prepared with hydrazine giving small yellow rosettes from glacial acetic acid, m.p. 170–171°. Calcd. for $C_{19}H_{16}O_8N_2$: N, 6.80. Found: N, 6.70.

Vanilloylformic Acid.—Hydrolysis of formaldehyde-bis-vanilloylformic acid acetal was carried out by boiling 2.02 g. with 67.5 ml. of 2 *N* hydrochloric acid for 22 hours. On cooling, a small amount of black solid separated and was removed, and the light brown colored solution was extracted with ether. After drying, the ether solution was evaporated to dryness giving a brown oil admixed with pale yellow solid. This residue was taken up in hot dry benzene and on cooling, pale yellow granular crystals separated; yield 0.8 g., m.p. 130–133°. Recrystallization from dry toluene gave pale yellow needles, m.p. 132.5–133°. Calcd. for $C_9H_8O_5$: C, 55.11; H, 4.11; CH_3O, 15.82. Found: C, 55.40; H, 4.20; CH_3O, 15.49.

Improved Vanilloylformic Acid Synthesis.—In a second preparation of vanilloylformic acid similar to that described above, 49.8 g. of acetovanillone was added to 11.7 g. of potassium in absolute ethanol and the solution refluxed with 40.2 g. of methylene iodide and 1 g. of copper powder for 12 hours. Another 40.2 g. of methylene iodide was added and reflux continued for a total of 36 hours. The reaction mixture was worked up as before to give 29.5 g. of chloroform-soluble material, m.p. 159–161°, a 57% yield. No starting material was recovered. Oxidation of the formaldehyde-bis-acetovanillone acetal (29.1 g.) suspended in 1200 ml. of water and 190 ml. of 2 *N* sodium hydroxide as before yielded 24.5 g. of crude formaldehyde-bis-vanilloylformic acid acetal, a 72% yield. Hydrolysis of 20.2 g. of formaldehyde-bis-vanilloylformic acid acetal by refluxing with 600 ml. of 2 *N* hydrochloric acid for 26 hours and workup as before gave 13.2 g. of crude vanilloyformic acid, 67% yield. Recrystallization from dry benzene followed by *n*-hexane–ether gave almost colorless needles, m.p. 131–132°. A conductometric titration on 4.70 mg. of this compound gave values for weak and strong acid equivalents of 190 and 210, respectively. Calcd. for $C_9H_8O_5$: mol. wt., 196.1.

Derivatives of Vanilloylformic Acid.—The 2,4-dinitrophenylhydrazone was prepared[18] as red crystals which darkened at 192°, softened at 207–210° and became completely liquid at 230°. Calcd. for $C_{15}H_{12}N_4O_8$: N, 14.89. Found: N, 14.87. Methylation of synthetic vanilloylformic acid (0.392 g.) with dimethyl sulfate and alkali gave 0.39 g. of pale yellow solid veratroylformic acid, which was recrystalized from dry benzene as almost white granular crystals, m.p. 133–134°, and the melting point of these crystals was not depressed when mixed with authentic veratroylformic acid. Methylation of synthetic vanilloylformic acid (0.196 g.) with diazomethane in ether gave 0.1 g. of ester product and recrystallization from aqueous ethanol gave white crystals of methyl veratroylformate, m.p. 60–61°. Similar treatment of veratroylformic acid gave the ester, m.p. 58–60°, and a mixture of these two products melted at 59–61°.

Oxidation of Vanilloylformic Acid.—A typical reaction mixture was vanilloylformic acid (98 mg.), sodium hydroxide (12.4 g.), cupric sulfate pentahydrate (26.9 g.) and water (69 ml.) contained in a 130-ml. stainless steel bomb which was rotated end over end in an oil-bath at 178° for two hours. The resultant mixture was filtered, acidified, ether extracted and an aliquot of the ether soluble solids was analyzed electrophoretically. Yields of vanilloylformic acid and vanillic acid were estimated to be 78 and 13.5 mg., respectively, for the above reaction conditions and 59 and 21 mg. when the reaction time was three hours. Similar treatments of acetovanillone and of acetovanillone–vanillin mixtures produced no vanilloylformic acid detectable by electrophoretic examination of the various ether extracts obtained.

Absorption Spectra.—Ultraviolet absorption spectra were measured with a Beckman Model DU spectrophotometer. Neutral solutions were examined in 95% ethanol. The solvent for alkaline solutions was prepared by diluting 2.8 ml. of 0.1 *N* alcoholic potassium hydroxide with 95% ethanol to 50 ml. as described by Pearl.[21] Protocatechuoylformic acid gave opaque solutions in alkaline ethanol so its extinction coefficient was obtained in an alkaline aqueous solution of the same concentration.

Infrared absorption spectra were measured with a Perkin-Elmer Model 21 Spectrophotometer. Solutions in acetonitrile solvent evaporated films from acetonitrile solutions, and Nujol mulls were used with sodium chloride plates in all cases.

Acknowledgment.—The authors are indebted to Mr. Vincent Felicetta for assistance with certain analytical determinations. The authors appreciated the grant of the Hooker Electrochemical Research Fellowship to D.G.

(21) I. A. Pearl and E. E. Dickey, THIS JOURNAL, **74**, 614 (1952).

SEATTLE, WASHINGTON

[CONTRIBUTION FROM THE NORTHERN UTILIZATION RESEARCH BRANCH[1]]

Factors Affecting Molecular Weight of Enzymatically Synthesized Dextran[2]

BY H. M. TSUCHIYA, N. N. HELLMAN, H. J. KOEPSELL, J. CORMAN, C. S. STRINGER, S. P. ROGOVIN, M. O. BOGARD, G. BRYANT, V. H. FEGER, C. A. HOFFMAN, F. R. SENTI AND R. W. JACKSON

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The molecular weight distribution of dextran synthesized by dextransucrase preparations derived from *Leuconostoc mesenteroides* NRRL B-512 can be varied considerably. A bimodal distribution of molecular weight of dextran is generally found. Initial sucrose concentration in reaction mixture affects both yield and molecular weight of polymer synthesized. This may result from the fructose which is produced. Presence of maltose in a reaction mixture leads to synthesis of oligosaccharides and low molecular weight dextran. Low molecular weight dextran also functions as "primer" and can be grown to higher molecular weight polysaccharide. Formation of low modal dextran is enhanced by increase in enzyme concentration at 30° or decrease in reaction temperature. A possible mechanism of enzymatic action operative in dextran synthesis is proposed.

