THE STUDY OF SHEAR INACTIVATION OF DEXTRANSUCRASE PREPARATION FROM LEUCONOSTOC MESENTEROIDES

by

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I. INTRODUCTION

Enzymes, a category of proteins which catalyze a myriad of complex biochemical reactions, play a critical role in the working machinery of life. Hundreds of enzymes have been identified, and the specificity and catalytic power with which they act are far superior to most man-made catalysts. The factors responsible for the powerful catalysis observed in enzymes have been extensively studied, and many theories have been developed. It is generally believed that enzymes owe to their native state their effiency and precision which are in turn the result of specific energetic interactions with the environment.

When enzyme molecules are brought into adversary environments such as extreme pH, high ionic strength, or abnormal temperature, or subjected to high hydrodynamic stresses, their native state is altered and usually an inactivation phenomenon accompanies these structural changes of the enzymes. Inactivation of the enzyme by hydrodynamic shear force is the subject of this research project. Two lines of investigation are pursued in this dissertation which unify and motivate the various parts. The first is, what can be learned about the structure and chemical mechanism of the enzymes from their physical and chemical responses to the applied shear force? The second is, is any long range effect associated with shear-induced structural changes when these

macromolecules are subjected to moderate shear forces? Or to be more specific, among the molecular population of the sheared enzymes, does any group of molecules exist at a sub-denatured state which represents catalyzing the reaction with less fidelity, in contrast to the popular notion that enzymes exist only in two categories: active or denatured?

The bulk of the work of this dissertation is an attempt to obtain this information for one enzyme system: dextransurcrase (α-1, 6-glucan: D-fructose 2-glucosyl-transferase, EC 2.4.1.5) selected primarily because of its particular polymerization mechanism. Dextransucrase is one of the few enzymes that can catalyze a polymerization reaction by itself; i.e., no co-factor or any high-energy phosphorylated intermediate is involved. This is done by utilizing the high energy of the hydrolysis of the glucose-fructose bond in sucrose to transfer the glucosyl moiety to a growing chain of polymer, dextran. Since the synthesizing polymer chain remains covalently attached to the enzyme molecule, and the growing dextran chain has a very high molecular weight in the beginning of the reaction, the whole reacting complex will expand its hydrodynamic volume along its reaction coordinate.

There are some reports on the enzyme conformation changes on substrate binding, and also on how substrates protect enzymes against temperature denaturation. One of the interesting hypotheses associated with dextransucrase's

mechanism and closely related to substrate action is Ebert and Schenk's hypothesis (1968) on the reacting complex. They hypothesize that sucrose molecule and synthesizing dextran chain were interacting with the enzyme at active site by many coordination bonds. So it is my intention to answer the first imposed question by verifying the hypothesis through shear study on the dextransucrase system.

Because of this, the enzyme polymerizes the product rather than hydrolyzes polymer. Any change or modification on enzymatic specificity would be reflected on the structural features of the synthesized polymers, it offers a chance to answer the second imposed question by studying the structures of the synthesized dextrans from partially inactivated enzymes.

The methodology and underlying philosophy of this work has been to observe the shear effects on this enzyme system by comparing the remaining enzyme activities between the cases of shear in the absence of substrate and shear in the presence of the substrate, and to test any long range effect associated with enzyme molecules which have been subjected to shear by incubating the sheared enzyme and by verifying any structural variations on the products.

II. LITERATURE REVIEW

A. Protein Structure and Conformation

A.1. The making of native-state of proteins

Proteins are composed of long chains of amino acid residues linked by peptide bonds, the order and the number of residues being determined by the genetic code for each particular protein. In solution, a newly synthesized polypeptide chain would be subject to continuous bombardment by the solvent molecules due to Brownian motion. Without any stabilizing interactions this would tend to produce a "tangled string" in continuous conformational flux. Such a structure, described as "random coil", is approximately the state of proteins that have undergone complete denaturation. In the native state, the functional form of protein, it exhibits a unique and stable three dimensional structure which is referred to as its conformation. This structure is present, in an almost identical form, in every molecule of that protein which is recognized as a definable chemical species and as a biological entity.

In contrast to synthetic molecules, whose conformations are governed by entropic-Brownian motion considerations, the conformations of protein macromolecules are dictated by specific energetic interactions. This makes a general theory of mechanical deformation of proteins very difficult because

of the differences among proteins of these energetic interactions. Hydrogen bonding, van der Waal's forces, electrostatic forces, hydrophobic forces and intramolecular covalent crosslinks play important roles in the construction of a protein molecule.

Enzymes, in particular, have as part of their structure an "active site" which is complementary in size, shape, and chemical nature to the substrate molecule which undergoes the chemical reaction being catalyzed. However, this does not imply that the enzyme molecule is totally rigid. The modern flexible enzyme, or induced fit hypothesis of Koshland (Koshland, 1963), suggests that the active site need not be a rigid geometrical cavity, but rather a specific and precise spatial arrangement of R-groups of amino acid residues that is induced by contact with the substrate. A large protein can bend, twist, and fold back on itself, thereby fixing the positions of the essential R-groups in space. This provides fixed bond distances and bond angles, which allows the catalytic action to take place.

A.2. Levels of protein structure

For the convenience of subsequent discussion, it is helpful to differentiate between the levels of structure of proteins. Primary structure is defined as the covalent backbone structure of polypeptide chains, including the sequence of amino acid residues. The C-N bond of the peptide linkage between amino acid residues is relatively rigid and can not

rotate freely, a property of supreme importance with respect to the three-dimensional conformations of proteins. Secondary structure refers to those fairly-regular structural elements, such as the α-helix, that involve interactions between amino acid residues which are spatially close to each other on the chain. Secondary structure is particularly evident in the fibrous proteins, where the polypeptide chains have an extended or longitudinally coiled conformation; it also occurs in segments of the polypeptide chains in globular proteins. Tertiary structure refers to the three dimensional structure of the peptide chain that results from interactions between amino acid residues relatively far apart in sequence. Intramolecular disulfide bonding is often a crucial element of this tertiary structure. In proteins containing more than one polypeptide chain, the terms are used in reference to the structure within each chain independently. Quarternary structure refers to how individual polypeptide chains of a protein having two or more chains are arranged in relation to each other. Proteins with multiple polypeptide chains are known as oligomeric proteins; their component chains are called subunits. Here electrostatic and hydrophobic interactions are important.

To avoid confusion, the terms conformation, native state, shape, higher structure, or three-dimensional structure will

be used to include all types of structures above the level of primary structure.

A.3. Protein denaturation

Most protein molecules retain their biological activity only within a very limited range of temperature and pH. Exposing soluble or globular proteins to extreme pH or to high temperatures for even a short period of time causes most of them to undergo physical changes known as denaturation. Denaturation is a term that is difficult to define exactly because it refers merely to changes in the properties of a protein. One of the distinctive consequences of the denaturation of a protein is a decrease in solubility at its isoelectric point. Also, protein denaturation is accompanied by an increased reactivity of several of the side-chain groups such as the sulfhydryl group of cysteine, the disulfide group of cystine, and the phenolic group of tyrosine.

Denaturation may be caused in various ways. Among them are heating, or treatment with acid, alkali, organic solvents, concentrated solutions of urea or guanidine hydrochloride. Hydrodynamic forces or high pressures also cause denaturation.

Proteins show a wide diference in their sensitivity to any one of these methods of denaturation. If the treatment is not prolonged unduly, the denaturation may be reversed by restoring the conditions at which the protein is stable. Classical experiments carried out by F. White and C. B.

Anfinsen (Sela, White and Anfinsin, 1957; White and Anfinsen, 1959) and their colleagues on ribonuclease reported that treatment of native ribonuclease with 8M urea in the presence of the reducing agent β-mercatoethanol caused a complete unfolding of the ribonuclease molecule, yielding a random coil. In this process the four intrachain disulfide bridges contributed by the cystine residues of ribonuclease were cleaved by the β-mercaptoethanol, converting them into eight cysteine residues. The combined unfolding and cleavage of the cross-links caused complete loss of the enzyme activity, but when the urea and β-mercaptoethanol were slowly removed from the ribonuclease solution by dialysis, the enzymatic activity of the ribonuclease gradually returned. It was reported that this renaturation process was hours in duration. (Anfinsen, et al.; 1961).

Some proteins devoid of disulfide cross-links can also be refolded into the native, active conformation spontaneously and quickly after denaturation such as nuclease from staphylococcus cells (Taniuchi and Anfinsen, 1971). The renaturation process of these nuclease molecules can be shown to take place in at least two phases--an initial rapid folding with a half-time of about 50 milliseconds and a second, somewhat slower transformation with a half-time of about 200 milliseconds. Samejima (Samejima, 1959) denatured bovine liver catalase molecules in alkaline solutions and found changes in the sedimentation pattern and a reduction

of enzymatic activity when the pH of the environment was brought from 7.0 to 12.0. The splitting of the catalase molecule proceeded to completion at pH 12.0, where also the enzyme activity disappeared completely. The interesting observation was the partial recovery of the enzyme activity (up to 50%) where the catalase solution was neutralized to pH 7.0. Later Samejima and Yang (Samejima and Yang, 1963) reported the denaturation and regeneration of bovine liver catalase when it was first subjected to an acid medium and then returned to a neutral phosphate buffer solution. The renaturation process took time in minutes. In 0.1 M buffer, the % recovery of enzymatic activity essentially levels off within 30 minutes. At higher ionic strength, the recovery of activity is more gradual, but even in 0.5 M buffer, it approaches the same asymptote of about 50%.

