with an authentic sample of the 2,4-dinitrophenylhydrazone of acetone.

**Methyl 9-Hydroperoxydehydroabietate.**—Methyl dehydroabietate (40.0 g.) was oxidized in the presence of 2.00 g. of benzoyl peroxide. Reaction was allowed to continue until 30.0 mole per cent. of oxygen had been absorbed. The product contained 29.8 mole per cent. of hydroperoxide. Fractionation of the oxidate was accomplished by discontinuous countercurrent extraction in separatory funnels using hexane and methanol containing 10% of water as the solvent pair. The method involves the diamond pattern of phase combination which has been described fully by Bush and Denson.[10] The fraction recovered from hexane (29.0 g.) contained 3.4% of hydroperoxide and yielded 21.3 g. of unchanged methyl dehydroabietate upon crystallization from methanol. After combination, the methanol solutions were poured into water and the precipitated material recovered by ether extraction. The ether-soluble fraction weighed 12.2 g. and contained 82.8% of hydroperoxide. Crystallization from methanol gave 3.6 g. of slender needles having a m.p. of 132.5 to 133.5°, $[\alpha]^{24}D$ −14° (1%, ethanol). *Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.85; H, 8.75; active oxygen, 4.62. Found: C, 72.80; H, 8.73; active oxygen, 4.60.

**Methyl 9-Oxodehydroabietate.**—A ferrous sulfate solution (1.8 g. in 40 ml. of a 1:1 water–methanol solution) was added dropwise with agitation to a solution of 9-hydroperoxydehydroabietate (1.0 g. in 50 ml. of methanol) over a period of one hour. The reaction mixture was heated to 50° and stirring continued for 1.5 hours. Several volumes of water were added to the reaction mixture and the precipitated material recovered by extraction with ether. The ether soluble fraction weighed 0.96 g. and after flash distillation at 0.2 mm. pressure the distillate weighed 0.78 g. After recrystallization from aqueous methanol the product melted at 68 to 69°, $[\alpha]^{24}D$ +6.4° (2%, ethanol), $\lambda_{max}^{EtOH}$ 254 mμ, α 35.5. *Anal.* Calcd. for $C_{21}H_{28}O_3$: C, 76.80; H, 8.59. Found: C, 76.74; H, 8.75. A 2,4-dinitrophenylhydrazone was prepared and crystallized to constant melting point from ethanol, m.p. 184.5 to 185.5°. *Anal.* Calcd. for $C_{27}H_{32}O_6N_4$: N, 11.01. Found: N, 11.37. A sample (0.5 g.) of the ketone was saponified by heating under reflux for 2 hours with 0.2 g. of sodium hydroxide dissolved in 25 ml. of diethylene glycol and 1 ml. of water. The product was recovered and recrystallized to constant melting point (159.5–161°) from aqueous methanol. The mixed melting point of the material and a sample of 9-oxodehydroabietic acid[11] was unchanged. The X-ray diffraction patterns of the two specimens were identical.

(10) M. T. Bush and P. M. Denson, *Anal. Chem.*, **20**, 121 (1948).

(11) A sample of 9-oxodehydroabietic acid was provided by Dr. Yolanda T. Pratt of the University of Maryland.

**Methyl 9-Hydroxydehydroabietate.**—A solution of sodium sulfide (1.5 g. in 50 ml. of a 1:1 water–methanol solution) was added dropwise with agitation to 1.5 g. of 9-hydroperoxydehydroabietate in 50 ml. of methanol over a period of two hours. The reaction mixture was heated and stirred at 50° for 3 hours before adding water and extracting the product with ether. The ether extract was recrystallized from aqueous methanol to yield 1.43 g. of material melting between 103–105°, after recrystallization the white needles melted from 112 to 112.5°, $[\alpha]^{24}D$ +17° (1%, ethanol). *Anal.* Calcd. for $C_{21}H_{30}O_3$: C, 76.32; H, 9.15. Found: C, 76.74; H, 9.41. A 3,5-dinitrobenzoate was prepared and crystallized from ethanol to constant melting point (163.5 to 164.5°). *Anal.* Calcd. for $C_{28}H_{32}O_8N_2$: N, 5.34. Found: N, 5.30. A sample of the methyl 9-hydroxydehydroabietate (0.40 g.) was saponified by heating under reflux in 15 ml. of diethylene glycol containing 0.15 g. of potassium hydroxide for 3 hours. The free acid was recovered and crystallized to constant melting point (178.5 to 179.5°) from a mixture of benzene and hexane. Calcd., neut. equiv., 316; found, neut. equiv., 316.