Previous reports[3,4] on synthesis of dextran by the enzyme, dextransucrase,[5] produced by *Leuconostoc mesenteroides* have indicated that certain sugars and sugar derivatives, notably isomaltose, maltose and α-methyl glucoside, function as glucosyl acceptor substrates or "primers." Extension of these findings has revealed that dextran of low molecular weight also acts as acceptor. By adjusting concentrations of such dextran as primer, an appre-

(1) One of the Branches of the Agricultural Research Service, U. S. Department of Agriculture.

(2) Presented in part before 122nd Meeting of the American Chemical Society, Atlantic City, New Jersey, September, 1952, and 124th Meeting of the American Chemical Society, Chicago, Illinois, September 1953.

(3) H. J. Koepsell, H. M. Tsuchiya, N. N. Hellman, A. Kazenko, E. S. Sharpe, C. A. Hoffman and R. W. Jackson, *Bacteriol. Proc.*, 52nd Meeting, 23 (1952).

(4) H. J. Koepsell, H. M. Tsuchiya, N. N. Hellman, A. Kazenko, C. A. Hoffman, E. S. Sharpe and R. W. Jackson, *J. Biol. Chem.*, **200**, 793 (1953).

(5) It is recognized that more than one enzyme may be involved in synthesis but, for convenience, the term "dextransucrase" will be employed in the singular in this report.

ciable portion of polysaccharide having a weight average molecular weight[6] of 50,000 to 100,000 can be synthesized.[7-9] Dextran with average molecular weight in this range is presently considered suitable for clinical use as plasma-volume expander.

Hehre also has found independently[10,11] that low molecular weight dextran serves as a modifier of dextran synthesis. Carlson, *et al.*,[12] on the other hand, could detect no such effect with enzymatic preparations from *Leuconostoc dextranicum* strain *elai*. Nadel, *et al.*,[13] have subsequently reported that dextran with average molecular weight of 75,000 ± 25,000 could be obtained by incorporating low molecular weight dextran in culture media. This is a more complete report of our investigations of factors which affect molecular weight of enzymatically synthesized dextran. Based on findings described here, small scale, pilot plant studies have been conducted on engineering aspects of an industrial process for production of dextran in molecular weight range suitable for clinical use as plasma-volume expander. They will be reported elsewhere.[14]

TABLE I

MOLECULAR WEIGHT OF DEXTRAN ISOLATED[a] AT VARIOUS EXTENTS OF CONVERSION[b]

Extent of conversion, %	Wt. av. mol. wt., × 10^6 Clarified by filtration[c]	Clarified by centrifugation[d]	Loss on centrifugation, %[d]
5.7	105	20	22
11.4	94	23	22
15.8	70	22, 14[e]	51, 28[e]
20.7	53	17	40
30.0	59	17	47
39.3	77	26	54
55.9	74	20, 24[e]	59, 49[e]
71.8	81	23	57
79.5	97	23	66
94.5	105	32	72
96.1	115	32, 32[e]	76, 66[e]

[a] Dextran precipitated with 85% EtOH, purified by 2 reprecipitations. [b] Reaction conditions: enzyme 50 D.S.U./ml. reaction mixture, sucrose 10%, 30°, *p*H 5.0. [c] Solutions clarified by filtration through a fine-porosity, fritted-glass Corning[15] filter with essentially no loss. [d] Solutions clarified by ultracentrifugation at 30,000 × *g* for 15 minutes. [e] Parent solution used for first value was diluted fivefold and reautoclaved for second value.

(6) It is understood that dextran is not a single molecular species but is composed of molecules varying in composition as well as in molecular weight.

(7) H. M. Tsuchiya, N. N. Hellman and H. J. Koepsell, Abstracts of Papers, Am. Chem. Soc., 122nd Meeting, 15A (1952).

(8) H. M. Tsuchiya, N. N. Hellman and H. J. Koepsell, THIS JOURNAL, **75**, 757 (1953).

(9) H. M. Tsuchiya, N. N. Hellman, H. J. Koepsell, J. Corman, C. S. Stringer, F. R. Senti and R. W. Jackson, Abstracts of Papers, Am. Chem. Soc., 124th Meeting, 35A (1953).

(10) E. J. Hehre, Abstracts of Papers, Am. Chem. Soc., 122nd Meeting, 18A (1952).

(11) E. J. Hehre, THIS JOURNAL, **75**, 4866 (1953).

(12) W. W. Carlson, C. L. Rosano and V. Whiteside-Carlson, *J. Bacteriol.*, **65**, 136 (1953).

(13) H. Nadel, C. I. Randles and G. L. Stahly, *Appl. Microbiol.*, **1**, 217 (1953).

(14) N. N. Hellman, H. M. Tsuchiya, P. Rogovin, B. L. Lamberts, R. Tobin, C. A. Glass, C. S. Stringer, R. W. Jackson and F. R. Senti, *Ind. Eng. Chem.*, in press.

(15) The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

Results

Molecular Weight of Dextran at Various Extents of Conversion.—In order to determine the variability in molecular weight of dextran at various extents of conversion, it was isolated from a reaction conducted with initial sucrose concentration of 10% and 50 dextransucrase units (D.S.U.) per ml. (Table I).

Molecular weight was determined by the light scattering method.[16] Two methods of clarification, filtration and ultracentrifugation, were employed in the preparation of solutions for analysis. Clarification by filtration had the advantage of not removing any dextran and, hence, of not disturbing molecular weight distribution; but filtration may not have adequately removed foreign polymeric materials introduced with the enzyme preparation. Purity of dextran isolated at 5.7% conversion would approximate 95% if an assumption is made that none of the solids introduced with enzyme was removed by filtration. The apparently higher molecular weight of dextran initially synthesized—up to 20.7% conversion—in filtered solutions may be due to inadequate clarification. As conversion proceeded, purity of dextran improved proportionately. Although centrifugation removed solids more nearly completely, it doubtless removed some dextran of higher molecular weight and, hence, did disturb molecular weight distribution.