There is insufficient information in the literature to predict the reversibility of a denatured protein, and to state in what time scale the denatured protein will regain its active conformation. There is not sufficient knowledge at present to state with certainty that all the various means of denaturation cause the same chemical changes in a native protein molecule.

A.4. Physical methods for the study of protein structure

Scientists have developed the basic principles, and some applications of important physical methods for the study of

protein structure. Some methods are based on hydrodynamics for the study of a protein in motion; for example, the rate of movement of molecules from a higher to a lower concentration or from an oriented to a random state (diffusion), the rate of migration in a centrifugal field (sedimentation) or the effect of solute particles on liquid flow (viscosity) are all measurable phenomen that reflect structural properties of the molecules. Optical methods, based upon absorption of radiation and associated dispersive phenomena, are used in studying protein conformations in solution, and such methods include ultraviolet absorption, relating transitions of molecules from their ground state to higher electronic levels, fluorescence spectroscopy, optical rotary dispersion, and circular dichoism.

B. Shear Study on Enzymes Inactivation

B.1. General aspects on shear inactivation

There are many established documetns on the shear induced disruption of synthetic macromolecules, it is not surprising that the shear forces are often regarded as a possible cause of protein denaturation when these globular proteins are subjected to shear during many steps of processing such as mixing, centrifugation, and even pumping. For example, high shear was felt to be a cause of protein denaturation in human plasma fractionation (G.W.R. Dike, 1970). Nystrom and Andren (1976) suggested that cellulase activity was lost during a

conversion of cellulose to glucose might because of high sheer gradients near the turbine impeller, later Reese and Ryu (1980) showed deactivation of cellulase under various conditions of shear. Therefore a knowledge of the effect of shear on globular proteins is basic to a better biochemical approach to the processing of globular proteins which must be maintained in a native state. It is convenient to study enzymatic proteins since their loss of catalytic activity by denaturation provides a more readily measurable index of structural changes, through it was recognized that inactivation of an enzyme may require less severe conditions than gross protein denaturation.

Molecules in a shear field are subject to rotation, considering the macromolecule shown in figure 1. If this molecule is immersed in a viscous matrix, polymeric or consisting of small solvent molecules, and the matrix is subjected to a shearing action as illustrated, the viscous matrix will cause the molecule to rotate in a clockwise direction. The combined effect of this rotation and the flow of the pervading matrix is to stretch the molecule along the line OO' and compress it along the line II'. In the course of rotation, a critical bond may be oriented so that sufficient energy is absorbed for its disruption. The bond breaking energy (ΔHb) may be expressed in terms of the volume changes the molecule experiences just as breaking, ΔV, and the shear stress τ, or $\Delta Hb = \tau \Delta V$.

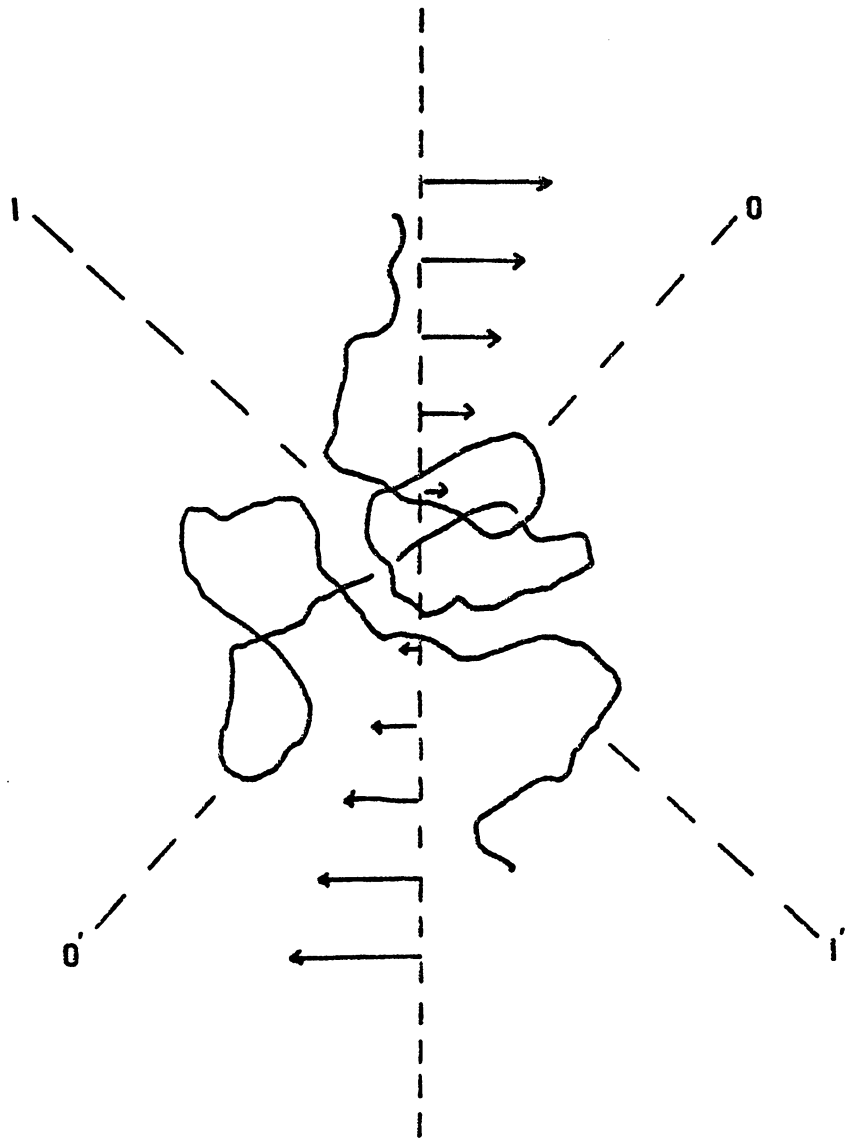


Figure 1. A typical macromolecule in a sheared liquid medium

It is clear that the forces holding the weakest bond together must be overcome before distortion can occur, and therefore at shear stresses below some critical value no inactivation occurs. In other words, molecules possess a certain shear strength that must be exceeded.

Denaturation through shear is similar to denaturation by heat. In both cases, the molecular population has an energy distribution and molecules being inactivated must be associated with an energy level greater than some critical value. In heat denaturation, the fraction of molecules capable of inactivation at any time is:

$$e^{-\Delta Ha/RT} \qquad \text{(Eq. 1)}$$

where RT is the average molecular energy and ΔHa is the energy of inactivation. Similarly, in shear denaturation this fraction is:

$$e^{-\Delta Hb/\tau_{av}\Delta V} \qquad \text{(Eq. 2)}$$

where τ_{av} is the average shear stress and ΔHb is the bond breaking energy. The thermal inactivation energy of many enzymes, ΔHa, is about 80,000 cal/mole (Morowitz, 1968), and the energy for breaking organic bonds is in the range of 100,000 cal/mole.

Shear indication follows a first order reaction which is typical of inactivation reactions caused by random bond-breaking such as occurs in thermal inactivation (Harrington

and Zimm, 1965, Charm and Wong, 1981).

It is generally believed that for macromolecules of sufficient molecular weight and concentration to experience entanglements with other macromolecules in fluid media, degradation is promoted by the mechanical energy stored in the chain links between entanglements. F. Bueche (1960) developed a theory which treats this phenomenon; one of its features is that the degradation is highly molecular-weight sensitive. There is a lower molecular weight limit below which no mechanical degradation occurs.

In dilute, non interactive solution, mechanical degradation has also been reported. Bowman and Davidson (1972) studied the rate of breakage of bateriophage DNA (molecular weight 25×10^6) by laminar flow through a capillary device at a concentration of 7.5 µg/ml. They found that at a constant temperature with varying solvent viscosity, controlled by added sucrose, the breakage rate is a function of the shear rate, not of shear stress. They also found that the shear rate required to achieve breakage is inversely proportional to molecular weight. Harrington and Zimm (1965) studied mechanical degradation of polystyrene in both good and poor solvents, and of DNA in two solvents. They also found limiting molecular weights for degradation in various shear devices.

B.2 Kinetics of shear inactivation

Charm and Wong (1970.a) conducted an experiment on enzyme solutions of catalase (227 units/ml), carboxypeptidase A (40 units/ml) and rennet (25 units/ml) in a Weissenberg Couette viscometer at 4°C. They subjected these enzyme preparations in the absence of substrates to shearing stresses from 0.02 to 2.6 Pascals at shear rates from 91.5 sec^{-1} to 1155 sec^{-1}, then determined the lost activity as a function fo time and shear rate. They found that all the enzymes were partially inactivated when subjected to shear. Other biologically active materials such as fibrinogen (Charm and Wong, 1970b), lysozyme, λ-phage, and heparin (Charm and Wong, 1975) exhibited similar inactivation patterns with respect to time at different shear rates. They also found in a number of studies quantifying shear inactivation that there was a correlation between the percent of remaining activity and the dimensionless group $\dot{\gamma}t$, where $\dot{\gamma}$ was the shear rate and t was the time exposure to the shear, over a range of $\dot{\gamma}$ and t values. The data for each material are correlated on a single line by plotting percent activity remaining vs. log $\dot{\gamma}t$ as illustrated in figure 2 for the catalase solution. To check the validity of the viscometer results, they constructed a flow system which permitted recycling of an enzyme solution through a tube in a streamline flow. The test section is a Teflon tube 560 μ in diameter, 1.88 meters long, and is designed to avoid an