A sample (0.70 g.) of methyl 9-oxodehydroabietate was reduced with hydrogen in ethanolic solution at room temperature using 0.60 g. of palladium catalyst. The sample rapidly absorbed 1 mole per mole of hydrogen and the reduction was terminated at this point. After removal of the catalyst the product (0.61 g.) was recovered by evaporation of the solvent. The product was dissolved in 25 ml. of pyridine containing 1.0 g. of succinic anhydride and the resulting solution heated under reflux for 2 hours. The pyridine was removed under reduced pressure and the residue dissolved in ether. The acidic material was isolated by extraction with 2% potassium carbonate solution. After neutralization, the carbonate solution was extracted with ether. The hydrogen succinate was obtained by removal of the ether and saponified by heating under reflux for 30 minutes with 25 ml. of methanol containing 1 g. of potassium hydroxide. The product was precipitated by addition of water and extracted with ether. After crystallization from isooctane there was obtained 0.42 g. of heavy prisms which were recrystallized from the same solvent to constant melting point, m.p. 92 to 93°, $[\alpha]^{24}D$ +56° (1% ethanol). *Anal.* Calcd. for $C_{21}H_{30}O_3$: C, 76.32; H, 9.15. Found: C, 76.56; H, 9.29. A 3,5-dinitrobenzoate was prepared from the alcohol and crystallized from ethanol to constant melting point (163–164°). On admixture of this dinitrobenzoate with that prepared from the alcohol melting at 112–112.5° the melting point was depressed to 149–152°. The X-ray diffraction patterns of the two dinitrobenzoates were distinctly different.

WILMINGTON, DELAWARE

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

# Correlation of Carbohydrate Structure with Papergram Mobility[1,2]

BY DEXTER FRENCH AND GENE M. WILD

RECEIVED DECEMBER 12, 1952

The regularity of the papergram mobilities of homologous oligosaccharide series leads to a straight line characteristic for each series when the logarithm of a partition function, $\alpha'$, is plotted against molecular size. The partition function is obtained from single or multiple ascent papergrams. These observations can be correlated into the generalization that increasing the size of a saccharide by one hexose unit will decrease the papergram mobility by an amount which depends on the type of hexose unit being added and on its mode of attachment. Examples given include oligosaccharides of the starch, dextran, levan, inulin and galactan types.

In a previous publication[3] it was observed empirically that the papergram mobilities of the starch series of oligosaccharides fall into a regular series, such that on plotting the logarithm of the apparent $R_f$ (from multiple ascent development) against the molecular size a curve was obtained which approached a straight line at low $R_f$ values. Oligosaccharides of the dextran series were found to fall on a similar curve of greater slope, while the presence of a single 1,6-link in an otherwise 1,4-linked saccharide decreased the logarithm of the mobility by a fairly constant amount. These observations have been used in this Laboratory in an empirical way to infer structural relationships between saccharides.[4] Development of the theory of paper

(1) Journal Paper No. J-2202 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project 1116.

(2) Presented before the Division of Sugar Chemistry of the American Chemical Society, April, 1952.

(3) D. French and D. W. Knapp, *J. Biol. Chem.*, **187**, 463 (1950).

(4) D. French, *Science*, **113**, 352 (1951).

chromatography,[5] especially the effect of substitution and homology on papergram mobility,[6] makes the application to oligosaccharides less empirical.