The data indicate that high molecular weight polymer was a principal product, even when only 5.7% of the sucrose had been converted to dextran. They are also taken as evidence for the molecular weight of dextran changing to only a limited degree during the course of conversion. The implications of these findings are discussed later.

Effect of Sucrose Concentration.—The first indication[3,7] that molecular weight distribution of dextran synthesized could be modified experimentally was obtained when the effect of varying initial sucrose concentration was examined (Fig. 1). The main product formed at 10% concentration was of high molecular weight; all of the dextran precipitated at 38% ethanol. Material isolated from such reaction mixtures had weight average molecular weight of many millions as determined by light scattering measurements. In contrast, the main product formed at 70% sucrose was of low molecular weight; no polymer precipitated below 51% alcohol. Infrared absorption spectra of isolated material were typical of dextran. Additional material, representing very low molecular weight polysaccharide, could be precipitated from 70% sucrose reaction mixture by 90% ethanol. Paper chromatography of the supernatant at 90% alcohol revealed a continuum of sugars extending down through the oligosaccharide range to free glucose and fructose. Oligosaccharides containing only glucose or both glucose and fructose were found. At intermediate sucrose levels (30 and 50%), dextran displayed a bimodal distribution of molecular weights. A portion of the polysaccharide precipitated at 38% ethanol; the remainder began to precipitate at 46 to 48% alcohol. Other sucrose con-

(16) F. R. Senti, N. N. Hellman, N. H. Ludwig, G. E. Babcock, R. Tobin, C. A. Glass and B. L. Lamberts, *J. Polymer Sci.*, in press.

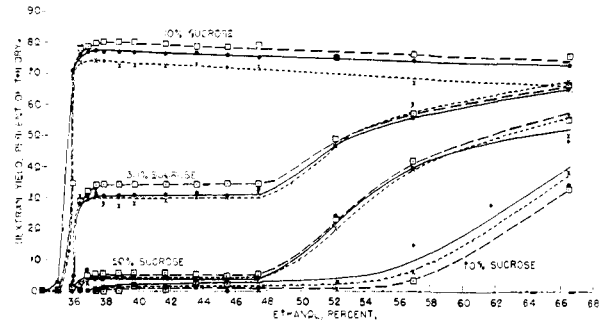

Fig. 1.—Ethanol precipitation characteristics of dextran synthesized at various sucrose concentrations; enzyme; ●, 38 D.S.U./g. sucrose; ⊡, 76 D.S.U./g. sucrose; X, 152 D.S.U./g. sucrose; 25°, *p*H 5.0.

centrations were also tested, as discussed below, but the low modal dextran[17] produced had molecular weights of less than 35,000 as determined by light scattering.

The bimodal distribution of dextran synthesized was also strikingly demonstrated by sedimentation studies in Model E Spinco Ultracentrifuge. Figure 2 shows sedimentation diagrams of reactions conducted at various concentrations of sucrose. Reaction mixtures were examined uniformly at dilutions calculated to contain 0.5% theoretical dextran concentration. All centrifugations were conducted under identical conditions and the diagrams are presented with equal magnification for the series so that the area under each peak corresponds to relative concentration of dextran in that component.

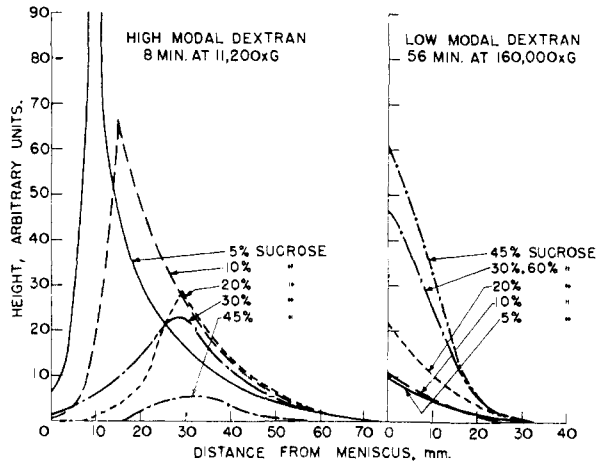

Fig. 2.—Sedimentation diagrams of reaction mixtures at various sucrose concentrations.

Ultracentrifugation of these reaction mixtures at a uniform concentration of high modal dextran of *ca.* 0.14% yielded sedimentation constants of 380, 485, 504, 471 and 140 Svedberg units at initial sucrose levels of 5, 10, 20, 30 and 45%, respectively. The sedimentation constant of high modal dextran first increased and then decreased as sucrose concentration was increased.

(17) For convenience, dextran with high molecular weight will be referred to as "dextran of the high mode" and dextran with low molecular weight as "dextran of the low mode." The former precipitates at *ca.* 40% alcohol concentration; the latter precipitates at higher concentrations.

The low modal dextran appeared very polydisperse and extended to molecular weights too low to be sedimented in the centrifugal field. Although its upper limit had a sedimentation constant of *ca.* 3 Svedberg units, which corresponded to dextran of about 100,000 molecular weight, average molecular weights did not exceed 35,000. Diagrams of the low modal component of both 30 and 60% sucrose lay below that of the 45% sucrose reaction mixture indicating that a maximum sedimentation constant of low modal dextran was also reached as a function of sucrose concentration. In reactions conducted at sucrose concentrations of 5 or 10%, the major product was of high molecular weight but a small and detectable portion of low modal dextran was synthesized.

The primary effect of change in sucrose concentration under conditions employed was variation in the relative amounts of high and low modal dextrans. It should be noted that three different enzyme-to-sucrose ratios at each sugar level were employed in these polymerizations (Fig. 1). Molecular weight distributions were dependent only on sucrose concentration. Secondary effects on molecular weights of high and low modal components were also observed. The relative sharpness of distribution of sedimentation of high modal dextran stood in contrast to that of the low modal fraction. Presence of two widely separated modes of molecular weight in the production necessitates caution in interpreting results[13] of molecular weight determination on dextran since values obtained may not correspond to any of the species present but rather to a mean of the two components.

