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The Effect of Synthesis Temperature on the Structure of Dextran NRRL B 512F

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ABSTRACT

The present study puts together the results from several types of experiments from which the effect of synthesis temperature on the primary structure and the macromolecular characteristics of native dextran NRRL B 512F has been deduced.

From light-scattering measurements, it has been concluded that the average molecular weight of non-associated molecules is almost independent of synthesis temperature and much smaller than all the values reported in the literature for non-fractionated native dextran. This result was made possible due to the use of a method which does not take into account the contribution of dextran aggregates. These aggregates usually lead to an over-estimation of dextran molecular weight.

By combining intrinsic viscosity measurements and hydrolysis of dextran with a glucoamylase, evidence is given that branching increases with synthesis temperature, particularly through secondary ramifications.

As a result, the conformation of dextran changes from an expanded random coil type at relatively low temperature (< 20°C), to a compact solid sphere type observed at higher temperature (30°C).

NOTATION

Dextrans are designated as, e.g. Dextran-3(D-3): dextran synthesized at 3°C. Dextran-3–20 (D-3–20): dextran synthesized between 3°C and 20°C inclusively.

INTRODUCTION

The term dextran was coined by Scheibler in 1869 for the highly viscous slimes which caused many problems in the beet-sugar industries; the slimes blocked filters and interfered with the crystallization process. Despite this early interest, the solution of the structure of dextran was only started in 1937 when sufficiently pure samples were obtained to allow a systematic investigation to be performed. A large amount of work was accomplished during the fifties when it was shown that dextran is a polysaccharide composed exclusively of the monomeric unit α-D-glucopyranose linked mainly by (1-6) glucosidic bonds.

Dextrans are produced by several strains of *Lactobacillaceae.* The dextran synthesized by *Leuconostoc mesenteroides* NRRL B 512F particularly was investigated, since this strain is chosen for industrial production. This dextran contains 95% of (1-6) bonds and 5% of (1-3) bonds which form the branch points (Sloan *et al.*, 1954).

At the same time, numerous studies were devoted to the determination of the molecular weight and the hydrodynamic volume of dextrans, particularly in order to establish the Mark–Houwink equation. With regard to the molecular weight of dextran in its native form, the values obtained by light-scattering or ultracentrifugation measurements ranged from 80 to 600 million daltons (Arond & Frank, 1954; Bovey, 1959). These extremely high values were questioned in 1967 by Ebert, who attributed their over-estimation to the presence of very stable aggregates in aqueous solution. Since that time, this problem has remained unsolved.

One aspect of dextran structure which has hardly been described in the literature, is the length of the side-chains. In 1971, Larm *et al.* found by methylation and partial hydrolysis of dextran that 85% of the side-chains contain only one or two glucose units. However, several studies

based on physico-chemical measurements (Wales *et al.*, 1953; Senti *et al.*, 1955, Antonini *et al.*, 1964) as well as on dextran hydrolysis with *exo*-dextranase (Walker & Pulkownik, 1973) led to the conclusion that dextran also contains some very long side-chains (> 30 units).

Commercial dextrans are produced at temperatures close to 25°C, and most of the studies concern dextrans synthesized at this temperature. However, the possibility of producing dextran in a cell-free medium allows the choice of the temperature from within a relatively large range (from 0°C to 30°C). This synthesis parameter seems to have a substantial effect on the structure and properties of dextran, according to the very different appearance and texture of the synthesis medium produced at low and high temperatures.

The aim of this study is to complete the knowledge on dextran structure and to focus on the effect of synthesis temperature on the structure and macromolecular characteristics of dextrans.

MATERIALS AND METHODS

Dextrans

Commercial dextrans of average molecular weight 500000 (T500) and 2000000 (T2000) were provided by Pharmacia (Uppsala, Sweden).

Dextran synthesis

Dextrans were synthesized using the enzyme dextransucrase highly purified from *L. mesenteroides* NRRL B 512F culture broth by a phase-partition process (Paul *et al.*, 1984). The synthesis conditions were chosen in order to obtain high molecular weight dextrans, and are described along with the dextran purification process in previous papers (Sabatie *et al.*, 1986*a*, 1986*b*). The synthesis temperature ranged from 0°C to 30°C.