The general equation relating the effect of substitution on distribution between phases is[6,7]

$$RT \ln \alpha = \Delta\mu_A + n\Delta\mu_x + m\Delta\mu_y + \ldots \quad (1)$$

where $\Delta\mu_A$, $\Delta\mu_x$, etc., are additive free energy terms which refer to the parent molecule and the various substituents, respectively, and which relate to the transport of one mole of the solute from the stationary to the mobile phase. The effective partition coefficient, $\alpha$, is related to the $R_f$ by the Consden equation[5]

$$\alpha = (A_m/A_s)(1/R_f - 1) \quad (2)$$

Since it is difficult to evaluate $A_m/A_s$ (the ratio of the areas of the mobile and stationery phases) especially for developing solvents which do not give macroscopic phase separations, we find it convenient[8] to use a partition function $\alpha'$, defined by the equation

$$\alpha' = R_f/(1 - R_f) = (1/\alpha)A_m/A_s \quad (3)$$

From eq. 3 it appears that as $R_f$ becomes small, $\alpha'$ approaches $R_f$. Combining equations 1 and 3, and using decimal logarithms, we have

$$\log \alpha' = \log A_m/A_s - (0.434/RT)\Delta\mu_A - \frac{0.434}{RT} n\Delta\mu_x - \frac{0.434}{RT} m\Delta\mu_y - \ldots \quad (4)$$

which may be written

$$\log \alpha' = C_1 - nC_2 - mC_3 - \ldots \quad (5)$$

$C_1$ is the value of log $\alpha'$ for the first member of a saccharide series and the other constants $C_2$, $C_3$, etc., are characteristics for the monosaccharide unit being added (glucose, galactose, arabinose, etc.), its position of attachment, ring form, anomeric configuration, etc.

In the study of compounds of low $R_f$ value, such as the higher molecular weight oligosaccharides, it is desirable to increase the apparent $R_f$ value and obtain better resolution by using the multiple ascent technique.[9] This is preferred over the downward flow technique, in which solvent and eventually the higher mobility solutes drip off the paper. It is possible, moreover, to calculate the true or single ascent $R_f$ value for a substance from its apparent value $R_f^a$, on a papergram of $a$ ascents, by the equation[9]

$$(1 - R_f)^a = 1 - R_f^a \quad (6)$$

Equation 6 depends on the assumption, verified independently by Jeanes, Wise and Dimler and also by the present authors, that the $R_f$ value for a given solute does not depend on the starting position of the solute on the paper.

By using a large number of ascents (15 or more) we have resolved the first 14 oligosaccharides of the linear starch series, and there seems to be no theoretical limitation to the size of saccharides which might be resolved in this way. A practical limitation, however, is that the number of ascents required to resolve each additional higher member increases in an exponential way, such that with the starch series it is necessary to use about twice as many ascents to resolve the next higher saccharide. This difficulty might be overcome by use of a chromatographic developer which promotes higher mobility for the larger saccharides, but so far no such solvent has been found which also gives sufficient resolution of the individual components.

Figure 1 shows the experimental points obtained for some representative oligosaccharide series when the papergram data are treated by equations 3 and 6. For each series, a straight line relationship is observed.