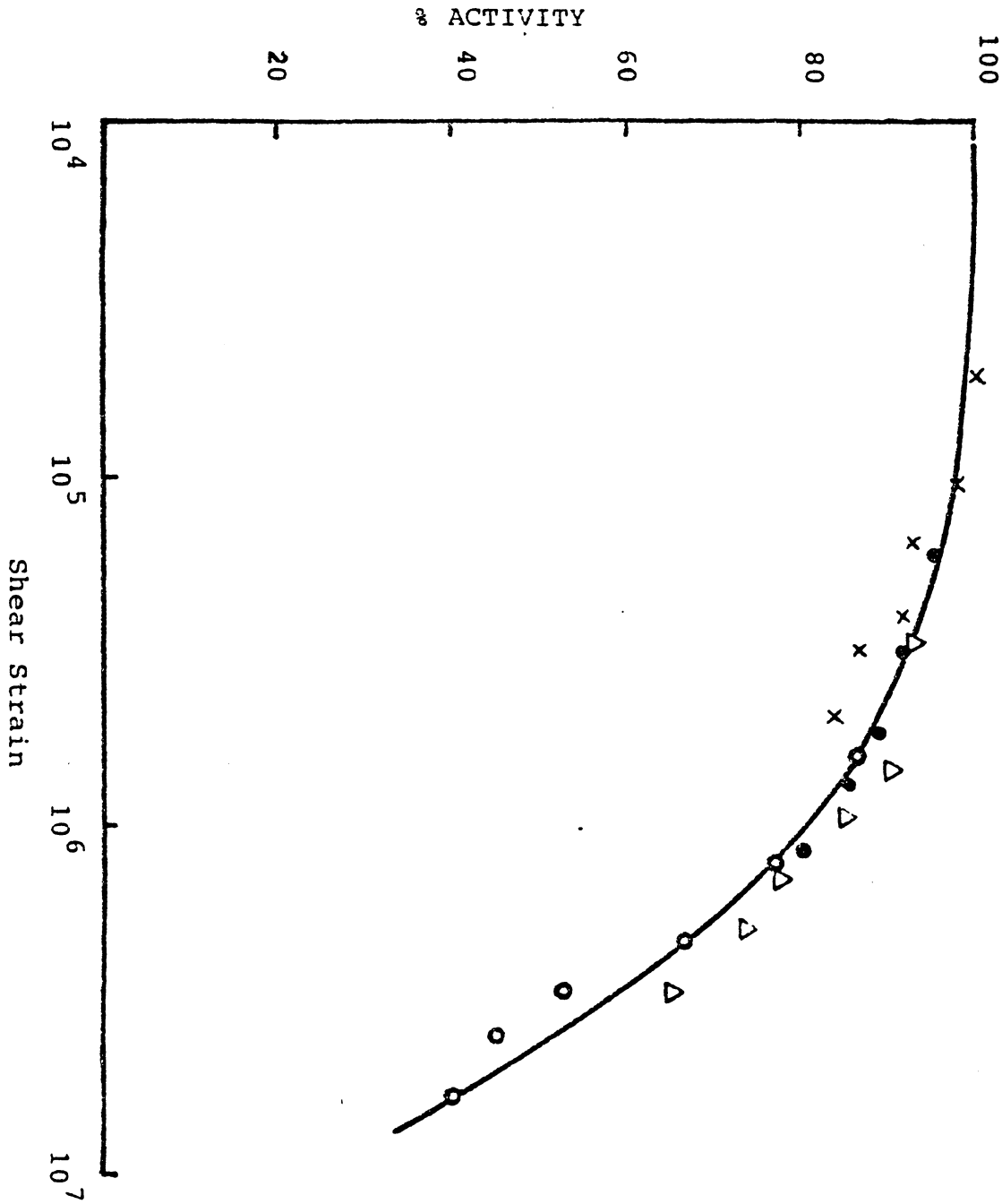


Figure 2. Activity of catalase as function of shear strain. Reproduced after Charm and Wong, Biotechnology and Bioengineering, vol. 12, 1970, p. 1107.

air-liquid interface in the flow of enzyme solution to eliminate the possible interference of the surface phenomenon. Catalase solution was tested and they found the percent remaining activity agreed with what predicted from the same correlation if the suitable $\dot{\gamma}t$ average was used. The correlation held whether the shear was applied continuously or intermittently. From their observations, the rule of thumb can be stated that there is little or no enzyme inactivation if the dimensionless group (shear strain) is less than 10^4. It was thought that the inactivation was caused by damaging the tertiary structure of the enzyme, when the molecule was oriented under the applied shear field in a certain manner. Their work is probably most directly related to the work of this dissertation.

This approach was taken by the same workers (Charm and Wong, 1970b) to propose a possible mechanism for loss of clottability of plasma fibrinogen in the circulatory system. Again, a correlation between percent remaining activity (clottability) and $\dot{\gamma}t$ was found. The results yielded a half-life for fibrinogen, in vitro, of 4 days, which was in excellent agreement with previous medical reports. The experiments were conducted at 4°C and 37°C, and the applied shear rate ranged from 290 $second^{-1}$ to 1155 $second^{-1}$.

Charm and Lai (Charm and Lai, 1971) extended the work to compare the filtration characteristics of four ultrafiltration systems using commercially available membrane on

suspensions and solutions: suspension of protein miscelles (casein), cell debris (E. coli) and catalase and rennet enzyme solutions. It was found that shearing inactivated enzymes in both recycle and vibration systems. Only rennet enzyme showed any partial recovery of activity upon standing after shearing.

B.3. Reaction rate influenced by shear

Shear conditions also cause changes in the kinetics of reactions by reducing the reaction rate. The most definitive study was carried out by Tirrell and Middleman (Tirrell and Middleman, 1975; Tirrell, 1977) with the enzyme urease. They measured the rate of hydrolysis of urea by urease, while the whole reaction mixture was under shear in a coaxial cylinder viscometer. The initial concentration of urea in all experiments was 160 mM. The urease concentration was 1.0 mg/ml in 0.2M phosphate-citrate buffer (pH 6.75). Their data shows the effect of varying conditions of shear rate on the kinetics of urease catalyzed urea hydrolysis. It was seen that at a given shear rate there was a continous decrease in the rate of urea hydrolysis as a function of shearing time. Moreover, this decay in hydrolysis rate occured more rapidly with increasing shear rate. It should be noticed that a fundamental difference exists between these experiments and Charm and Wong's; Charm and Wong measured the activity of an enzyme preparation

after shearing it to variable periods of steady shear, whereas Tirrell and Middleman studied the kinetics of a reaction occuring while under shear.

The most significant contribution of their study is the kinetic data obtained at zero shear after various periods of steady shear. The results, displayed in figure 3 indicated that partial inactivation of urease : by shear has both irreversible and reversible elements, and the former element could be correlated with $\dot{\gamma}t$. If entirely irreversible deactivation occured under these conditions, the data obtained after the cessation of shear should continue along a line having a slope equal to the instantaneous slope at the moment when the shearing force was removed.

In later studies, Tirrell and Middleman (Tirrell and Middleman, 1978) applied this kind of analysis to another enzyme: Lactic dehydrogenase (LDH) with molecular weight 140,000. Again, they drew the conclusion that at higher levels of shear stress, LDH inactivation was partially irreversible. Also, they found that it was not necessary to have the large aggregated structure of the enzyme in order for shearing force to modify the catalytic behavior. In the case of urease, inactivation was dependent on the presence of the large aggregated structure.