Enzymatic hydrolysis

Dextran hydrolysis was performed with *Rhizopus* sp., glucoamylase supplied by Sigma (St. Louis, MO, USA) (Cat. No. A725, Lot 124F-0369). The reaction mixture for dextran hydrolysis (10 ml) contained dextran (1%), acetate buffer (50 mM), sodium benzoate (1 g/litre) and enzyme (0·3%) at pH 4·5 and 40°C.

Assays of the percentage of hydrolysis, deduced from the reducing power in the digest, were performed using the dinitrosalicylic method (Sumner, 1935). The residues of hydrolysis were identified by the HPLC gel filtration technique using a Spherogel TSK1000 PW column.

Physico-chemical determinations

Light-scattering

The intensity of scattered light from dimethylsulfoxide (DMSO) or aqueous solutions of dextrans was measured between 30° and 150° using a commercial Sofica photometer at room temperature with non-polarized light (546 nm). The specific refractive index increment (dn/dc) of dextran was taken as 0·072 and 0·15 ml/g in DMSO and water, respectively (Dintzis & Tobin, 1978; Brandrup & Immergut, 1975).

The solutions were clarified by centrifugation at 28000 for 50 min. The molecular weight of non-associated molecules was determined using the logarithmic modification of the Zimm plot and according to the data analysis proposed by Burchard & Pfannemuller (1969). As dextran-30 is insoluble in DMSO measurements were performed in aqueous solution.

Viscosimetry

Measurements of intrinsic viscosity were carried out in aqueous solutions with concentrations in the range 0·5–2 mg/ml, using a Low-Shear 30 viscometer (Contraves, Zurich).

RESULTS

Light-scattering

A typical Zimm plot drawn following the classical (linear) method, is shown in Fig. 1. The same curve shape has been observed with all the dextran-3–25 in DMSO and with the dextran-30 in water. This representation yields very high molecular weights ($\bar{M}_w > 50 \times 10^6$) which certainly correspond to the apparent molecular weight of aggregates. In order to avoid an over-estimation of $\bar{M}_w$, we have used the logarithmic modification of the Zimm plot as proposed by Guinier for globular proteins. In this case, the particle scattering function $P(\theta)$ is given by

$$P(\theta) = \exp\left[\left(\frac{-16\pi^2 n_o^2}{3\lambda^2}\right)\sin^2(\theta/2)\langle s^2\rangle\right]$$

with n_o = specific refractive index of the solvent

λ = wavelength of the incident light

$\langle s^2 \rangle$ = radius of gyration.

The logarithmic modification of Fig. 1 gives the graph shown in Fig. 2. The extrapolation to zero angle is performed from the data obtained at wide angles, according to the method proposed by Burchard & Pfannemuller (1969). These authors consider dextran solution as a blend of two species, dextran molecules in solution and associated molecules. In such a case, the general equation becomes

$$K^*c/R(\theta) = 1/[W_a M_a P_a(\theta) + (1 - W_a) M_s P_s(\theta)] + 2A_2 c$$

where K^* $= 2\pi^2 n_o^2 (dn/dc)^2 / N_a \lambda^4$

M = molecular weight

(dn/dc) = specific refractive index increment

a subscript = associated molecules

s subscript = molecules in solution

W_a = mass fraction of associated molecules

A_2 = second virial coefficient

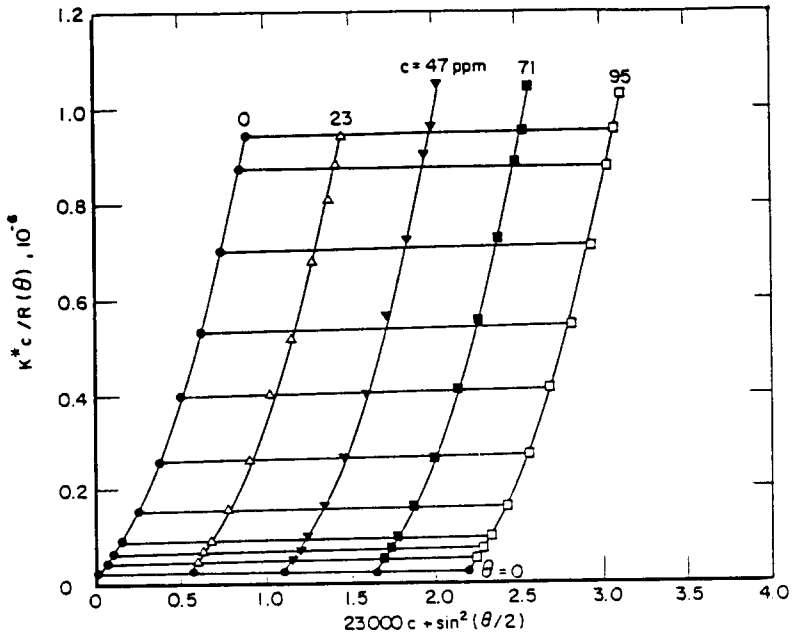

Fig. 1. Linear representation of Zimm plot for dextran-3 in DMSO.