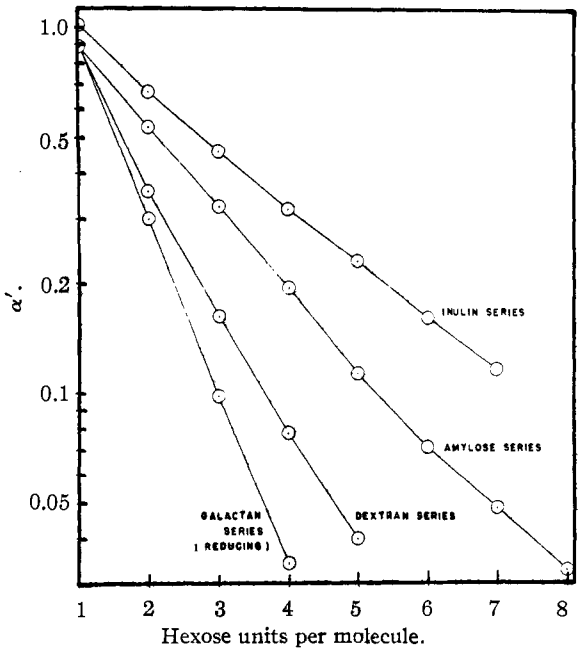


Fig. 1.—Papergram mobilities of oligosaccharide series: inulin series (fructose, inulobiose, inulotriose, etc.) (obtained by hydrolyzing inulin in 0.01 $M$ $H_2SO_4$ at 70° for 15 min.); amylose series (glucose, maltose, amylotriose, etc.) (obtained by hydrolyzing amylose in 0.1 $M$ $H_2SO_4$ at 100° for 15 min.); dextran series (glucose, isomaltose, dextrantriose, etc.) (obtained by hydrolyzing dextran in 0.1 $M$ $H_2SO_4$ at 100° for 60 min.); galactan series (glucose, melibiose, manninotriose, etc.) (obtained by hydrolyzing the water-soluble carbohydrates of *Teucrium canadensis* rhizomes by 0.01 $M$ $H_2SO_4$ at 100° for 10 min.); chromatographic solvent butanol:pyridine:water, 6:4:3 parts by volume; Eaton and Dikeman paper No. 613.

Figure 2 gives comparable data for a few series based on the amylose type of chain. Partial acid hydrolysis of amylose gives the series glucose, maltose and higher homologous members. By using coupling reactions with *macerans* amylase, cyclohexaamylose and suitable co-substrates[10] it is possible to synthesize short amylose chains with the reduc-

(5) R. Consden, A. H. Gordon and A. J. P. Martin, *Biochem. J.*, **38**, 224 (1944).

(6) A. J. P. Martin, *Biochem. Soc. Symp.*, **3**, 4 (1949).

(7) A similar relationship was derived by I. M. Langmuir, *Colloid Symposium Monograph*, **3**, 48 (1925).

(8) E. C. Bate-Smith and R. G. Westall, *Biochem. Biophys. Acta*, **4**, 427 (1950), have suggested use of the term $R_m$ which is defined as log $(1/R_f - 1)$.

(9) A. Jeanes, C. S. Wise and R. J. Dimler, *Anal. Chem.*, **23**, 415 (1951); D. French, D. W. Knapp and J. H. Pazur, THIS JOURNAL, **72**, 5150 (1950).

(10) D. French, J. Pazur, M. L. Levine and E. Norberg, *ibid.*, **70**, 3145 (1948).

ing ends of the molecules substituted by a variety of groups derived from the co-substrates used. It is apparent that in each series log $\alpha'$ decreases by a constant amount on going to the next member of the series.

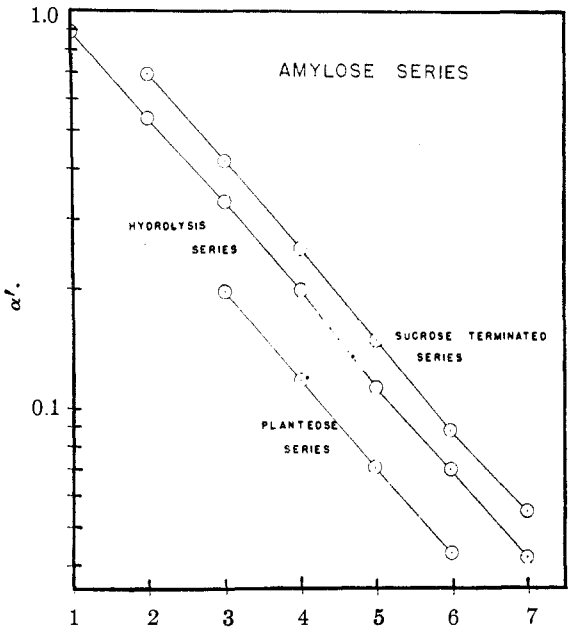


Fig. 2.—Effect of chain length and termination on papergram mobilities of amylose-type oligosaccharides: hydrolysis series (glucose, maltose, amylotriose, etc.); sucrose-terminated series (sucrose, 4-α-D-glucopyranosyl-sucrose, 4-α-maltopyranosyl-sucrose, etc.) (obtained by the coupling reaction[10] with sucrose, cyclohexaamylose and *macerans* amylase); planteose-terminated series (planteose, 4-α-D-glucopyranosyl-planteose, 4-α-maltopyranosyl-planteose, etc.) (obtained by the coupling reaction with planteose,[19] cyclohexaamylose and *macerans* amylase).