B.4. Reversible, irreversible inactivation and recovery

The reversible character of shear inactivation revealed

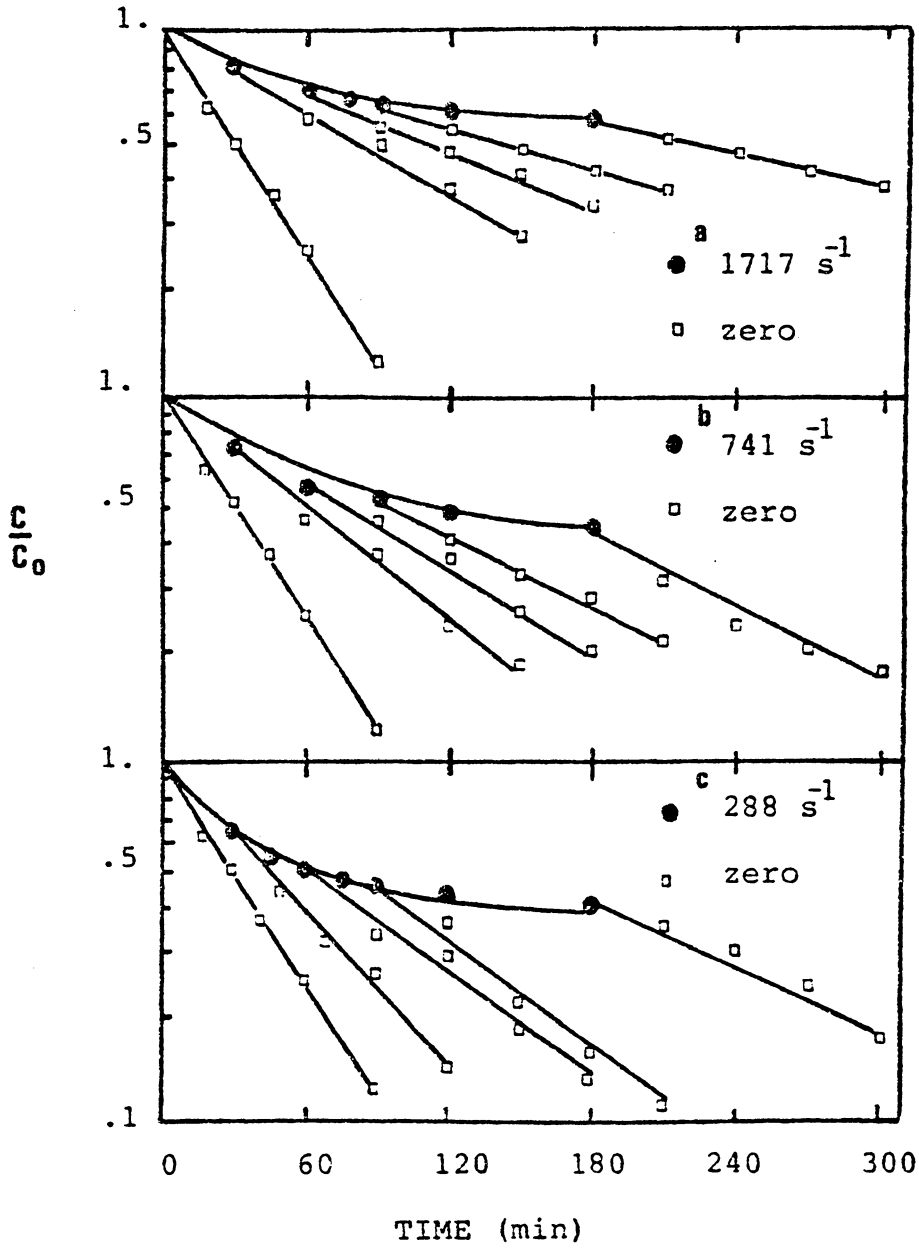


Figure 3. Urea concentration as a function time at zero shear after variable periods of steady shear. Reproduced after Tirrell's Ph.D. dissertation, University of Massachusetts, 1977, p. 86.

by Tirrell's experiments was considered to be instantaneous as far as the assay technique could discern although the limit of its sensitivity would be on the order of several minutes. It was thought that under less drastic shear action, shear caused the enzyme molecule to stretch without breaking within an elastic limit that permitted recovery when the shear field was removed. So this reversible inactivation was tied to the conformation of the protein which implied that there was no breaking of bond involved, while the permanent inactivation was attributed to damages of tertiary structure of the enzyme molecule involving disruption of one or several bonds. Some discrepancies about the recovery of the inactivated molecules appear in the literature. Charm and Wong (1970a, 1971) reported that rennet enzymatic activity showed partial recovery after shear. Such recovery was not rapid but occurred on standing for up to 1 1/2 hours as shown in figure 4. For example, rennet recovered from 60% activity to 80% in 100 minutes after removal of shear. They also showed that for carboxypeptidase A, intermittent shear with 1 minute interruptions between 1 minute or 5 seconrds of shear had the same effect as steady shear. This result is not compatible with instanteous recovery.

Just like renaturation process, no prevailing statement in the literature addresses how fast the recovery is. It should not be expected that the effect of shear on kinetics would be the same for enzymes of different size and shapes.

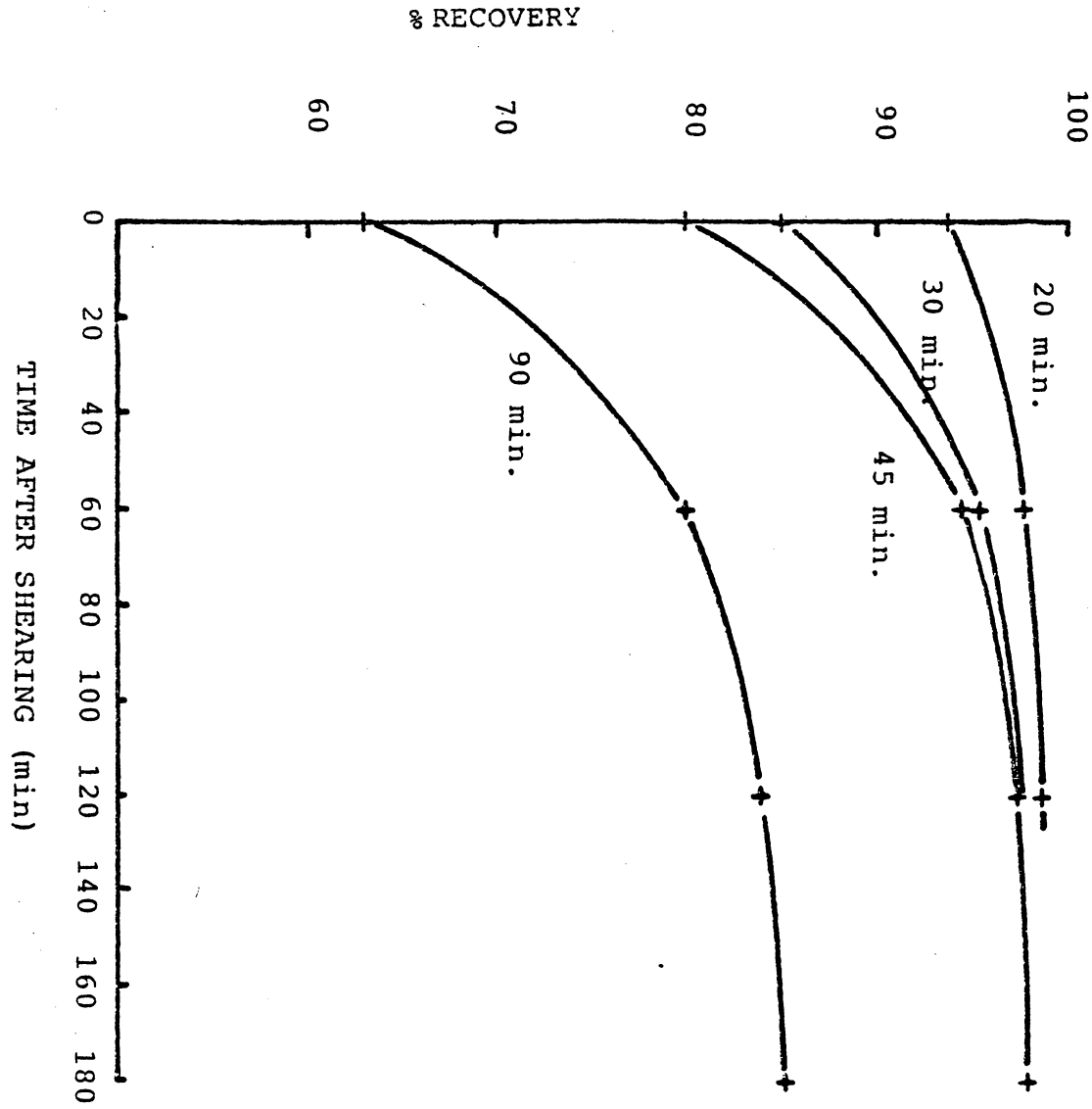


Figure 4. Recovery of rennet activity after shearing. Reproduced after Charm and Wang, Biotechnology and Bioengineering, vol. 12, 1970, p. 1109.

B.5. Shear induced conformational changes

The first significant research addressing the question of shear induced conformational changes of proteins was that of Joly and Barbu (Joly, 1948, 1949; Joly and Barbu, 1949; Barbu and Joly, 1949). Their work examined the aqueous solution of horse serum albumin (HSA) and tobacco mosaic virus (TMV), at concentrations from as low as 0.025 weight per cent to as high as 2.5% for HSA. The solutions were subjected to velocity gradients, i.e. shear rates, from 27 second^{-1} to 9900 second^{-1}, in a circular Couette device fitted to measure flow birefringence. At low velocity gradients (e.g. 2000 second^{-1}), they found a flow induced aggregation process. For shear rates above 2000 second^{-1}, the shear force broke up the aggregates. This technique has been suggested as a way of studying the magnitude of the physical forces involved in the protein quarternary structures of aggregates and isozyme (Reithel, 1963).

Tirrell (1977) applied a size-exclusion charmatography column packed with CPG-10 porons glasses to ascertain the nature of the structure changes accompanying the inactivation of urease solution. He eluted the column with urease preparations after exposing it to shear at a shear rate of 1717 sec^{-1} for several periods of exposure time and monitored the protein concentration and urease activity. He found a shear induced aggregation phenomenon and the loss in

activity of the aggregated isozymes. He also applied circular dichroism spectroscopy to learn something about any changes in secondary structures that might occur upon shearing. The sheared samples showed some differences from the non-sheared sample after two hours exposure. The concentration of urease in both studies is fifty times higher than the concentration of kinetic study. Due to the limitation of the instrument, he could study the irreversible conformational change only after removing the shear field. No data are available on protein structural changes during shear.

B.6. Conflicting reports on shear inactivation.

Thomas et al. (Thomas, Nienow and Dunnill, 1979) studied shear effect on yeast alcohol dehydrogenase in a sealed coaxial viscometer. They found little inactivation at 30°C for prolonged shear. Thomas and Dunnell (1979) then tried to study the influence of shear upon catalase and urease using the same techniques as applied to alcohol dehydrogenase. They found essentially no inactivation as would be expected from the data of Tirrell and Middleman, Charm and Wong. They felt the presence of air might enhance the effect of shear inactivation and caused the difference.

C. Dextransucrase Production and Its Purification

C.1. Culture conditions for dextransucrase production

Several species and strains of Leuconostoc and Streptococcus elaborate dextransucrase, an enzyme that synthesizes the polysaccharide, dextran, from sucrose. It was Hehre and Sugg (Hehre, 1941; Hehre and Sugg, 1942) who demonstrated that the cell free broth filtrate would synthesize dextran and proved their identity with the corresponding dextrans produced in growing culture. Since dextransucrase's discovery, it has gained importance because dextran and modified dextrans have many industrial and medical uses.