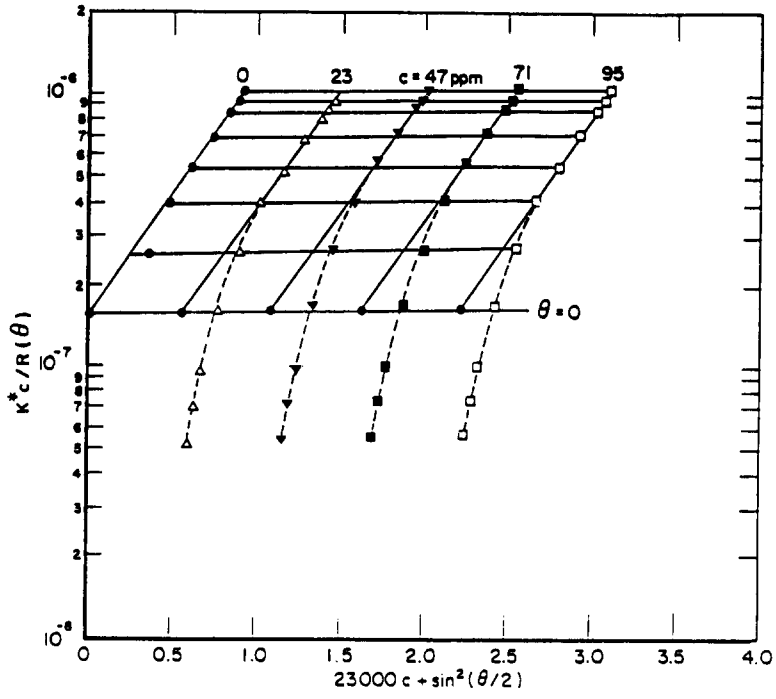

Fig. 2. Semi-logarithmic representation of Zimm plot for dextran-3 in DMSO.

N_a = Avogadro number
$R(\theta)$ = Rayleigh ratio
c = dextran concentration

Burchard & Pfannemuller justified this method by measuring the molecular weight of a tricarbanylated dextran completely dissolved in dimethyl formamide, which was equal to the molecular weight of the corresponding uncarbonylated dextran, obtained by their method described here. From the equation of $P(\theta)$ the radius of gyration of the non-associated molecule can also be deduced; $\langle s^2 \rangle$ is calculated from the slope K_s of the extrapolated line at zero concentration and at a wide angle:

$$\langle s^2 \rangle = \frac{3K_s\lambda^4}{16\pi^2 n_o^2}$$

Thus, the values indicated in Table 1 are obtained for the dextrans dissolved in DMSO and in Table 2 for the dextrans in aqueous solution. It appears that the molecular weights of dextran-3–25 are very similar to

TABLE 1
Molecular Weight and Radius of Gyration of Dextran-3-25

	Synthesis temperature (°C)			
	3	*10*	*20*	*25*
$\bar{M}_w(\times 10^6)$	6·2	6·2	6·6	7·1
$\langle s^2\rangle^{0.5}$ (Å)	726	725	712	668

Solvent: DMSO.

TABLE 2
Molecular Weight and Radius of Gyration of Dextran-3 and Dextran-30

	Synthesis temperature (°C)	
	3	*30*
$\bar{M}_w(\times 10^6)$	6·9	13·5
$\langle s^2\rangle^{0.5}$ (Å)	714	863

Solvent: water

and much smaller than all the values reported in the literature for native dextran. However, the dextran-30 has a higher molecular weight. This difference cannot be attributed to the solvent since the molecular weights of dextran-3 in both solvents are almost equal.