Figure 3 shows the group of oligosaccharides produced by the action of a fungal transglucosidase on maltose, in the presence of radioactive glucose[11] (fructose, turanose and the tri- and tetrasaccharides of the amylose series are included for reference). It may be seen that the compounds fall into two series: glucose, isomaltose, dextrantriose, which are radioactive, and maltose, panose[4,12] and a homologous tetrasaccharide (4-α-dextrantriosyl-D-glucose), which are not. A fast-moving radioactive disaccharide, present in only small amounts in the enzyme digests, bears the same chromatographic relationship to glucose that turanose bears to fructose and may be 3-(α-D-glucopyranosyl)-D-glucose.

Saccharide series showing the effect of α-D-galactopyranosyl groups on mobility are presented in Fig. 4. Sucrose, raffinose, stachyose and higher homologous saccharides have been found in several plants.[13] In our experience ash manna (*Fraxinus ornus*), *Stachys tuberifera* tubers, *Teucrium canadense* rhizomes, *Verbascum thapsus* roots, *Soja hispida* seeds and other plant tissues give chromatograms which show the first four or more of such a series. The structure for stachyose reported by Onuki[14] on the basis of methylation studies is galactose < 1,6 galactose < 1,4 glucose < 1,2 > fructose. Similarly Murakami[15] has investigated verbascose, the pentasaccharide from *Verbascum thapsus* and has concluded that it is galactose < 1,6 galactose < 1,6 galactose < 1,4 glucose < 1,2 > fructose. However, the regularity with which sucrose, raffinose, stachyose and verbascose fall into a chromatographic series, together with the very well substantiated structure of raffinose and melibiose, leads us to suggest that the structure of stachyose is galactose < 1,6 galactose < 1,6 glucose < 1,2 > fructose and that verbascose is the higher homologous compound, similarly 1,6-linked between the galactose and glucose units. It is of interest that Armstrong[16] proposed the 1,6-linked formulation for stachyose