Effect of Fructose Concentration.—Since fructose is a major product of the dextransucrase reaction, the observed effect of sucrose might be due to high fructose concentration arising during the reaction. To demonstrate the effect of this hexose independently of sucrose concentration, an experiment was performed in which sucrose level was kept at a low steady state concentration and fructose concentration maintained at approximately 2.5, 5.0 and 10.0%. Eighteen hundred ml. of buffered, sucrose solutions containing 4.75, 9.5 or 19.0% sucrose was added dropwise over 24 hours to 200 ml. of reaction mixtures containing 70,000 D.S.U. and 2.5, 5.0 and 10.0% fructose, respectively, and 0.01 *M* acetate buffer (*p*H 5.0). These sucrose concentrations of the "feed" solutions were selected so as to maintain fructose concentrations at the above levels. Sufficient enzyme was present to prevent accumulation of sucrose. This was verified by reducing powers of samples taken during the course of this experiment. Reactions were conducted at 25° ± 1°. The reaction products (Fig. 3) were characterized by means of ethanol precipitation.

At 2.5% fructose concentration, essentially all of the product was of high molecular weight. At 5.0% fructose, most of the polymer synthesized was high modal dextran, but a small amount of low modal component was formed. However, at 10% fructose, only 35% of the product synthesized was of the high mode and the remainder was of the low mode. It thus appears that high fructose concentration favors synthesis of low modal dextran and explains,

at least in part, the effect of high initial sucrose concentration. The action of fructose is quite complex, however. Samples of reaction mixtures were examined in the ultracentrifuge during the course of synthesis. In the reaction mixture containing 10% fructose, high modal dextran was formed primarily in the first 4 hours of reaction. The sedimentation constant of low modal fraction increased to approximately 1.6 in the first 12 hours of sucrose addition; thereafter, it remained constant. Thus, in spite of essentially constant fructose and sucrose concentrations, molecular weight distribution of synthesized dextran varied during the course of polymerization.

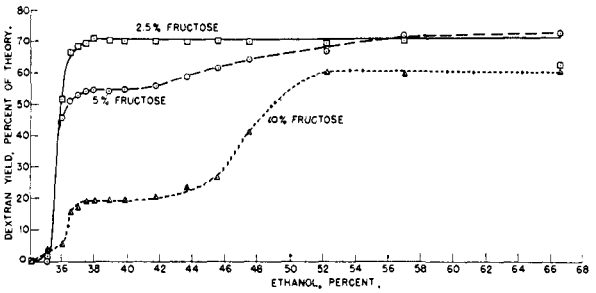

Fig. 3.—Ethanol precipitation characteristics of dextran synthesized at various fructose concentrations; 25°, *p*H 5.0, see text for reaction conditions.

Maltose as Primer.—The glucosyl acceptor role played by auxiliary sugars in dextran synthesis has already been reported.[4] Maltose had proved to be an exceptionally active acceptor substrate; it increased reaction rate and decreased yield of dextran precipitable at 50% ethanol. An experiment was performed in which dry sucrose was added incrementally to a reaction mixture containing 50 D.S.U. of enzyme and 50 mg. of maltose per ml. Acetate buffer at 0.02 *M* and *p*H 5.0 was used and synthesis was conducted at 30 ± 1.0°. Sufficient time was allowed for complete conversion of each portion of sucrose before the next was added. After fructose concentration had increased to about 11%, additional portions of enzyme solution were added in order to maintain the reducing power at this level and the enzyme concentration at 50 D.S.U. per ml. Samples, removed at various sucrose to maltose ratios (S/M), were examined by paper chromatography (Fig. 4).

Maltose decreased in concentration as sucrose was added and an oligosaccharide, identical in mobility to that of panose,[18] was formed. This trisaccharide would be the anticipated product if a glucosyl radical were linked through an α-1,6-bond to the non-reducing end of maltose. As more sucrose was added, the trisaccharide disappeared with concomitant formation of a still higher oligosaccharide presumed to be a tetrasaccharide. When S/M reached 26.5, no detectable amounts of oligosaccharides which could be moved on paper by the solvent employed were found. Apparently the oligosaccharides had grown to higher molecular weight polysaccharides. The oligosaccharides seen in this chromatogram represent, of course, only a portion of the bimodal distribution.

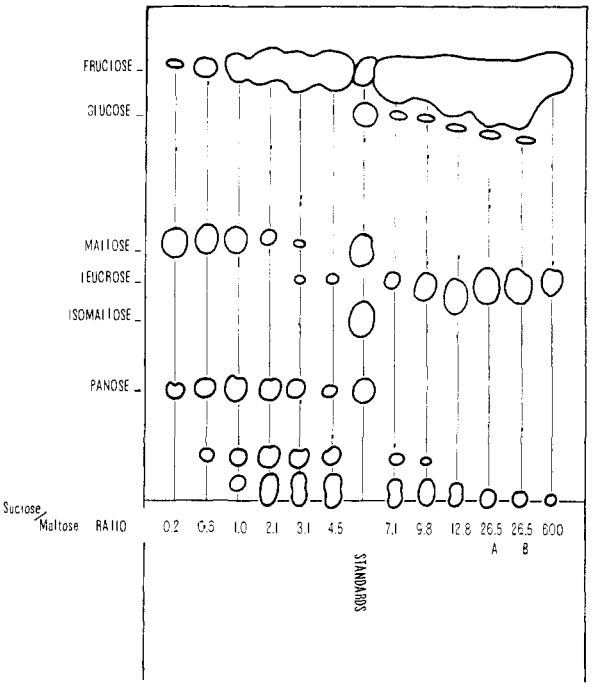

Fig. 4.—Oligosaccharides formed from reaction of sucrose and maltose; 30°, *p*H 5.0, see text for reaction conditions.