The variation of the particle scattering function $P(\theta)$ with the product $u^2\langle s^2\rangle$ (where $u^2=[16\pi^2 n_0^2/\lambda^2\sin^2(\theta/2)]$) can give some information about the shape of the molecules. In the case of dextran-3-25, the variation of $P^{-1}(\theta)$ with $u^2\langle s^2\rangle$ (i.e. dextran-3 in water; Fig. 3) cannot be interpreted because of the interfering contributions of branching and molecular weight distribution. However, the dextran-30 gives a particle scattering function very similar to that of the sphere model (see Fig. 3). This agreement shows that this dextran has a more compact conformation than the other dextrans.

A more complete analysis of these results will be achieved in the light of the primary structure of the dextrans.

Hydrodynamic volume

The intrinsic viscosities of dextrans in aqueous solutions are detailed in Table 3. For the sake of clarity, Fig. 4 shows the data reported by Senti *et*

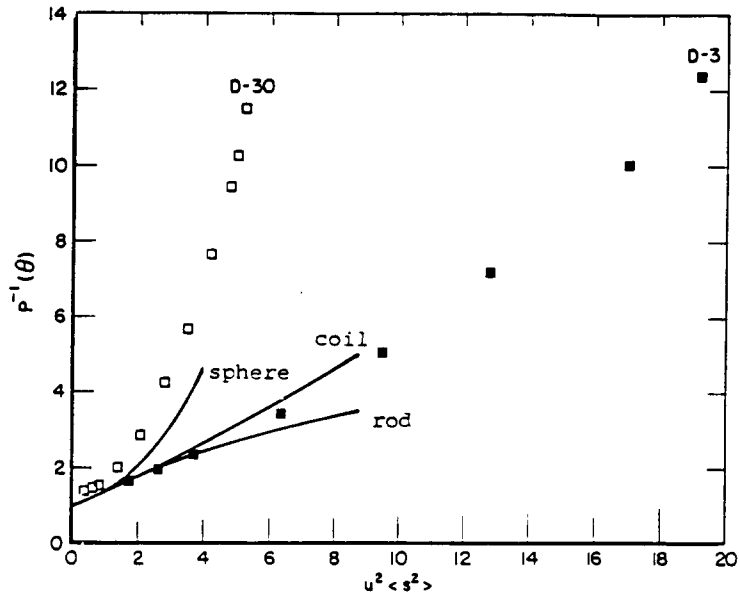

Fig. 3. Particle scattering function versus $u^2\langle s^2\rangle$, $u^2=(16\pi^2n^2/\lambda^2\sin^2(\theta/2))$. Dextran-3 and dextran-30 in water. Models of sphere, coil and rod from Benoit & Doty (1953).

TABLE 3
Intrinsic Viscosity in Water and Limiting Percentage of Hydrolysis by the Glucoamylase from *Rhizopus* sp.

	Synthesis temperature (°C)					
	0	*3*	*10*	*20*	*25*	*30*
$[\eta]$(ml/g)	—	470	480	460	120	52
% Hydrolysis	41	39	37·6	32·7	28·4	27·3

al. (1955) on dextrans synthesized at 25°C, the Mark–Houwink equations proposed by Granath (1958) for the same type of dextran, the intrinsic viscosities of the commercial dextrans and the present values for the dextran-3–30 in water. This figure shows that the intrinsic viscosity of dextran-25 lies on the Mark–Houwink straight line obtained with dextrans synthesized at the same temperature (Granath, 1958), where

$$[\eta]=1{\cdot}3\bar{M}_w^{0{\cdot}28}$$

Flory (1953) has shown that the Mark–Houwink exponent a equals 0·5 for linear random coils in Θ conditions. A lower value such as 0·28 is