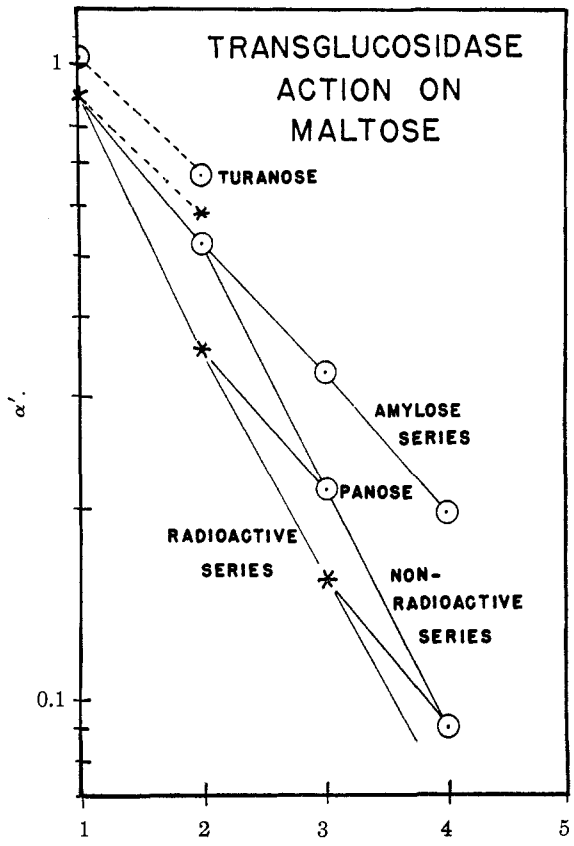


Fig. 3.—Papergram mobilities of oligosaccharides produced by action of *Aspergillus oryzae* transglucosidase on maltose in the presence of a trace of radioactive glucose: amylose series (glucose, maltose; amylotriose and amylotetraose are also included for reference though they are not produced in the reaction); radioactive series (glucose, isomaltose, dextrantriose); non-radioactive series (maltose, panose, 4-α-dextrantriopyranosyl-D-glucose); the radioactive disaccharide at $\alpha' = 0.59$ is a new compound which bears the same chromatographic relationship to turanose ($\alpha' = 0.67$) that glucose bears to fructose ($\alpha' = 1.03$).

(11) J. H. Pazur and D. French, THIS JOURNAL, **73**, 3536 (1951); *J. Biol. Chem.*, **196**, 265 (1952).

(12) M. L. Wolfrom, A. Thompson and T. T. Galkowski, *ibid.*, **73**, 4093 (1951).

(13) B. Tollens and H. Elsner, "Kurzes Handbuch der Kohlenhydrate," fourth ed., Johann Ambrosius Barth, Leipzig, Germany, 1935, pp. 477, 502 and 509.

(14) M. Onuki, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, **20**, 201 (1933).

(15) S. Murakami, *Acta Phytochem. (Japan)*, **11**, 213 (1940).

(16) E. F. Armstrong and K. F. Armstrong, *Chemistry and Industry*, **53**, 912 (1934).

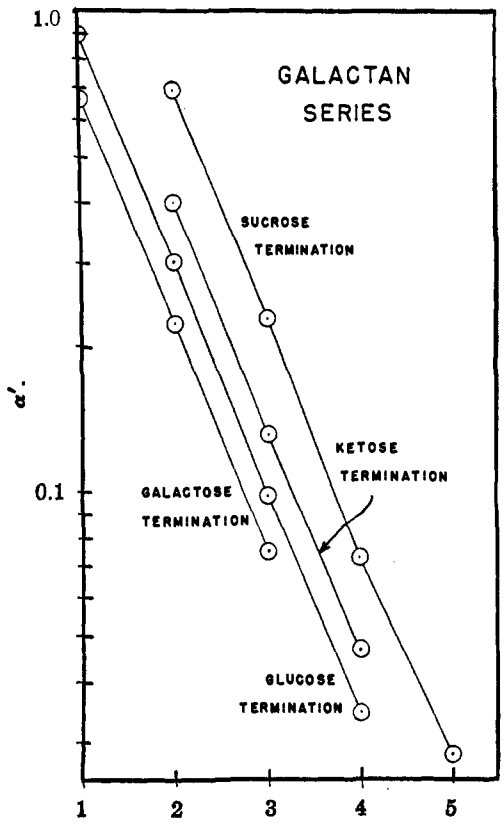


Fig. 4.—Influence of chain length and termination on papergram mobilities of galactan-type oligosaccharides: sucrose terminated series (sucrose, raffinose, stachyose, verbascose) (obtained from *Teucrium canadensis* rhizomes); ketose terminated series (planteobiose, etc.) (obtained from the acid inversion of ash manna, identified by the phloroglucinol–hydrochloric acid and alkaline copper–phosphomolybdic acid sprays, not oxidized by hypoiodite solution, present in only small amount); glucose terminated series (glucose, melibiose, manninotriose, verbascotetraose); galactose terminated series (galactose, galactobiose, galactotriose) (obtained by oxidizing the glucose-terminated series with bromine water and subsequently hydrolyzing in 0.1 *M* $H_2SO_4$ at 100° for 30 min.).

