDROLYZATES OF ETHYLATED, MODIFIED DEXTRANS

Modified dextran	*Number of degradation*	*2,3,4,6-Tetra-O-methyl-D-glucose* (%) (T = 1.00)	*6-O-Ethyl-2,3,4-tri-O-methyl-D-glucose* (%) (T = 1.00)	*3-O-Ethyl-2,4-di-O-methyl-D-glucose* (%) (T = 2.16)	*2,3,4-Tri-O-methyl-D-glucose* (%) (T = 2.50)	*2,4-Di-O-methyl-D-glucose* (%) (T = 5.10)
1	0	5.5			89.0	5.5
11[a]	1		3.0	2.4	86.0	3.1
15[b]	2		~1[c]	5.1	84.5	0.7

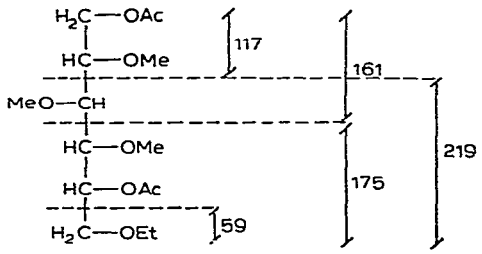
[a]Recalculated values, assuming that 5.5% of the D-glucose residues have been eliminated. [b]Recalculated values, assuming that 9% of the D-glucose residues have been eliminated. [c]Only partially separated from a contaminating, unidentified, non-sugar component.

tion (formation of 6-*O*-ethyl-2,3,4-tri-*O*-methyl-D-glucose, but no 2,3,4,6-tetra-*O*-methyl-D-glucose). It further demonstrated that a considerable percentage of D-glucose residues having a free hydroxyl group on C-3 had been formed during the degradation (formation of 3-*O*-ethyl-2,4-di-*O*-methyl-D-glucose). As expected, the combined

percentage of these two sugars corresponded to the percentage of terminal units in the original dextran.

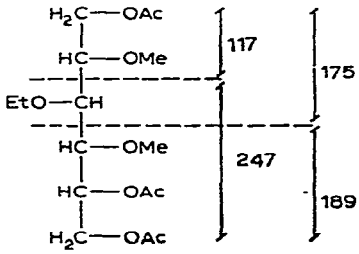
Mass spectrometry of the O-ethyl derivatives. — The mass spectra of alditol derivatives containing both *O*-methyl and *O*-ethyl substituents have not been studied previously. The replacement of a methoxyl group by an ethoxyl group should not, however, significantly influence the fragmentation reactions. The two derivatives containing *O*-ethyl groups were thus readily identified by using the principles previously outlined for partially methylated alditol acetates.

One of the derivatives, *A* (*T* 1.00), gave signals at *m/e* 43 (base peak), 59, 80, 83, 85, 87, 99, 101, 115, 117, 129, 145, 159, 161, 175, and 219, corresponding to all the primary and many of the secondary fragments expected from the 6-*O*-ethyl-2,3,4-tri-*O*-methyl-D-glucose derivative.



A

The second derivative, *B* (*T* 2.16), gave signals at *m/e* 43 (base peak), 80, 87, 89, 101, 103, 115, 117, 121, 129, 131, 175, 187, and 189, in agreement with the expected fragmentation of the alditol acetate derived from 3-*O*-ethyl-2,4-di-*O*-methyl-D-glucose. The primary fragment at *m/e* 247 was not observed, but, instead, a secondary fragment at *m/e* 187, most probably derived by elimination of acetic acid, could be identified.



B

Second degradation. — The material (**11**) obtained after the first degradation contains residues having a free primary hydroxyl group that correspond to the penultimate residues in the original side chains. It also contains chain residues having a free hydroxyl group at C-3 arising from those branches consisting of single D-glucose residues. It should have been possible, by tritylation, methylation, and detritylation, to methylate the latter positions selectively.