Molecular weight distribution of polymers synthesized in reaction mixtures of this type was examined by the methanol precipitation procedure and ultracentrifugal sedimentation. A bimodal distribution resembling those occurring in the 30% sucrose as well as in the 10% fructose syntheses was found. Similarly, average molecular weight of dextran of the low mode was too low for use as a plasma-volume expander. Paralleling the 10% fructose reaction mixture, the average sedimentation constant of the low modal component rose with addition of sucrose to *ca.* 1.5. Above S/M of 75, it remained constant.

Experiments have also been performed at 30°, in which S/M of 6.6, 66 and 660 were tested at sucrose concentrations of 10 and 20%. These sucrose levels yield theoretically 5.26 and 10.5% fructose, respectively, at completion of synthesis. In these reactions, lower S/M was required for demonstration of bimodal distribution at 10 than at 20% sucrose. The bimodal distribution was present at a S/M of 6.6 but not at 66 or 660 when 10% sucrose was used, whereas it was obtained at all S/M values with 20% sucrose.

Low Molecular Weight Dextran as Primer.—We have reported earlier[7-9] that low molecular weight dextran also serves as primer and that increase in concentration of this acceptor substrate (or more specifically, high ratio of primer to sucrose) decreases molecular weight of low modal dextran. Unlike other primers, this primer leads to appreciable synthesis of low modal dextran with no apparent upper limit to molecular weight. The molecular weight of the product can be readily controlled over a wide range. A dialysis technique was em-

(18) We are indebted to Dr. S. C. Pan (E. R. Squibb and Sons) for providing the sample of panose and to Dr. Allene Jeanes (Northern Utilization Research Branch) for the sample of isomaltose.

ployed in our earlier work[7,8] as follows: a mixture of enzyme and low molecular weight dextran was placed inside a dialysis membrane sack, and the sack immersed in a sucrose solution. Subsequently,[9] we found that sucrose, dextran primer, and enzyme could be mixed together at initiation of synthesis. Results (Fig. 5) obtained under these conditions confirmed our earlier findings.

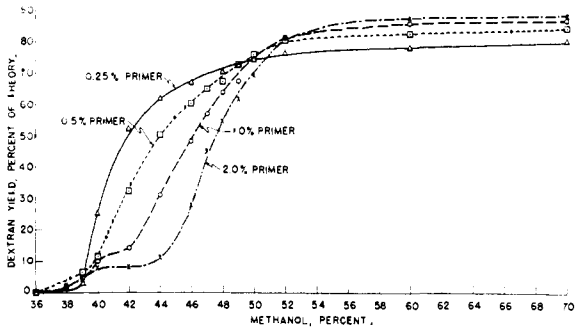

Fig. 5.—Methanol precipitation characteristics of dextran synthesized with varying concentrations of low molecular weight dextran as primer; enzyme 40 D.S.U./ml. reaction mixture; sucrose 10%, 15°, *p*H 5.0.

Rate of Sucrose Addition.—Three methods of adding sucrose to mixtures of enzyme and primer (low molecular weight dextran) have been investigated: Dialysis technique, mixing of reactants at initiation of synthesis, and slow, dropwise addition of sucrose solutions to enzyme-primer mixtures over periods of time extending to 31 hours. Rate of sucrose addition appeared to have a slight, but definite, effect. The dialysis procedure and the dropwise addition of sucrose solution at the slowest rate led to sharpest distribution of low modal dextran. However, enhancement of sharpness was not sufficient to warrant the inconvenience involved. Hence, all reactants were mixed at initiation of experiments in subsequent work reported here.

Effect of Temperature.—The temperature for maximal rate of dextransucrase activity, as measured by increase in reducing power, is in the range of 32 to 34°. A noteworthy finding is that temperature, in addition to altering reaction rate, affects markedly molecular weight distribution in primed syntheses. Lowering reaction temperature from 30 to 15° reduced yield of dextran of the high mode and increased yield of dextran of the low mode (Fig. 6). Furthermore, average molecular weight of low modal dextran was slightly increased at 15°. As anticipated, rate of reaction was lower at 15 than at 30°; time required for complete conversion at 10% sucrose was approximately doubled at the lower temperature. A further increase in yield of dextran of the low mode occurred in polymerizations conducted at 10 and 4°. Formation of high modal dextran, however, was not eliminated.

The temperature phenomenon has also been observed, but to a lesser degree, in unprimed reactions with 30% sucrose and in syntheses primed with maltose. Indeed, dextran with molecular weight in the range of 50,000 to 100,000 was formed to some extent in reactions primed with maltose at lower temperatures.

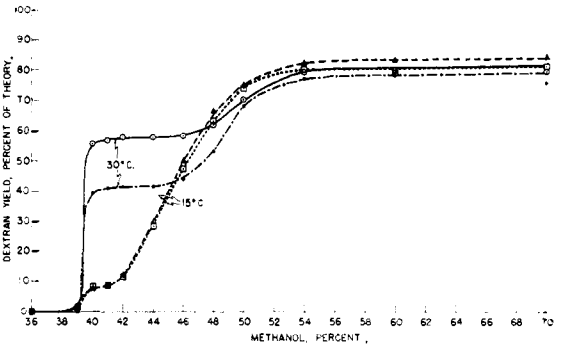

Fig. 6.—Methanol precipitation characteristics of dextran synthesized at two enzyme concentrations and at 15 and 30°; enzyme: ● and ⊡, 40 D.S.U./ml. reaction mixture; ⊙ and △, 10 D.S.U./ml. reaction mixture; sucrose 10%; dextran primer 1%; *p*H 5.0.

Effect of Enzyme Concentration at 15 and 30°.—We have reported[7] that yield of low modal dextran increased as enzyme concentration was increased in dialysis experiments at 25°. This effect was reexamined under conditions whereby all reactants were mixed at initiation of synthesis. Data obtained at 30° (Fig. 6) confirmed our previous observation.