but cited the Onuki work as evidence for such a structure. Also, a recent report by Bradfield and Flood[17] states that partial acid hydrolysis of stachyose gives melibiose, a result which could be obtained only if there is a 1,6-linkage uniting the galactose and glucose units. On partial hydrolysis of the sucrose-terminated series by acid or by yeast invertase, one obtains the series of reducing saccharides glucose, melibiose, manninotriose, etc. Again the regularity of this series is strongly indicative of a continuity of structure type on passing from melibiose to manninotriose, although the classical formulations for these compounds would suggest a break at this point. By carrying out either bromine oxidation or borohydride reduction[18] the reducing group can be destroyed; on subsequent partial acid hydrolysis one obtains the galactose-terminated series of 1,6-linked saccharides: galactose, galactobiose, galactotriose, etc. A series of fructose-containing saccharides, apparently terminated by planteobiose[19] (6-(α-D-galactopyranosyl)-D-fructose, *syn.* melibiulose), has been detected in ash manna. From the exact parallelism with the other series, with which it occurs in nature, it appears likely that it is a corresponding homologous series of 1,6-linked units.

The levan series type is illustrated in Fig. 5. In the reducing series, there is a sharp discontinuity on passing from fructose to the disaccharide, but thereafter a perfectly regular gradation in mobility.

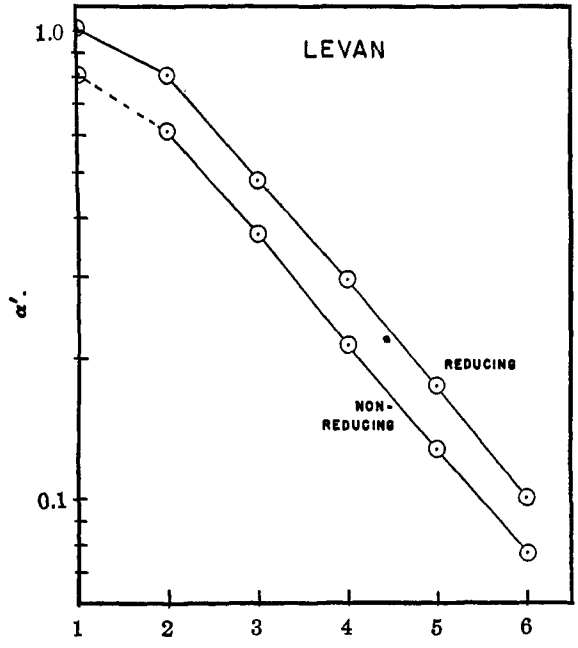


Fig. 5.—Influence of chain length and termination on papergram mobilities of levan oligosaccharides: reducing series (fructose, levanbiose, levantriose, etc.) (obtained by hydrolyzing levan in 0.01 *M* $H_2SO_4$ at 70° for 15 min.); non-reducing series (sucrose, 6′-β-D-fructofuranosyl-sucrose, 6′-β-levanbiofuranosyl-sucrose, etc.) (obtained by concentrating the mother liquor from the precipitation of levan from a sucrose–*Bacillus subtilis* fermentation).

The reason for this discontinuity is most likely found in a change of allowed ring structures on passing from fructose to the disaccharide; fructose can exist in either pyranose or furanose ring forms but the reducing group of a 2,6-linked disaccharide or higher homologous saccharide is limited to the furanose or open chain forms. It has been shown by Isherwood and Jermyn[20] that furanose sugars have considerably higher mobilities than the corresponding pyranoses. A similar break is noted in going from fructose to planteobiose, which is similarly restricted to furanose or open-chain form. The non-reducing series of levan oligosaccharides, terminated by sucrose, is obtained from the bacterial digest of sucrose after the bulk of the levan has been removed by alcohol precipitation. It shows a regularity and parallelism with the reducing series which one would expect if the reducing group of each terminal fructose unit is joined to glucose

(17) A. E. Bradfield and A. E. Flood, *Nature*, **166**, 264 (1950).

(18) M. Abdel-Akher, J. K. Hamilton and F. Smith, THIS JOURNAL, **73**, 4691 (1951).

(19) D. French, G. M. Wild, B. Young and W. J. James, *ibid.*, **75**, 709 (1953).