It was found that the bacterial strain Leuconostoc mesenteroides NRRL B-512 produced dextransucrase in relatively large amounts into the culture supernatant solution with a minimum number and quantity of related contaminating enzymes, and it formed a high-molecular-weight, soluble dextran (A. Jeanes, 1966). This may be contrasted with other strains of L. mesenteroides and with the dental-plaque streptococci, which form both soluble and insoluble dextrans and elaborate more than one type of dextransucrase, together with relatively large amounts of such related, contaminating enzymes as invertase and levansurcrase. For these reasons, L. mesenteroides B-512 serves as an important model in studying the structure of dextran and the mechanism of

dextran biosynthesis by dextransucrase. This strain is commonly used in scientific studies and is the choice of this research.

Tsuchiya and coworkers (Tsuchiya, et al., 1952) studied the cultural factors that affect dextransucrase production by NRRL B-512. They found the sucrose concentration in the medium should be lowered to a point such that viscosity of the culture is sufficiently low to permit the separation of bacterial cells. With this strain, the optimal sugar level was 2 per cent. The source of nitrogen and other nutrients was supplied by yeast extract and mineral salts. The optimal pH for production of dextransucrase was 6.7. Koepsell and Tsuchiya (1952) studied the dextransucrase yield in vat fermentation with the temperature at 25°C and pH maintained at 6.7 throughout fermentation with 5 N NaOH; the aeration rate was 0.05 volume of air per volume of culture per minute; and moderate agitation of 90 rpm. They found the maximum yield occurred at eight hours of fermentation time.

Robyt and Walseth (1979) published a paper on production, purification and properties of dextransucrase from NRRl B-512F which was the guideline for the preparative procedure of this research. The organism was grown on the medium described by Hehre (1955) and Tsuchiya et al. (1952), which was identical to the medium used in this research (please refer to medium E, in Chapter III, Experimental Methods and Material). Cultures were incubated on a rotary

shaker for 12 hours at 25°C. Successive transfers (10%) were made into larger volumes until 1 liter was used to inoculate 10 liters of medium in a New Brunswick fermentor that was incubated at 25°C with 150 rpm agitation and an aeration rate of 0.5 liter per 10 liter of culture per minute. Cells were removed by centrifugation and the supernatant solution was used as the source of dextransucrase. They found the production of dextransucrase was stimulated two-fold by the addition of 0.005% of calcium chloride to the medium, but the undesirable levansucrase levels were not affected. This data also revealed that dextransucrase was not produced until near the beginning of the stationary phase. At this point, the enzyme was secreted rapidly into the culture medium and reached a maximum after about 3 hours.

C.2. Purification work for dextransucrase from culture fluid

To purify the dextransucrase preparation, Tsuchiya et al. (Tsuchiya, Hellman et al., 1955; Stringer and Tsuchiya, 1958) used prechilled ethanol up to a concentration of 35% v/v to precipitate the enzyme from solution. The precipitate was resuspended in pH 5.0 water and dialyzed against tap water. The resulting suspension held 75% of the enzyme activity of the initial culture liquor. Dextransucrase from this organism is an inducible enzyme that requires sucrose in the culture medium and results in some quantity of dextran being formed in the culture supernatant. The

precipitation of the enzyme by alcohol carried a significant amount of dextran with it.

Hehre (Hehre, 1955) applied 370 grams of ammonium sulfate to each liter of chilled culture fluid to achieve purification of dextransucrase and keep preformed dextran and sucrose out of the enzume preparation. Braswell (Braswell, et al., 1962) improved the purification strategy of alcohol precipitate by absorption onto calcium phosphate gel, such that the eluted enzyme was free of dextran, as judged by a serological test. Ebert and Schenk (Ebert, Schenk, 1962) studied dextransucrase purification extensively, and developed a scheme that produces a nascent enzyme. However, this scheme was tedious and resulted in low yield.

Robyt and Walseth (Robyt, Walseth, 1979) applied modern separation equipment to this system. Dextransucrase was purified by concentration and dialysis of the culture supernatatn with a membrane device, Bio-Fiber 80 miniplant, and by subsequent treatment with dextranase followed by chromatography on Bio-gel A-5m.

The chromatography of the culture supernatant concentrate on Bio-Gel A-5m showed that dextransucrase and levansucrase had migrated in the void volume. After treatment with dextranase, dextransucrase was retarded by Bio-Gel A-5m.

After the three-step purification work, they claimed

an overall 240-fold purification, wth no contaminating enzymes detectable. Robyt and Walseth probably were the first investigators who looked at these contaminating enzymes seriously and tested their existence by incubating with different substrates, though dextransucrase production and purification had long been in the literature. The decrease of the molecular size of dextransucrase after dextranase treatment indicated that the enzyme in the culture supernatant concentrate is in a high-molecular weight form. Aggregation of dextransucrase seems to be a fairly general phenomenon. A gel electrophoresis technique was applied on the purified enzyme which revealed two protein bands, both of which possessed dextransucrase activity. They concluded that the fast-moving band could be a monomer of relatively low molecular weight, rather than the slow-moving, somewhat diffuse band, which was probably an aggregate of the monomer.

Kaboli and Reilly (Kaboli and Reilly, 1980) purified dextransucrase by a similar procedure. Three lieters of supernatant were concentrated to 100 ml with an Amicon 402 ultrafilter containing an XM-100A membrane and were added to a 4.8 x 105 cm column packed with LKB Ultrogel AcA 34. Cumulative purification of that preparation was 247 fold with a 54% yield. The contaminating enzymes such as invertase or levansucrase were determined by the amount of glucose

after prolonged incubation with the adequate substrates respectively. Enzyme solutions as various stages of purification produced less than 3% of their total reducing sugar as glucose, with no clear trend appearing as purification proceeded.

D. Dextransucrase and its Polymerization Mechanism

Since the discovery of dextransucrase in 1941, considerable interest in its mechanism of action has arisen. The catalytic properties of dextransucrase depend on its source and on reaction conditions such as pH, temperature, substrate and product concentration, and enzyme-to-substrate ratio.

Dextransucrase (E.C. 2.4.1.5) is classified as a transglycosidase and has a molecular weight of 280,000 by ultracentrifuge. (Ebert and Schenk, 1968). The enzyme first hydrolyzes sucrose and then transfers the glucose unit to a growing chain of dextran while releasing free fructose as shown in figure 5. The general reaction can be written as:

$$\underset{\text{Sucrose}}{n\text{-}(C_{12}H_{22}O_{11})} + \underset{\text{Acceptor}}{HOR} \xrightarrow[\text{dextransucrase}]{} \underset{\text{Dextran}}{H(C_6H_{10}O_5)_nOR} + \underset{\text{Fructose}}{n\text{-}C_6H_{12}O_6} \qquad \text{(Eq. 3)}$$

The second molecule: acceptor has been found to govern the molecule size of product dextran and rate of fructose

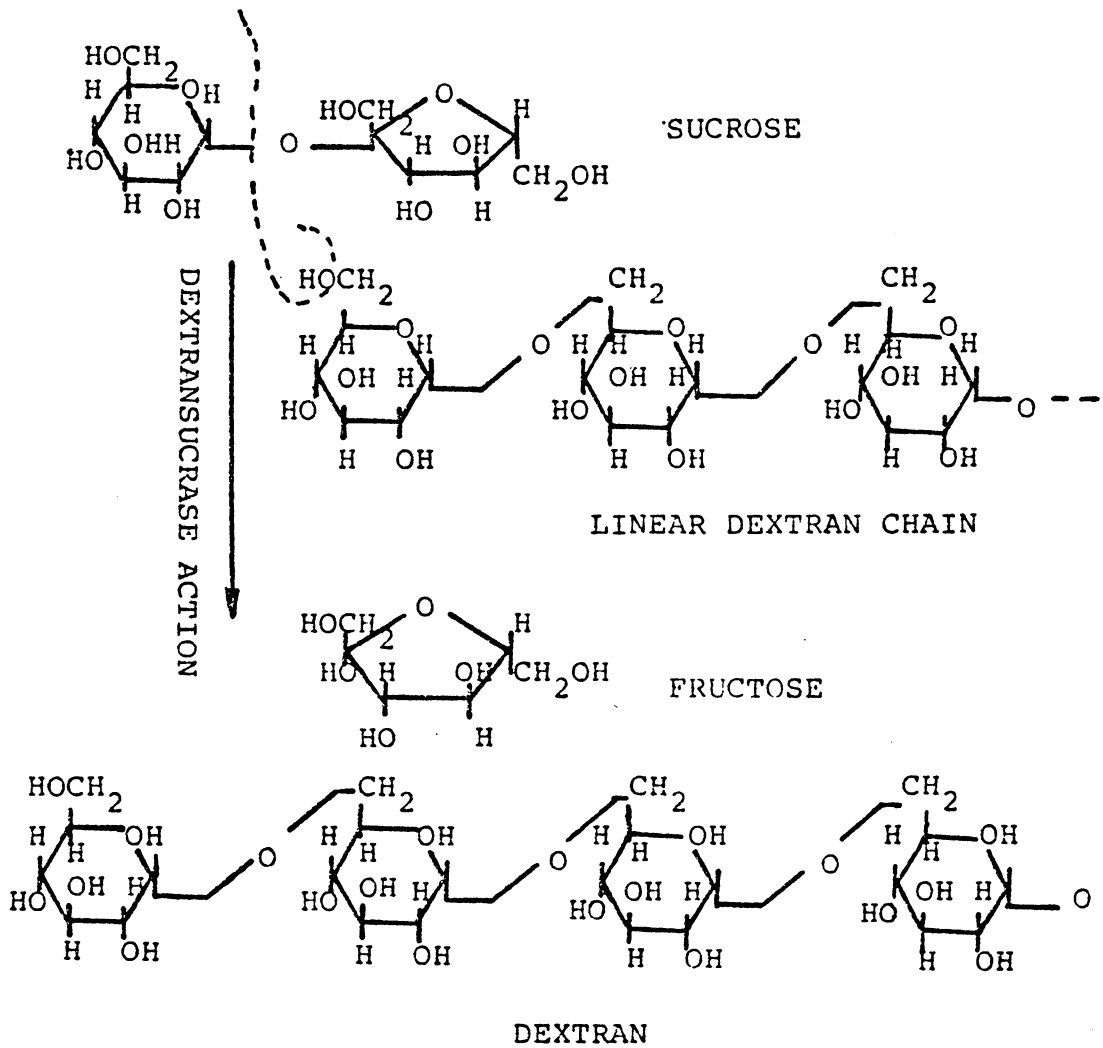


Figure 5. Synthesis of dextran from sucrose catalyzed by dextransucrase.

production. The following subsections are to abstract the information pertinent to its kinetics and mechanism.