The effect manifested at 30° might suggest that the yield of high modal dextran is reduced at higher enzyme level because of a depolymerative activity present in our enzyme preparation. However, higher enzyme concentration affects yield of high modal dextran only if present during the time that dextran is being synthesized from sucrose. Addition of fresh enzyme in high concentration after completion of synthesis, with further reaction time, had only slight effect on molecular weight distribution. At 15°, however, molecular weight distribution was affected much less by variation in dextransucrase level. Syntheses have been conducted at enzyme levels from 1.25 to 160 D.S.U. per ml. of reaction mixture at this temperature; essentially identical precipitation data were obtained.

Effect of Sucrose Concentration in Primed Reactions.—Since initial sucrose concentration influences molecular weight distribution in unprimed syntheses, this factor was also examined in the dextran-primed reactions. Sucrose levels were varied from 5 to 20%, and sucrose-to-primer ratio held constant at 10:1. As sucrose concentration was increased, the average molecular weight of the major dextran component decreased gradually (Fig. 7). This may be due to the sucrose (or fructose) effect described previously.

Sucrose concentration was also varied over the same range, but primer concentration held constant at 1.0%. It is of interest that in this experiment the sucrose (or fructose) effect was not observed in syntheses conducted at higher sucrose levels (Fig. 8). This may be due to lower primer-to-sucrose ratio at these levels, a condition which has been shown above to lead to higher molecular-weight polysaccharide. The two factors—the high sucrose (or fructose) concentration and low primer-to-sucrose ratio—may have counteracted each other.

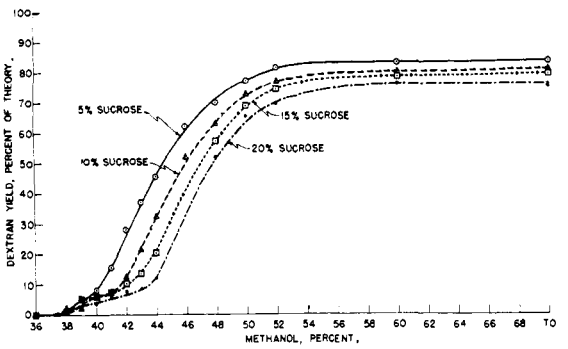

Fig. 7.—Methanol precipitation characteristics of dextran synthesized at various sucrose concentrations and at constant primer to sucrose ratio; enzyme 40 D.S.U./ml. reaction mixture; sucrose dextran primer 10%; 15°, *p*H 5.0.

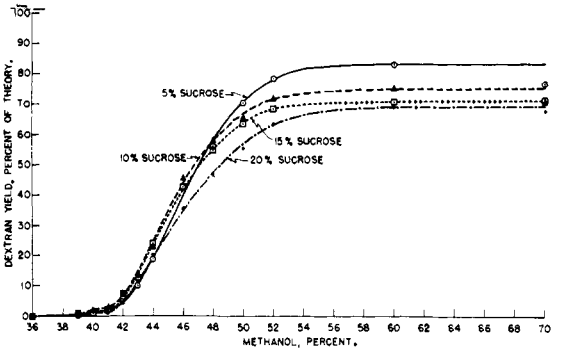

Fig. 8.—Methanol precipitation characteristics of dextran synthesized at various sucrose concentrations and constant primer concentration; enzyme 40 D.S.U./ml. reaction mixture; dextran primer 1%; 15°, *p*H 5.0.

Effect of *p*H.—Within the *p*H limits of 4.7 and 5.6, molecular weight distributions were only slightly affected by hydrogen ion concentration. As anticipated, syntheses at *p*H 4.7 and 5.6 did not proceed as rapidly as did the polymerization at optimal *p*H of 5.0.

Discussion

Any mechanism proposed to explain the enzymatic synthesis of dextran must account for: the magnitude and relative uniformity of molecular weight of dextran at various extents of conversion, in reaction to which no primer had been added (Table I); the occurrence of two dextran components of different molecular weight under most reaction conditions; the dependence of molecular weight of low modal dextran on concentration of primer (Fig. 5); and the influence of reaction temperature and enzyme concentration on molecular weight distribution (Fig. 6).

The bimodal distribution and the temperature effect suggest the presence of two enzymatic activities; for example, dextran from one of the modes might be derived from polymer of the other. However, our attempts to demonstrate such activities have failed. Hence, we are compelled for the present to attempt an explanation of the data in terms of a single enzymatic activity.

According to the glycosyl–enzyme complex hypothesis[19-21] for polysaccharide synthesis, dextran formation would involve: (1) a reaction between sucrose and enzyme with formation of a glucosyl–enzyme complex, and (2) a subsequent reaction between this complex and acceptor with transfer of a glucosyl unit to the latter and release of free enzyme. This mechanism is analogous to a restricted type of condensation polymerization. It has already been suggested[22] that polysaccharide syntheses may be polymerizations of this type in which one elementary step, chain propagation, occurs.

The primer (acceptor) effect on molecular weight of low modal dextran (Fig. 5) is compatible with this concept. An increase in primer concentration, *i.e.*, increase in primer to sucrose ratio, should lower the molecular weight of the synthesis product. Moreover, occurrence of the bimodal distribution, or more explicitly, formation of the high modal fraction, implies that some acceptor molecules react more frequently than do others. Such primer chains may be assumed to have high affinity for enzyme, and, therefore, grow to extremely high molecular weight.

However, the relatively small change in molecular weight of dextran at various extents of conversion (Table I) is not readily explained by this mechanism. If the number of original primer molecules (inherent in the system) is assumed not to increase during the polymerization, molecular weight should increase as a linear function of extent of conversion. If, on the other hand, primer concentration increased as fructose and other reaction products were formed during synthesis, the average molecular weight at 96.1% conversion should be lower than at 20.7% conversion. Instead, molecular weight of material isolated at 96.1% conversion was double that of dextran at 20.7% conversion.

Again, the effects of temperature and enzyme concentration (Fig. 6) are not immediately understandable within limitations of the glycosyl–enzyme complex mechanism. These reaction conditions would not be expected to influence molecular weight distribution of the product.