Even though the yields of most of the individual reaction steps were good, ten steps had already been carried out, and the decrease in material was considerable. This sequence of reactions was, therefore, omitted and the modified dextran (**11**) was converted into the 6-deoxy-*p*-tolylsulfonyl derivative **14**. Alkaline degradation of **14**, and ethylation of the product, were performed as already described, and the resulting material (**15**) was hydrolyzed and the product analyzed (see Table II).

A low percentage of 2,4-di-*O*-methyl-D-glucose (0.7%) and a corresponding proportion of 6-*O*-ethyl-2,3,4-tri-*O*-methyl-D-glucose were found, demonstrating that the material resulting from the second degradation still contained some branches. 3-*O*-Ethyl-2,4-di-*O*-methyl-D-glucose (5.2%) was also found, representing backbone residues to which, in the original dextran, side chains consisting of one or two D-glucose units had been attached. The high percentage of this residue indicates that few secondary positions had reacted during *p*-toluenesulfonylation of **11**, or that secondary *p*-tolylsulfonyl groups had been replaced by ethyl groups in subsequent reactions. Secondary *p*-tolylsulfonyl groups do not normally react with iodide or *p*-toluenesulfinate, and would, presumably, be hydrolyzed off during the treatment with the strong base. Such alkaline hydrolysis of "isolated" *p*-toluenesulfonates without Walden inversion is well known[13].

The gas–liquid chromatogram of the alditol acetate mixture obtained from **15** was more complicated than that obtained from ethylated **11**. Several minor peaks were derived from contaminating, noncarbohydrate material, as was revealed by mass spectroscopy. The minor component derived from 2,4-di-*O*-methyl-D-glucose and 6-*O*-ethyl-2,3,4-tri-*O*-methyl-D-glucose were not perfectly resolved from the other peaks, containing contaminants, and the percentages given for these components therefore represent upper limits.

DISCUSSION

The percentage of branched D-glucose residues in dextran B-512 decreased from 5.5 to 3.3 during the first degradation, demonstrating that ~40% of the side chains consist of a single D-glucose residue. These values were determined by methylation and ethylation analyses of different dextran derivatives, and there is reasonably good agreement between these analyses; this is in agreement with previous results[1], which gave a value of ~25% by use of a less accurate method.

A finite proportion of branched residues, at most 0.7%, remained after the second degradation. Although a more accurate determination of this value proved difficult, it seems reasonable to conclude that at least 45% of the side chains contain two D-glucose residues, and that 15%, at most, contain more than two D-glucose residues. The molecular weight of the dextran is high ($\bar{M}_w = 2.5 \times 10^7$), and the contribution from the terminal unit of the backbone can thus be neglected, especially as it was demonstrated that no significant degradation occurred during the reactions.

The recovery of the material after each reaction step was good (~80%) and the main losses were probably mechanical. Selective enrichment of any given type of

dextran molecule seems to be excluded, and the final analyses may be presumed to be representative of the original sample.

It is essential that the different reactions proceed to completion. Therefore, higher concentrations and longer reaction-times than those generally used for the corresponding preparative reactions were chosen. The absence of 2,3,4,6-tetra-*O*-methyl-D-glucose revealed by analysis of **3** and **4** demonstrates that complete tritylation of the primary hydroxyl groups had been achieved.

Incomplete *p*-toluenesulfonylation, or incomplete replacement of *p*-tolylsulfonyloxy groups by iodide followed by alkaline hydrolysis of remaining *p*-tolylsulfonyl groups during the degradation, is a potential source of error. Its occurrence is not detected in the analyses of the degraded product, and the results could be misleading as, at the start of the second degradation, some of the primary hydroxyl groups would be derived from original terminal units and not from the penultimate terminal units. The iodine analysis of **9** was, however, in good agreement with that expected for complete reaction, and these errors are not considered significant.

When the first degradation (**6–11**) was repeated, substantially the same results were obtained.

The reaction sequence **1–7**, yielding a fully methylated 6-*O*-*p*-tolylsulfonyldextran, should also be applicable to other polysaccharides, which could then be degraded in the same way.