D.1. Solution kinetics

The kinetic study of the enzyme-catalyzed reactions are generally expressed by the rate equations proposed by Michaelis-Menten (Lehninger, 1970).

$$V = \frac{V_{max} \cdot S}{K_m + S} \qquad \text{(Eq. 4)}$$

V = initiation reaction rate

V_{max} = maximum reaction velocity

K_m = Michaelis constant which is an indication of the affinity between the substrate and the enzyme molecule.

S = substrate concentration

Hehre (1946) undertook the kinetic study by monitoring the reducing sugar contents of the reaction mixtures after the proper intervals of incubation at different concentrations of sucrose and found that when the sucrose concentration was below 200 mM, the reaction rate followed what would be a typical Michaelis-Menton curve. With higher substrate concentrations, reaction rate was much lowered. He also found that after relatively long periods of time of incubation, the conversion of sucrose to dextran and fructose went nearly to completion.

Stringer and Tsuchiya (1958) had the same observation and reported that the Michaelis constant, K_m, typically

varied from 15 mM to 350 mM (Neely, 1959a), while the maximum reaction velocity usually ranged from 58.3 mg fructose/ℓ enzyme-minute (Neely, 1958) to 76.5 mg reducing sugar/ℓ enzyme-minute (Hehre, 1946) when sucrose concentration was below 0.2M. The deviation from the Michaelis-Menten rate expression was originally explained by Hehre as follows: he postulated that water molecules were bound during the formation of colloidal solution of dextran and not available for reaction; later this was explained by substrate inhibition phenomenon (please refer to subsection on Inhibition). A new modified rate equation was derived as

$$V = \frac{V_{max}\, S}{S+K_m+S^n/K_2} \qquad \text{(Eq. 5)}$$

where K_2 is a dissociation constant for sucrose. In the modified reaction scheme, excess substrate could bind to the substrate-enzyme complex SE to form an inactive complex SES, and K_2 simply is

$$[SE][S]/[SES] \qquad \text{(Eq. 6)}$$

The subscript n was assigned the integer value of 2. K_2 values ranged from 1.6 M (Stringer and Tsuchiya) to 3.9M (Neely).

D.2. Substrate specificity

One of the main characteristics of the dextransucrase enzyme is the unique role played by sucrose as the donor of

D-glycosyl groups. None of a wide variety of sugars and sugar derivatives has been found to serve as substrate in the absence of sucrose (Koepsell et al., 1953). The specificity of dextransucrase may be attributed to the structural conformation of the enzyme. Neely (1959a) studied the variation of the Michaelis constant and maximum initial reaction velocity with temperature (Table 1); he found the plot of log K_m against 1/T showed a sharp break at 30°C with a rapid increase in the value of K_m. This increase is probably related to a greatly reduced affinity of the enzyme for substrate sucrose. Neely did demonstrate that the substrate specificity may be alternateable when the enzyme is incubated at 33°C for 4 hours with maltose. The results indicated that the mild heat treatment of the enzyme tended to open up the protein structure to the extent that maltose was able to reach the catalytic site.

D.3. Acceptor molecules

Koepsell and coworkers (Koepsell et al., 1953) observed that dextran having an average molecular weight of several millions was produced at the beginning of the reaction, and the molecular weight of dextran changing to only a limited degree during the course of reaction. They found that in the presence of certain sugar, notably maltose and isomaltose, the enzymatic reaction resulted in mixtures of oliogosaccharides as major products insteady of the usual high molecular weight polymer product.

TABLE 1. Michaelis constant and maximum initial reaction velocity of dextransucrase as function of temperature.

Temp°C	K_m (mM)	V_m
35.0	340	0.28
34.0	298	.33
33.0	180	.32
32.5	155	.32
32.0	80	.31
31.0	51	.30
30.5	42	.28
30.0	30	.28
25.0	24	.24
21.0	25	.16
17.0	23	.16
12.0	20	.11
5.0	18	.08

V_m expressed as mg. fructose liberated in 30 minutes per ml.

Reproduced after W. B. Neely, Journal of the American Chemical Society, p. 4417, vol. 81, (1959a).

It was thought such auxiliary sugars acted as glucosyl acceptors, and thus competed with the normal acceptors. Though dextransucrase is specific toward the substrate, it is much less specific for the type of molecules that will function as the glucosyl acceptor. Intensive research in this area in the 1950's was pursued for the purpose of the production of direct enzymatic synthesis of clinical dextran. A survey of a large number of sugars and sugar derivatives indicated that isomaltose, maltose, α-methyl glucoside and glucose can act as efficient glucosyl acceptors as indicated by an increase in the fructose production rate and a decrease of long dextran chain with a corresponding increase in oligosaccharide formation, while fructose and galactose behave much less efficiently. They reduce the reaction rate and have little effect on the yield of high molecular weight dextran.

Dextrans can serve as acceptors, too. They fall into both the efficient and less efficient classifications depending on their molecular weight. Native dextrans (usually those of molecular weights of millions) do not appear to influence either the rate or the product distribution. But dextrans of lower molecular weights, in the range of 5000 to 60,000, have been observed to be efficient acceptors, and they lead to appreciable synthesis of low modal dextrans. (Tsuchiya, et al., 1953)

D.4. Factors affecting molecular weight distribution--enzyme/substrate ratio

Tsuchiya et al. (1955) did a thorough investigation on how the reaction conditions and various acceptors affect the molecular weight of the product dextran. They used ethanol or methanol precipitation techniques to generate molecular weight distribution data. Initial sucrose concentration affects both yield and molecular weight of the synthesized dextran. The main product formed at 10% sucrose concentration was of high molecular weight; all of the dextran precipitated at 38% ethanol. In contrast, the main product at 70% sucrose was low molecular weight.

Temperature not only alters the reaction rate of dextransucrase activity, it also affects markedly the molecular weight distribution. Lowering the reaction temperature from 30° to 15°C reduces the yield of dextran of the high molecular weight.

In this shear study of dextran-sucrase preparation, an important parameter will be the enzyme to substrate ratio. Tsuchiya, et al., (1955) found that the enzyme-substrate ratio also affects the molecular weight distribution. At 30°C and sucrose concentration at 10%, pH 5.0, a decrease of enzyme/substrate ratio from 4 to 1 increases the molecular weight of dextran, the yield of molecular weight dextran

larger than 100,000 increases 15 to 20%.

D.5. Inhibition

Any substance that reduces the velocity of an enzyme-catalyzed reaction can be considered to be an inhibitor. The inhibition of enzyme activity is one of the major regulatory devices in living cells, and is one of the most important diagnostic procedures of the enzymologist. Sucrose at a level lower than 0.2 M does not affect the reaction rate, but when sucrose concentration is higher, the reaction rate decreases. Stringer and Tsuchiya (1958) found that the addition of α-methyl glucoside alleviated the sucrose inhibition phenomenon and shifted the optimal sucrose concentration to a higher value. They believed that α-methyl glucoside competes with sucrose molecles for the acceptor site preventing the active site from being saturated with sucrose.

The end product fructose also shows inhibition when its concentration reaches a certain level.

Several researchers (Neely, 1961; Robyt and Walseth, 1979) found the chelating agent EDTA (tetrasodium ethylenediamine tetraacetic acid) strongly inhibited the activity of purified dextransucrase. But the addition of calcium ion would completely restore the activity.

D.6. The effect of pH

It is generally believed that pH will influence the

velocity of an enzyme-catalyzed reaction. The active sites on enzymes are frequently composed of ionizable groups that must be in proper ionic form in order to maintain the conformation of the active site, to bind the substrates, or to catalyze the reaction. The pK values of the prototropic groups of the active site can often be determined by measuring the pH dependence of the reaction rate. Neely (1958) has revealed that dextransucrase has a bifunctional catalytic site, the pK's of which correspond to a carboxyl and an imidazole group.

Stringer and Tsuchiya (1958) reported that dextransucrase had a narrow optimal pH window around ph 5.2 and was independent of the nature of th pH buffer.

D.7. The effect of temperature

Temperature has a dual effect on the enzyme catalyzed reaction. An increase in temperature imparts more kinetic energy to the reactant molecules resulting in more productive collisions per unit time, but an increase in temperature also hastens the denaturation of enzyme molecules, and the enzyme suffers loss of activity.

Dextransucrase catalyzes the formation of dextran over a wide range of temperatures (3° to 37°) but is very heat labile. For example, it shows high activity and stability at 30°, yet is destroyed in a few minutes at 40°C (Hehre, 1955).

The variation of reaction rate with temperature for dextransucrase revealed that the activation energy for the catalyzed reaction was 8.57 ± 1.23 kcal/g mole from 15°C to 30°C (Kaboli and Reilly, 1977). Other data available are 11.0 kcal/g mile for 5° to 35°C by Neely (1959a) and 10.3 kcal/g mole for 0° to 30°C by Ebert and Schenk (1968).