The above considerations lead us to propose an alternative mechanism for dextran synthesis. The magnitude and relative uniformity of molecular weight of dextran throughout the reaction to which no primer had been added (Table I) is suggestive of a chain reaction type of polymerization.[23] This type is characterized by three elementary steps: chain initiation, chain propagation and chain termination. Chain transfer which influences both initiation and termination may also occur. Application of this polymerization type to the action of dextransucrase would identify: (1) initiation with formation of an enzyme–acceptor complex, (2) propagation with reaction between this complex and sucrose with transfer of a glucosyl residue to the former, and (3) termination with dissociation of enzyme from the primer chain. Chain transfer would be identified with transfer of an enzyme mole-

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cule from one acceptor chain to another. This concept allows for the possibility that more than one propagation step may occur before enzyme is transferred from the first growing chain to another. The chain, of course, grows only during the time it is associated with enzyme. It should be noted that Stacey[24] has already expressed the point of view that "the synthesizing enzyme remains in combination with the polysaccharide." Hehre's experiments[22] prompted him to state that "dextran may be contained as an integral part of the dextransucrase molecule."

The primer effect on molecular weight of low modal dextran (Fig. 5) is compatible with our proposed mechanism. Increase in primer concentration could enhance transfer activity and the concomitant operation of propagation and transfer steps would lead to uniform distribution of glucosyl units to acceptor molecules. Formation of some high modal dextran, in reactions to which primers have been added, necessitates an assumption that enzyme is less readily transferred from acceptor chains with high molecular weight than from those with low molecular weight.

Data on the effect of temperature on molecular weight distribution (Fig. 6) imply that a greater proportion of added primer molecules react in synthesis at 15° than at 30°. This might result from a change in ratio of rates of transfer to propagation steps. If lowering the temperature decreases propagation rate more than transfer rate, the ratio would be higher at 15° than at 30°. This situation would allow relatively more acceptor chains to undergo propagation.

The manner in which enzyme concentration might influence molecular weight distribution at 30° is not clear. The definitive experiments on this effect remain to be performed. Possibly, a study of dependence of high modal dextran formation on extent of conversion in reactions primed with low molecular weight dextran might be enlightening.

Our alternative mechanism for action of dextransucrase would appear to explain more of our findings than would the glycosyl–enzyme complex concept. However, it obviously requires testing by further experimental work. No attempt has been made in the above discussion of the two hypothetical mechanisms to take into account the effect of branching on molecular weight distribution.

In view of the complex manner in which enzyme and sucrose concentrations affect synthesis, consideration of the simple enzyme to sucrose ratio proposed by Nadel, *et al.*,[13] would appear not to provide adequate explanation for formation of low modal dextran. Indeed, data presented (Figs. 1 and 6) directly contradict their basic contention.

The concept of chain transfer reaction may apply to reversible systems such as the phosphorylase system, or to depolymerative reactions like β-amylase action, if the enzymes are associated with the polymers. A controversy[25-28] currently exists between proponents of the single- and multi-chain degradation hypotheses. Reaction conditions favorable for chain transfer might modify single-chain depolymerization to multi-chain depolymerization.[29]

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Experimental

Dextransucrase.—Enzyme preparations were obtained from *L. mesenteroides* NRRL B-512 propagated in a sucrose–corn steep liquor–salts medium.[30-31] The initial *p*H of the medium was 7.2; after acid production had lowered the *p*H to 6.7, automatic alkali addition was begun. The *p*H was maintained at 6.7 ± 0.1 until reducing power of cultures had just passed its peak, whereupon addition of NaOH was discontinued. The reducing sugar content was of the order of 0.1% (fructose equivalent) and the *p*H was about 5.0 to 5.5 at conclusion of fermentations. Culture liquors with activities of 40 to 50 D.S.U. per ml. were obtained.[30,31] The cultures were adjusted to *p*H 7.0–7.2 to precipitate extraneous materials and passed through a filter press as rapidly as possible since dextransucrase is relatively unstable at this *p*H. One pound of filter aid was added per 100 pounds of liquor and 0.1 pound filter aid was used as precoat per square foot of filter area. The filtrates, from which at least 85% of bacterial cells had been removed, were immediately adjusted to *p*H 5.0–5.3. The enzyme is most stable in this *p*H range. The filtrates were cooled to 4° and all subsequent operations were conducted in a room at this temperature. Ethanol, prechilled to −18°, was added slowly and with agitation to a concentration of 35% v./v.; the temperature of mixture was not allowed to rise to over 8° during the addition. The mixture was allowed to stand overnight, whereupon enzymatically active precipitates settled out. The clear supernatants were pumped off and discarded; the precipitates were compacted by centrifugation. They were suspended in water and adjusted to *p*H 5.0. The milky suspensions were dialyzed against running tap water. The *p*H of the enzyme preparation was adjusted to *p*H 5.0–5.2 frequently during all operations by careful addition of H_2SO_4.

The resulting enzyme suspensions, which generally assayed about 700 D.S.U. per ml. and represented about 75% of the activity of initial culture liquors, were held either at 4° or in frozen state at −18°. One D.S.U.[30,31] is defined as that amount of enzyme which will convert, under defined conditions, 1 mg. of sucrose to dextran in 1 hour at 30° and *p*H 5.0, as determined by increase in reducing power calculated as fructose.

The partially purified enzyme preparation employed in the experiment described in Table I assayed 350 D.S.U. per mg., about tenfold the activity of the enzyme preparation used in other experiments reported here. It was obtained by ammonium sulfate precipitation of supernatants derived from centrifuge-clarification of an enzyme suspension prepared in the manner described above. Enzyme preparations assaying as high as 1000 D.S.U. per mg. have been obtained in this Laboratory.