The combined methylation–ethylation analysis may find other applications in structural polysaccharide chemistry.

EXPERIMENTAL

General methods. — Evaporations were performed under diminished pressure at bath temperatures not exceeding 40°. G.l.c. was conducted at 170° on columns (200 × 0.3 cm) containing 3% (w/w) of ECNSS-M on Chromosorb G (80–100 mesh). A Perkin–Elmer Model 900 gas chromatograph with flame-ionization detector was used. It was observed that the response of the detector, on a molar basis, was the same, within ±5%, for several alditol acetates of different degrees of methylation and, consequently, differences in response factors were disregarded. For the quantitative evaluation of the g.l.c., a Hewlett Packard 3370 A integrator was used. For mass spectrometry, the mixture of alditol acetates, dissolved in chloroform, was injected into a Perkin–Elmer Model 270 combined gas chromatograph–mass spectrometer. The mass spectra were recorded at a manifold temperature of 200°, ionization potential of 60 eV, ionization current of 80 μamp, and an ion-source chamber-temperature of 80°.

Methylation and ethylation analysis. — The polysaccharide (10 mg) in a 5-ml serum bottle, sealed with a rubber cap, was dissolved in methyl sulfoxide (2 ml). Nitrogen gas was flushed through the bottle, and a solution of 2M methylsulfinylsodium in Me_2SO (2 ml) was added dropwise by use of a syringe. The resulting, gelatinous solution was agitated in an ultrasonic bath (40 kHz.sec^{-1}) for 1 h, and then

kept overnight at room temperature. Methyl iodide (or ethyl iodide) (0.5 ml) was then added dropwise with a syringe, with cooling in ice–water, and the turbid solution was agitated for 30 min in the ultrasonic bath, resulting in a clear solution which was poured into water. This solution was dialyzed overnight against tap water, and evaporated to dryness. The methylated polysaccharide was treated with 90% formic acid (3 ml) for 2 h at 100°, the solution concentrated, and the product hydrolyzed in 0.12M sulfuric acid (3 ml) for 12 h. The hydrolyzate was made neutral with barium carbonate, and the sugars were converted into their alditol acetates, which were analyzed by g.l.c.–m.s.

Tritylation of dextran (**1**). — To a solution of **1** (9 g) in Me_2SO (150 ml) were added pyridine (150 ml) and trityl chloride (42 g). The mixture was kept for 2 days at room temperature, diluted with Me_2SO (250 ml), and poured, with stirring, into methanol (5 liters). The precipitated *O*-trityldextran was collected on a sintered-glass filter, washed with methanol, and dried over phosphorus pentaoxide *in vacuo*; yield of **2**, 12 g.

Methylation of **2**. — To a solution of **2** (3.6 g) in Me_2SO (240 ml) in a sealed bottle (under nitrogen) was added 2M methylsulfinylsodium in Me_2SO (250 ml). The mixture was stirred for 2 h, and kept overnight at room temperature. Methyl iodide (70 ml) was added portionwise under external cooling; the resulting solution was stirred for 1 h, poured into water, dialyzed, and evaporated to dryness. The residue was dissolved in chloroform, and precipitated by addition of light petroleum (b.p. 60–80°); yield of **3**, 4.5 g. Part of this material (10 mg) was hydrolyzed, and the resulting mixture of methylated sugars was analyzed as already described (see Table I).

Detritylation of **3**. — To a suspension of **3** (4.5 g) in methanol (750 ml) was added 4M hydrochloric acid (10 ml). The mixture was stirred vigorously for 21 h at room temperature and filtered, and the solid was successively washed with methanol and water; yield of **4**, 2.5 g. Part of the product was subjected to methylation analysis (see Table I).

Tritylation of **4**. — To a solution of **4** (2.5 g) in chloroform (20 ml) were added pyridine (20 ml) and trityl chloride (12.5 g). The solution was kept for 64 h at room temperature, freed of chloroform by distillation, and the resulting pyridine solution poured into water. The precipitated product was successively washed with methanol and water; yield of dry product (**5**), 1.3 g.