Kaboli and Reilly (1977) studied the thermal stability of dextransucrase in soluble form as well as in immobilized form. The activation energy for denaturation was 53 ± 7.6 kcal/g mole between 30°C and 35°C. A slower denaturation rate was evident when the temperature was below 35°C. Hence, the optimal operating temperature for this enzymatic reaction was 30°C or less (Itaya and Yamamoto, 1975).

D.8. Polymerization mechanism

Historically it took a long time to elucidate the polymerization mechanism of dextransucrase. At the beginning, it was thought to be a condensation type of polymerization reaction. Hehre (Hehre, 1951) and Edelman (Edelman, 1956) discussed these particular enzymes thoroughly. They hypothesized that there was a mediation step of a glucosyl-enzyme complex in which dextransucrase would first hydrolyze the glucosidic linkage between the glucosyl and fructose moiety, and then transfer the glucosyl radical to the C6 hydroxyl group of the terminal glucose unit of a growing dextran chain, at the nonreducing end.

Later, Tsuchiya and coworkers (1955) proposed an alternative mechanism based upon a chain type of polymerization that accounted for the relative uniformity of the molecular weight of dextran at various extents of conversion along the reaction coordinates and all the factors mentioned above. Application of this mechanism to the action of dextransucrase identifies (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 the enzyme from the primer chain. It should be noted that Stacey (Stacey, 1943) and Hehre (Hehre, 1951) had already mentioned that the synthesizing enzyme remained in combination with the polysaccharides.

Ebert and Schenk (1968) proposed an insertion mechanism for the biosynthesis of dextran that was based on the theoretical interpretation of the action of acceptor molecules and known kinetic data. In this type of polymer synthesis a termination reaction is possible if the permanent bond between the enzyme and the polymer chain is broken by the acceptor molecule in such a way that it cannot be reformed. A mechanism which includes an insertion type of polymer growth and the termination of the polymerization by an acceptor can be formulated as follows.

$$E + S \rightleftharpoons ES \longrightarrow EPI + X \qquad \text{(Eq.7)}$$

$$EPn + S \underset{k-1}{\overset{k_1}{\rightleftharpoons}} ESP_n \longrightarrow EPn+1 + X \qquad \text{(Eq.8)}$$

$$ESP_n + A \underset{k-3}{\overset{k_3}{\rightleftharpoons}} AESP_n \xrightarrow{k_4} APn + ES \qquad \text{(Eq.9)}$$

Equation (8) is a growing cycle.

The reaction between the free enzyme, E, and the substrate, S, occurs only at the beginning of the reaction, when the free enzyme is present in the reacting system. According to this proposed mechanism, the deviation of dextransucrase action from Michaelis-Menten reaction at higher concentrations of sucrose is explained by the fact that sucrose itself could act as an acceptor. It also gave the most reasonable account for the action of acceptor molecules. Strong acceptors result in the formation of low molecular weight dextrans, and their influence on the rate is small. Weak acceptors have a strong negative effect on the rate, but the decrease of the molecular weights is considerably less. They also proposed that dextran can also react as an acceptor and form branches of different lengths, a proposal which will be discussed in the next section.

This excellent theoretical derivation of the insertion type of polymerization mechanism was demonstrated by Robyt, Kimble and Walseth (1974). In their study, the immobilized dextransucrase on Bio-Gel was labeled by incubating the dextransucrase-gel with [^{14}C] sucrose, then the label was released by treating with heat. Two types of labeled carbohydrates were found to be released: glucose and dextran. The direction of biosynthesis was also determined by pulse and chase experiments with [^{14}C] sucrose. Their data provided evidence that biosynthesis of dextran by dextransucrase is performed by the addition of glucose units to the reducing ends of the growing chains. They proposed that the enzyme forms a covalent complex with the reducing end of the dextran chain and that the glucosyl unit from sucrose forms a covalent intermediate with the enzyme and is inserted between the enzyme and the reducing end of the growing dextran chain. The proposed insertion mechanism is shown in figure 6.

Robyt and Walseth (1978) examined the acceptor reaction step in the presence and absence of sucrose. This was done using D-^{14}C glucose, D-^{14}C fructose and ^{14}C- reducing end labeled maltose as acceptor. It was found that all three of the acceptors were incorporated into the products at the reducing end. They proposed that the acceptor reaction was proceeded by a nucleophilic displacement of glucosyl and

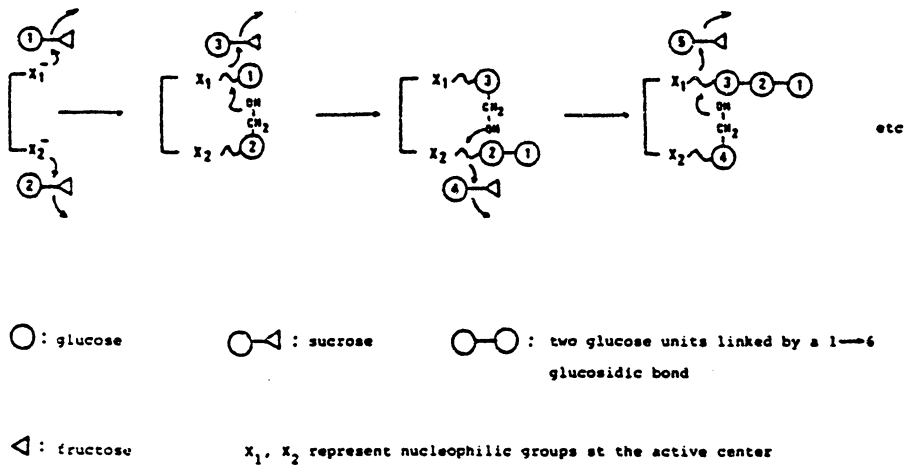


Fig. 6 Proposed dextransucrase mechanism by Robyt, Kimble and Walseth. This figure is reproduced after their article in "Archives of Biochemistry and Biophysics", volume 164 (1974). The enzyme has two equivalent catalytic groups X1 and X2. Starting with the nascent enzyme both groups attack sucrose to give glycosyl complexes. The C_6-OH of one of these glucose units makes a nucleophilic attack onto the C_1 of the other glucose unit thereby forming an $\alpha(1\rightarrow 6)$ glucoside linkage; this releases one of the nucleophilic groups which then attacks another sucrose forming a new enzyme-glucosyl complex. The process continues with the two catalytic groups alternatively forming covalent complexes with glucose and dextran.

dextranosyl groups from a covalently bonded enzyme-complex by a specific, acceptor hydroxyl group, and that this reaction effected a glycosidic linkage between the D-glucosyl and dextranosyl groups and the acceptor. This work provided experimental data for the justification of the insertion mechanism proposal with some insight into the acceptor reaction.

Further work was carried out by Kindler and Ludwig (1975) using Ebert and Schenk's insertion mechanism. In pre-steady state reaction kinetics, they were able to determine the rate constants associated with the insertion mechanism given in the equations below:

$$EPn + S \underset{k-1}{\overset{k_1}{\rightleftharpoons}} ESPn \xrightarrow{k_2} EGPn \xrightarrow{k_3} EPn+1 \quad \text{(Eq.11)}$$
$$\downarrow F$$

E is dextransucrase

Pn is dextran attached to the enzyme

S is sucrose

F is fructose

and G is glucose

Here the enzyme-sucrose-dextran complex undergoes two steps: first to liberate fructose and then to inset the glucosyl unit to the dextran chain. Values for these rate constants were experimentally determined to be k_1=35 ℓ/mole-s; k-1, k_2 and k_3 = 1 sec^{-1}.

D. 9. Ebert and Schenk's structurally determined growth hypothesis

One of the most unusual features of Ebert and Schenk's proposed insertion mechanism is designated as structurally determined growth. They think the propagation reaction

$$ESPn \longrightarrow EPn+1 + X$$

as shown in equation (8) can be directed by structural properties of the enzyme-polymer complex. These polymerization reactions must contain a great number of additional elementary reactions implying more enzymatic reactions with complicated regulative functions. From kinetic studies it follows that the reaction of sucrose with the enzyme-dextran complex is fast compared with the propagation step. As shown in figure 7, they hypothesized that the growing polymer chain and the enzyme were combined in a complex through many coordinate bonds. However, the carbon 1 atom of the end unit closest to the active center was bound to the enzyme by a covalent bond. At the same active center, a substrate molecule was absorbed, and initially fixed by several coordinate bonds. At this point in the sequence, at least three subreactions occur: (1) the splitting of sucrose into glucose and fructose, (2) the addition of a new glucosyl unit to the polymer chain, and (3) the movement of the polymer chain along the enzyme. As soon as these subreactions were complete, the structure of the enzyme-dextran