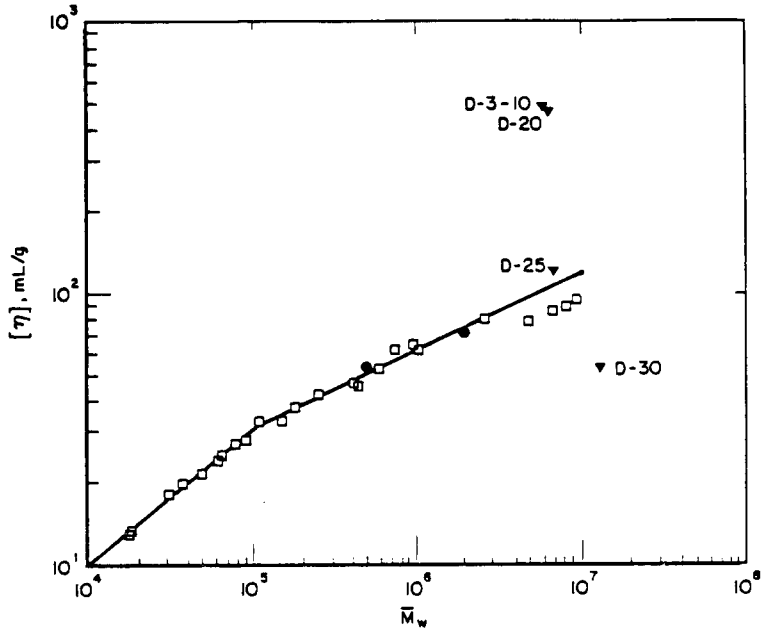

Fig. 4. Intrinsic viscosity versus molecular weight of dextran NRRL B 512F in water: (▼) present data; (□) data from Senti *et al.* (1958); (●) T500 and T2000, data from Pharmacia; straight lines according to the Mark–Houwink equations from Granath (1958); $\bar{M}_w < 100\,000\ [\eta] = 9{\cdot}78 \times 10^{-2} \bar{M}_w^{0{\cdot}5}$; $\bar{M}_w > 100\,000\ [\eta] = 1{\cdot}3 \bar{M}_w^{0{\cdot}28}$.

indicative of a highly branched structure. Since it is likely that short side-chains would not change chain conformation, this confirms that commercial dextrans and dextran-25 contain long side-chains.

The lower intrinsic viscosity obtained with dextran-30 despite a higher molecular weight, suggests that the structure of this dextran is even more branched. This result is in agreement with light-scattering data for dextran-30 and shows, once again, the compactness of its molecular stucture.

In contrast, dextran-3–20 shows a much more expanded conformation which probably results from a less branched structure. In spite of the lack of Mark–Houwink equation for these dextrans, it is expected that the coefficient *a* would be much higher than 0·28 and consequently, that dextran-3–20 would behave nearly as a random coil (Fig. 4).

Enzymatic hydrolysis of dextran

Numerous hydrolytic enzymes have been described in the literature as *endo-* and *exo*-dextranase (Walker & Pulkownik, 1973; Sawai *et al.*, 1976; Kobayashi & Matsuda, 1978; Ohya *et al.*, 1978; Sawai *et al.*,

1978). However, no $\alpha(1\text{-}3)$ debranching enzyme has been yet identified. This kind of activity would allow the determination of the length distribution of the side-chains. A simple way to get information on side-chain length is the use of commercial glucoamylase as an *exo*-dextranase. In fact, Kobayashi & Matsuda (1978) have demonstrated that the glucoamylase from *Rhizopus niveus* can be used to remove D-glucose residues from the non-reducing ends of dextran, its action being stopped at the branch points.

Firstly, it has been verified that the glucoamylase from *Rhizopus* sp. supplied by Sigma does not contain any *endo*-activity, by observing only a slow decrease of medium viscosity during hydrolysis (Fig. 5). Secondly, glucose residues were identified as the only hydrolysis product.

The kinetics of hydrolysis deduced from the reducing power in the digest is shown in Fig. 6. The arrow indicates a second addition of enzyme. It is clear that this addition of enzyme on completion of the reaction produces no more glucose, showing that a true limit of conversion is attained.

It appears that the maximum percentage of hydrolysis decreases when the temperature of dextran synthesis increases (see Table 3). The percentage hydrolysis (28·4%) obtained with dextran-25 is very close to