(20) F. A. Isherwood and M. A. Jermyn, *Biochem. J.*, **48**, 515 (1951).

through a sucrose linkage. The presence of sucrose-terminated oligosaccharides as the low molecular weight components in the levan synthesizing system suggests that levan is produced by a *trans*-fructosidation mechanism, the first step of which may be

$$\underset{\text{(sucrose)}}{2\ \text{G–F}} \longrightarrow \underset{\text{(sucrose-terminated triose)}}{\text{G–F–F}} + \underset{\text{(glucose)}}{\text{G}}$$

Subsequent mechanistically similar reactions lead eventually to the very large levan molecules.

Bacon and Edelman have shown the presence in Jerusalem artichokes and other Compositae of a series of oligosaccharides containing glucose and fructose, sucrose being the first member of the series and higher members derived by substitution of fructofuranose units on position 1 of the fructose unit of sucrose or the terminal fructose unit of higher oligosaccharides. Inulin then may be represented as a sucrose-terminated chain of β-2,1-fructofuranoside units. Such a structure accounts nicely for the known presence of glucose in the inulin structure.[21]

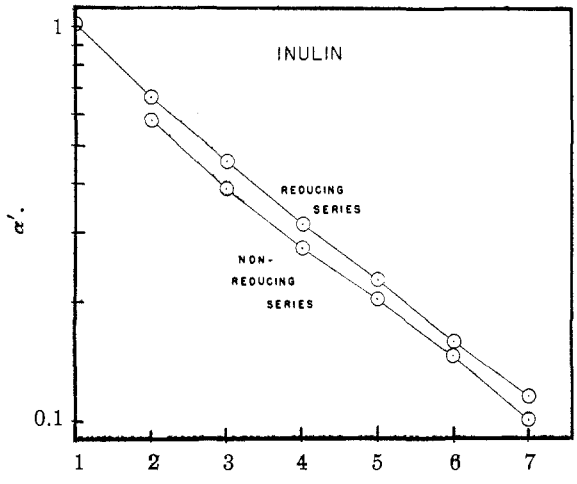


Fig. 6.—Influence of chain length and termination on papergram mobilities of inulin oligosaccharides: reducing series (fructose, inulobiose, inulotriose, etc.); non-reducing series (sucrose, 1′-β-fructofuranosyl-sucrose, 1′-β-inulobiofuranosyl-sucrose, etc.) (obtained from the juice of dahlia bulbs).

If one compares the chromatographic mobilities of the reducing inulin series (produced by acid hydrolysis of high molecular weight inulin) with the low molecular weight non-reducing oligosaccharides ("inulide"), one finds that the chromatographic mobilities fall on slightly separated parallel lines (Fig. 6). The chromatographic series are easily distinguished, however, since compounds of the hydrolysis series respond to the alkaline copper spray for reducing sugars whereas those in the inulide series (from plant extracts) do not.

We believe that the data given here illustrate the following principle, which was derived empirically but might have been anticipated from theoretical considerations[6]: that the logarithm of the partition function $\alpha'$ is an additive property of the various structural features of a given oligosaccharide molecule, and that given suitable reference compounds one may predict with reasonable accuracy the papergram mobility for a saccharide of any given structure. Conversely, the papergram mobility may be a very useful indication as to possible structural features of incompletely known saccharides, and thus facilitate the more rigorous structure analysis.

**Acknowledgment.**—We wish to thank the Corn Industries Research Foundation for support which made this study possible.

AMES, IOWA

(21) J. S. D. Bacon and J. Edelman, *Biochem. J.*, **48**, 114 (1951). E. L. Hirst, D. I. McGilvray and E. G. V. Percival, *J. Chem. Soc.*, 1297 (1950). Isolation of 2,4,6-trimethylglucose from the hydrolysis of methylated inulin must not be construed as indicating the presence of glucose as a non-terminal unit, within the inulin chain, as has been suggested, but rather as indicating incomplete methylation of a terminal glucose unit. It is well known that methylation of sucrose to the heptamethyl stage proceeds rapidly, and that the products of hydrolysis of heptamethylsucrose are mainly 2,4,6-trimethylglucose and 1,3,4,6-tetramethylfructose. Similar results might be expected even from a "completely" methylated inulin, *i.e.*, a preparation which has a methoxyl content approaching the theoretical value.