Methylation of **5**. — Compound **5** (1.3 g) was methylated according to the procedure already described; yield of **6**, 1.3 g. Part of this material was hydrolyzed and the hydrolyzate analyzed (see Table I).

Detritylation of **6**. — Compound **6** (1.3 g) was detritylated according to the procedure already described; yield of **7**, 1.0 g. Part of this material was methylated, and the product analyzed as already described (see Table I).

p-*Toluenesulfonylation of* **7**. — *p*-Toluenesulfonyl chloride (5 g) was added, in portions, to a solution of **7** (1.0 g) in 1:1 (v/v) chloroform–pyridine (20 ml). The resulting, clear solution was kept for 3 days at room temperature, and poured into

water (100 ml). The aqueous phase was extracted with chloroform (3 × 50 ml) and the extracts were combined, washed successively with water, cold 0.25M sulfuric acid, aqueous sodium hydrogen carbonate solution, and water, and evaporated to dryness, yielding **8** (0.90 g).

Reaction of **8** *with iodide.* — To a solution of **8** (0.90 g) in 1:2 (v/v) in chloroform–DMF (75 ml) was added 12 g of sodium iodide. The solution was kept for 18 h at 100° and then poured into M sodium thiosulfate (100 ml). The two-phase system was dialyzed, and the chloroform phase was then evaporated to dryness, yielding **9** (0.70 g).

Anal. Calc. for $C_6H_7O_2(OMe)_{2.95}\ I_{0.05}$: I, 3.0. Found: I, 2.95.

Reaction of **9** *with* p-*toluenesulfinate.* — To a solution of **9** (100 mg) in 1:3 (v/v) chloroform–DMF (50 ml) was added 2 g of sodium *p*-toluenesulfinate. The mixture was kept for 18 h at 100°, and then partioned between water and chloroform. The chloroform phase was successively washed with aqueous sodium thiosulfate (1M) and water, and evaporated to dryness, to yield **10** (90 mg).

Alkaline degradation of **10**. — Compound **10** (90 mg) was dissolved in Me_2SO (2 ml) in a serum bottle sealed with a rubber cap. Nitrogen was flushed through the sealed bottle, and a solution (2M) of methylsulfinylsodium in methyl sulfoxide (4 ml) was added dropwise. The resulting mixture was agitated in an ultrasonic bath for 1 h, and kept for 18 h at room temperature. The solution was poured into water (50 ml), made neutral with acetic acid, dialyzed, and concentrated to dryness. The degraded polysaccharide was dissolved in 2:1 (v/v) chloroform–acetone, and the solution was applied to the top of a column (80 × 3 cm) of Sephadex LH 20, which was then eluted with 2:1 (v/v) chloroform–acetone. The optical rotations of the fractions were determined, and the modified dextran **11** (80 mg) was eluted with the void volume. Part of this degraded material (5 mg) was subjected to methylation analysis (see Table I), and another portion (5 mg), to ethylation analysis (see Table II).

p-*Toluenesulfonylation of* **11**. — Compound **11** (70 mg) was *p*-toluenesulfonylated as described for **7**, yielding **12** (70 mg).

Reaction of **12** *with iodide.* — Compound **12** (70 mg) was treated with sodium iodide (3 g) as described for **8**, yielding **13** (65 mg).

Reaction of **13** *with* p-*toluenesulfinate.* — Compound **13** (65 mg) was treated with sodium *p*-toluenesulfinate (2 g) as described for **9**, yielding **14** (50 mg).

Alkaline degradation of **14**. — Compound **14** (50 mg) was treated with methylsulfinylsodium in Me_2SO, affording **15**, which, on addition of ethyl iodide to the reaction mixture, was transformed into its ethylated derivative. This was isolated by chromatography on a column of Sephadex LH 20. Part of the material was hydrolyzed and the hydrolyzate was analyzed (see Table II).

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