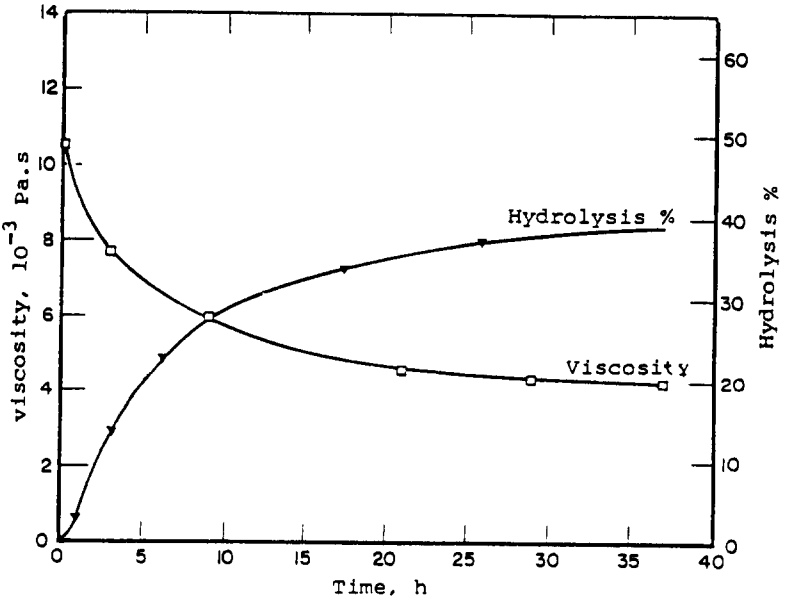

Fig. 5. Viscosity evolution of the medium during dextran hydrolysis with the glucoamylase from *Rhizopus* sp.

those published by several authors for native dextran NRRL B 512F, using different enzymes (see Table 4). Although the synthesis temperature was not specified in these studies, it is likely that these dextrans, produced at the Northern Regional Research Laboratories, are synthesized at a temperature close to 25°C.

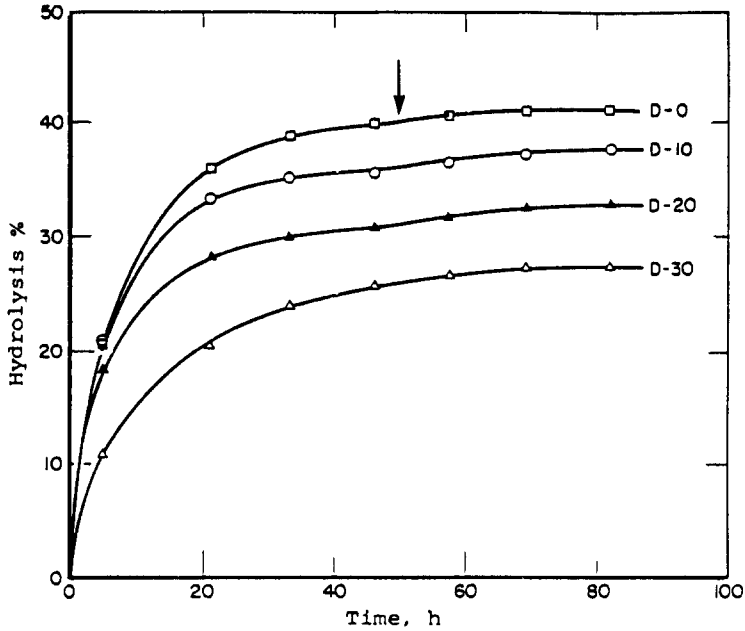

Fig. 6. Kinetics of hydrolysis of dextran-0-30 with the glucoamylase from *Rhizopus* sp. The arrow indicates the second addition of enzyme.

TABLE 4
Limiting Percentages of Hydrolysis of Native Dextran NRRL B 512F Obtained with Different Enzymes

Enzyme	*% Hydrolysis*	*Reference*
Exo-1,6-α-glucosidase *Arthrobacter globiformis* I42	30–32	Sawai *et al.* (1976)
Isomaltodextranase *Arthrobacter globiformis* T6	29	Sawai *et al.* (1978)
Exo-1,6-α-glucosidase *Arthrobacter globiformis* I42	29	Ohya *et al.* (1978)

DISCUSSION

Hydrodynamic volumes of dextrans, as indirectly estimated by their intrinsic viscosity with respect to their molecular weight, indicate that the conformation of dextran-30 molecules is more compact that those of dextran-3–20. This is also illustrated by the ratio $\langle s^2 \rangle / \bar{M}_w$ in water which is 0·055 for dextran-30 against 0·074 for dextran-3. Since it is unlikely that the flexibility of linear segments does vary, this difference is to be ascribed to a different branching, dextran-30 being assumed to be more branched than dextran-3. This is in agreement with previous data using periodate oxidation (Tsuchiya *et al.*, 1955) and methylation (Braswell *et al.*, 1962) methods, which show that the branching degree increases with the synthesis temperature. We have already reported that the percentage of (1-3)glucosidic bonds is 3·4% and 4·8% for dextran-5 and dextran-30, respectively (Sabatie *et al.*, 1986*b*). These data were obtained using dextran permethylation, acid hydrolysis and HPLC analysis methods.