Low Molecular Weight Dextran Primer.—Low molecular weight dextran employed as primer was obtained from a previously conducted primed synthesis of dextran by precipitation between 51 and 60% v./v. methanol. The precipitate was slurried in absolute methanol, collected on coarse filter paper, and dried below 33° under vacuum. The weight average molecular weight of this product was 22,500 as determined by light scattering procedure. Prior to use, the white powdered dextran was dissolved by suspending in sufficient water to make a solution of about 10% w./v. This suspension was steamed for a few minutes at 121° in an autoclave and stirred while hot to achieve complete solution. The solution was filtered after cooling and the *p*H which was about 4.5, adjusted carefully to 5.0 with NaOH. The dextran content of this solution was determined by polarimetry. The $[\alpha]^{25}D$ was taken to be +200.°

Enzymatic Syntheses.—All reactions with exceptions specifically noted above were conducted as follows. One volume of enzyme solution and 3 volumes of sucrose-primer–buffer solution were each brought to reaction *p*H and tem-

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perature. The buffer consisted of HOAc–NaOAc to give an acetate concentration of 0.01 *M* in reaction mixture. The reactants were mixed, the *p*H checked, and a small amount of toluene added to prevent contamination by microörganisms. At termination of syntheses, reaction mixtures were steamed in an autoclave at 100 to 105° for 20 minutes, cooled and stored at 4° until analysis.

In all experiments reported, reactions achieved reducing values at least equal to that for complete conversion of sucrose to dextran and fructose. Hehre[32] has previously noted that reducing values slightly in excess of theory are obtained in enzymatic synthesis of dextran.

Paper Chromatography.—A major portion of dextran was precipitated with 50% ethanol and removed by centrifugation. Supernatant liquors were spotted on paper. The butanol–pyridine–water (3:2:1) solvent[33] was employed to develop chromatograms and ammoniacal silver nitrate was used as spray to detect reducing sugars and dilute urea–phosphoric acid solution[34] to detect fructose-containing oligosaccharides.

Molecular Weight Distribution and Yield of Dextran Synthesized.—Molecular weight distribution and yield of dextran were obtained from alcohol precipitation data. The alcohol concentration at which dextran precipitates from water–alcohol solutions decreases as molecular weight of dextran increases. The relation between precipitation behavior and molecular weight was established by precipitation of enzymatically synthesized, carefully fractionated dextran of known molecular weights. Either ethanol or methanol was employed as precipitant. Reaction mixtures, adjusted to *p*H 5.0, if necessary, were steamed at 105° in an autoclave for 20 minutes to redissolve any precipitated dextran. After mixtures had cooled to 25°, volumes were adjusted so that dextran content was 2.0%. Aliquots (generally 20 ml.) were transferred to test-tubes and warmed to 45–50°. Graded amounts of alcohol were added with shaking and test-tubes stoppered immediately. The mixtures were allowed to equilibrate in a constant temperature room at 25 ± 1.0° for 44–48 hours. After centrifugation, polarimetric measurements were made on supernatant liquors. The $[\alpha]^{25}D$ of dextran in mixtures of water and alcohol was taken to be +200°. Some solutions were opalescent but could be cleared by addition of formamide. In such cases, a correction was made for change in specific rotation on the basis that the $[\alpha]^{25}D$ of dextran in pure formamide is +215°. Weight of dextran precipitated was calculated from decrease in optical rotation. It is recognized that factors other than molecular weight affect alcohol precipitation characteristics of dextran, but the effects are minor under conditions used. Hence this method of analyzing molecular weight distribution was employed because of its relative simplicity.

Under our experimental conditions, it was found that dextran with average molecular weight of 50,000–100,000 precipitated in the methanol range of 44 to 50%.

Yields of dextran are reported as per cent. of dextran anticipated theoretically from sucrose converted, and where added, low-molecular-weight dextran.

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[CONTRIBUTION FROM THE ROSS CHEMICAL LABORATORY, ALABAMA POLYTECHNIC INSTITUTE]

The Reaction of *m*- and *p*-Phenylene-bis-diazonium Fluoborates with Phosphorus Trichloride

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In an attempt to prepare the isomeric phenylenediphosphonic acids, none of which have been described in the chemical literature to date, we examined the reaction of the *meta* and the *para* isomers of phenylene-bis-diazonium fluoborates with phosphorus trichloride as one of the more promising routes to these acids. Neither the Friedel–Crafts nor the Grignard reactions afforded the desired compounds, although both these reactions are used advantageously in the synthesis of arylphosphonic acids.

Numerous preparations have been reported through the fluoborate route since the original publication by Doak and Freedman[1] but in all cases only the monodiazonium salts have been employed.

We found that the fluoborate route, as applied to the *m*- and the *p*-phenylene-bis-diazonium salts failed to yield the expected diphosphonic acids. Rather unexpectedly, the products isolated from the reactions were the corresponding isomers of the chloro- and the fluorophenylphosphonic acids. Evidently, while one of the diazonium groups reacted normally, the other one underwent not the Doak–Freedman reaction, but a form of the Sandmeyer reaction, and, in part, a Schiemann reaction.

Experimental Part

Reaction of *m*-Phenylene-bis-diazonium Fluoborate.—Since the available descriptions of the preparation of the bis-diazonium salts are devoid of some important experimental details, the preparation is given in some detail below; it follows that given by Täuber and Walder.[2] *m*-Phenylenediamine dihydrochloride (15 g.) was tetrazotized, according to Täuber and Walder, and the resulting solution was treated with 87.5 ml. of sodium fluoborate solution.[3] The resulting fluoborate was obtained in 45.3–52% yield in the form of light yellow powder which decomposed at 206°.

This (36.7 g.) was suspended in 250 ml. of anhydrous ethyl acetate, treated with 32.5 ml. of phosphorus trichloride, followed by 8 g. of cuprous chloride, and the stirred mixture was heated carefully to 50°, since no reaction was evident at room temperature after prolonged stirring. At approximately 50° the mixture acquired a transient violet color, which turned to gray, after which a vigorous reaction took place and the viscosity of the solution decreased. After 2.5 hr. at 55–60° the mixture was cooled, treated with 50 ml. of cold water and steam distilled. After the usual treatment[2] the copper-free, concentrated solution deposited a crop of colorless crystals which was augmented by saturation of the solution with dry hydrogen chloride. After nine recrystallizations from dilute hydrochloric acid the material showed a constant melting point (131–131.5°) and was appreciably hygroscopic after drying *in vacuo*. Further recrystallization of this product from concentrated hydro-

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