Enzymatic experiments were performed to describe more accurately such structural differences. However, a decrease in the limiting percentage of hydrolysis when the synthesis temperature increases is apparently not in line with the conclusions drawn from viscosimetric measurements. In fact, it should be mentioned that data from enzymatic hydrolysis must be interpreted assuming a certain type of branching. This problem is not yet clearly elucidated for dextran. Thus, Sidebotham (1974) reported that data from partial acid hydrolysis experiments could not differentiate laminated, comb-like and ramified structures (Fig. 7).

Assuming a comb-like structure, the average length of the side-chains containing more than 3 glucose units which represent 15% of the total branching in the case of dextran synthesized at 25°C can be calculated (Larm *et al.*, 1971). The present limiting percentage hydrolysis of dextran-25 would yield an average chain length of 36 glucose units for these long side-chains. On a similar basis, Walker and Pulkownik (1973) estimated an average length of 33 glucose units.

The comb-like structure seems, however, to be too simplistic. There are several pieces of evidence for a ramified structure (see Fig. 7). As a matter of fact, electron microscopic observations by Titova *et al.* (1974) and light-scattering data by Beke & Gilanyi (1971) support the hypothesis of a ramified structure. The mechanism of dextran synthesis by dextransucrase as described by Robyt *et al.* (1974); Robyt & Taniguchi (1976); and Robyt & Walseth (1978) is also in line with this type of structure. Branching originates from the acceptor reaction and it is evident that such a reaction cannot differentiate the identical structures

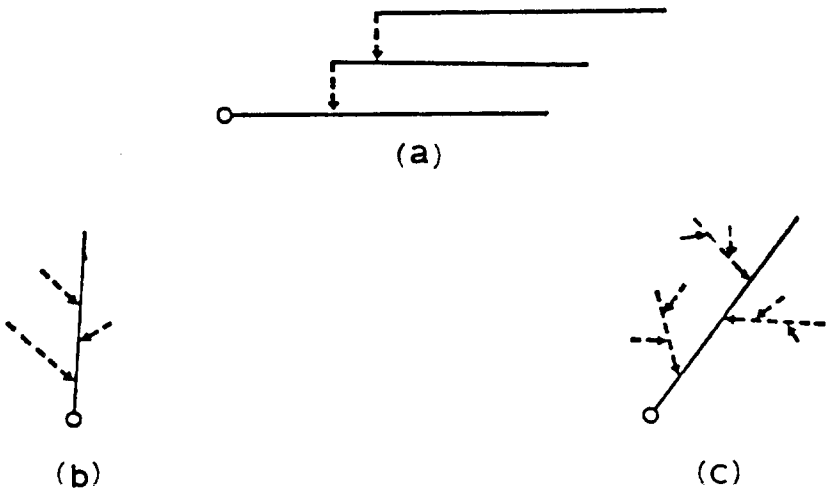

Fig. 7. Possible structures of dextran (from Sidebotham, 1974); (a) laminated structure; (b) comb-like structure; (c) ramified structure.

of the backbone and the long side-chains. The result is multibranched macromolecules.

Assuming a ramified structure with primary and secondary branchings (see Fig. 7), the limiting percentage of hydrolysis corresponds to the amount of glucose units included between the non-reducing end of long side-chains and the first secondary branch point which would stop the action of glucoamylase. The decrease of the limit of conversion suggests that secondary branching becomes more important when synthesis temperature increases. It is clearly shown from enzymatic hydrolysis data that the higher the synthesis temperature, the more compact the dextran molecule.

The behaviour of dextran in solution results from this primary structure. This is particularly clear for dextran-30 in aqueous solution, which exhibits light-scattering properties typical of the solid sphere model as illustrated in Fig. 3. This contrasts with the other dextrans which are close to random coil type macromolecules.

Thus, by a combination of different methods, i.e. light-scattering, intrinsic viscosity determination and enzymatic hydrolysis with glucoamylase, further information on the structure of the native dextran NRRL B 512F was obtained. There is clear evidence that branching increases with synthesis temperature, particularly through secondary ramifications. As a result, conformation of dextran changes from an expanded random coil type if synthesis temperature is relatively low (D-

3–20) to a compact solid sphere type if synthesized at 30°C (D-30), dextran-25 exhibiting intermediate characteristics. Since most of these differences originate from the branched structure, it would be worthwhile to investigate this question more accurately. On the other hand, such different structures and behaviour in aqueous solution would yield large differences in rheological properties. This will be discussed in another publication.

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