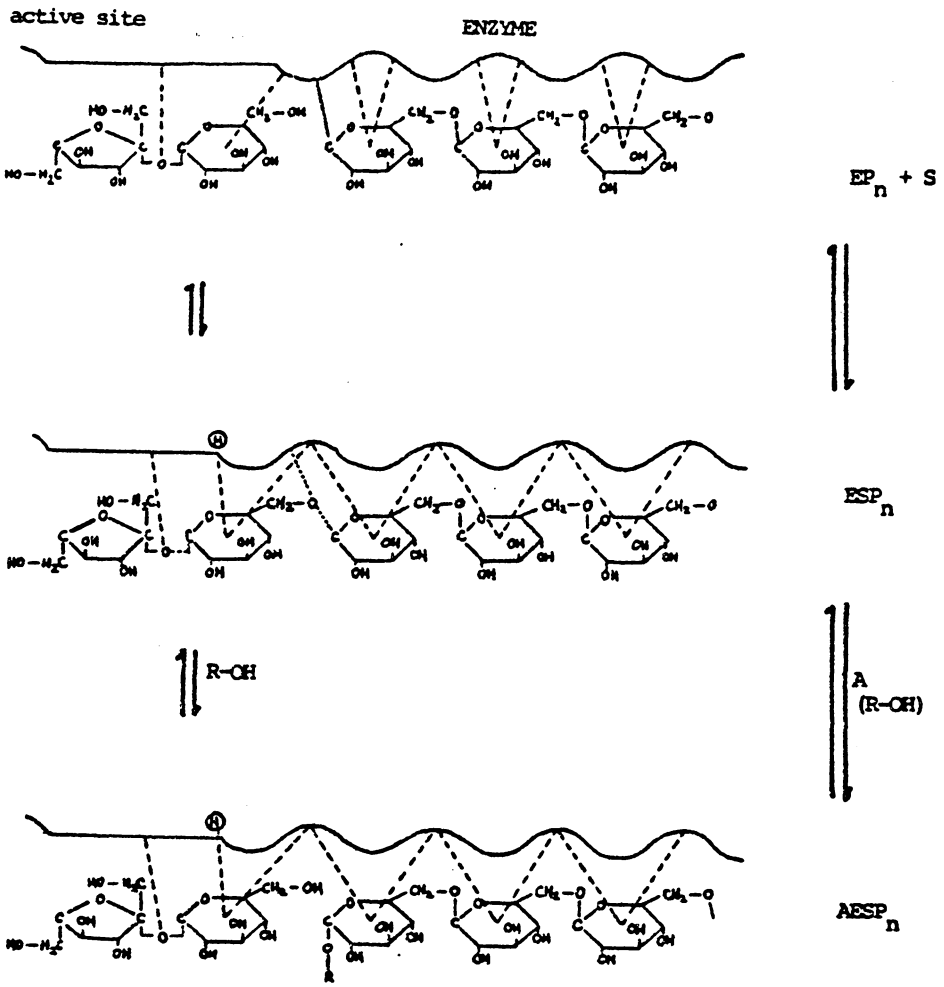


Fig. 7 Proposed "structurally determined insertion-type growth" of dextransucrase action by Ebert and Schenk. This figure is reproduced after their article in Advance Enzymology, volume 30, 1968.

complex was restored, a new substrate was absorbed, and a new propagation step took place.

The hypothesis assumes that in principle the subreactions conserve their physicochemical characteristics, but they are connected to each other in such a way that they always react together in certain temporal correlations. It assumes that there is a regulating factor for this activity of enzyme with respect to the different subreactions. Such a regulating function can be carried out by the conformation or rather by conformational changes of the enzyme, which would have to occur periodically. The author thinks this hypothesis deserves much attention and should be highly appreciated. One of the major tasks is to discover whether I can find some structural changes on the dextran molecules after I subject the dextransucrase to a moderate shear for different periods of time. If this regulating activity is through the enzyme's higher structures, and there is a long lasting state of the sheared-induced conformation changes, then I should be able to perturb the dextransucrase's conformation by hydrodynamic shear force and then observe some changes. Furthermore, I can test the existing stage of the sucrose-dextransucrase-dextran complex by comparing the effect of shear on the enzyme with and without the substrate's presence in a well-defined shear field.

E. Dextran Structure and Branch Mechanism

E.1. Dextran structure

As mentioned before, dextran is a bacterial polysaccharide of glucose units linked at the 1,6 position with a small, variable number of non-1,6 linkages, and all these non-1,6 linkages belong to triple bonded unites, which therefore constitute branching points. One of the factors controlling the extent of branching in the polysaccharide that is obtained is the strain of the bacterium used. The early chemical and bacteriological work on dextran has been reviewed in detail (Hucker and Pederson, 1930; Tarr and Hibbert, 1931). Nearly 100 strains have been studied and structure of the dextran formed determined (Jeanes, et al., 1954).

To elucidate the structural aspects of the polysaccharide, the following chemical analyses have been performed on NRRL B-512 dextran:

a) Methylation studies - Van Cleve and co-workers (1956) fully methylated B-512 dextran in liquid ammonia with sodium and methyl iodide, then completely hydrolyzed it by heating it in an acetic acid solution containing aqueous hydrochloric acid. The resulting mixture of reducing sugars gave 2,4-di-O-methyl-D-glucose, 2,3,4-tri-O-methyl-D-glucose and 2,3,4,6-tetra-O-methyl-D-glucose in a molecular ratio of 1:21:1. The isolation and identification of 2,4-di-O-

methyl-D-glucose in the products demonstrated that NRRL B-512 dextran contained the 1,3-glucosidic linkage, and that this linkage occurred at the branch point.

b) Periodate Oxidation - In 1945, Brown et al., (1945) proposed a method for determining chain length by the measurement of the amount of formic acid liberated in the periodate oxidation of the polysaccharide. This was improved for greater accuracy by Jeanes and co-workers (Jeanes and Wilham, 1950; Rankin and Jeanes, 1954). They utilized a combined estimate of the production of formic acid and the consumption of periodate for classifying dextran linkages into three groups, (1⟶6), "(1⟶4)-like" and "(1⟶3)-like" structures. They carried out the oxidation using sodium metaperiodate at 25°C. The concentration of formic acid was determined by titration with 0.01 N NaOH, with phenolphthalein as an indicator. Periodate was determined by the method of Fleury and Lange (1933). They determined that B-512 dextran had a structure of 95% (1⟶6) linkage and 5% (1⟶4)-like linkage. Smith and coworkers (Smith, et al., 1952; Hamilton and Smith, 1956) extended the periodate oxidation procedure to a concomitant reduction step, so the oxidized polysaccharides were reduced to the corresponding alcohols, then subjected to hydrolysis. The resulting fragments would be indicative of the types of linkages present in the parent polymer. Sloan, et al.,

(1954) applied Smith's procedure, and showed that B-512 dextran contained 5% (1 ⟶ 3)-linkage. These results were consistent with the methylation study. (Van Cleve, et al., 1956). However, it should be noted that this method could not differentiate between (1⟶6)-linked units and non-reducing end units, so that a highly branched molecule having many non-reducing end groups was indistinguishable from a linear molecule with high proporiotn of (1⟶ 6) linkages.

c) Acid Hydrolysis-- Partial acid hydrolysis of dextran resulted in the isolation and characterization of the disaccharide iosmaltose as 6-O-α-D-glucopyranosyl-D-glucopyanose. Jones, et al., (1954) analyzed the yield of D-glucose and di-, tri-, and tetrasaccharides on limited acid hydrolysis, and concluded that 80% of the external branches were only one unit long.

d) Dextranase Treatment - Jeanes and co-workers (Jeanes, et al., 1953) used dextranase to hydrolyze dextran, which gave isomaltose and isomaltotriose in 50% and 20% yield respectively. This furnished excellent evidence that the main structural linkage in B-512 dextran was α-D-(1⟶6), and the structure of the side chain was the same as the main chain.

In addition to chemical studies, physical measurements of the properties of NRRL B-512 dextran were conducted.

(1) Polymer studies--Early works were conducted using electron microscopy, ultracentrifugation and flow birefringence, and X-ray analysis. The most important aspects of the studies were the interpretations of intrinsic viscosity data in solvents. It is well known that the intrinsic viscosity of polymers is a measure of the volume pervaded by the polymer molecule in solution. This volume is affected by the geometrical characteristics of the polymer chain, by restrictions on free rotation, and by the solvent environment. Flory and Fox (1951) developed a quantitative theory of the intrinsic viscosity of linear polymers which took into account all of these factors. Zimm and Stockmayer (1949) have developed methods of calculating the radii of the gyration of branched molecules. They pointed out that a factor, termed g value, representing the ratio of the squares of the unperturbed radii of gyration of a branched molecule to those of an unbranched molecule having the same chemical constituion, could be derived from intrinsic viscosity data.

$$g = \overline{So}^2/\overline{Soo}^2$$

Wales, Marshall and Weissberg (1953) studied the intrinsic viscosity and molecular weight relationship for dextran solution. They were able to fit the observed data for B-512 dextran to a suitable model. Branch lengths of 5 to 6 glucosyl residues with a spacing of 9 units between branches were found to make the most satisfactory fit to

experimental data. Senti and co-workers (Senti, et al., 1955) made an extensive study on B-512 dextran and fitted the observed g value to the model containing 77% of the branches as one unit long, a finding already suggested by Jones (Jones, et al., 1954). In this model, the remaining branches extended to lengths greater than 50. The viscosity-molecular weight relationship observed could be accounted for if it was assumed that most of the one-unit-long branches were arranged on the main chain in the Staudinger or "comb" form, and that a few of the long branches were joined in a "macro-branched" structure. From the relationship between the viscosity of a native dextran solution and the molecular weights of the dextrans, Ebert (1967) proposed that native dextran had a helical conformation. He proposed that the dextran chains form a helix with two glucose units per turn. In this model, alternate glucose units were situated adjacent to each other, and all hydroxyl groups of C2 and C4 atoms of the glucose ring were connected by hydrogen bonds which stabilized the helix. The hydroxyl groups of the C3 atoms were fixed on the helix at equal intervals of about 7 Å, an it was assumed that superposed structures were formed by intermolecular hydrogen bonds between these hydoxyl groups.

(2) Infrared adsorption spectra-- Barker and co-workers (1953; 1954) confirmed that dextran produced by

different bacterial strains showed different amounts of light adsorption at 794 cm^{-1}. They felt this was due to the presence of (1——→ 3) linkages.

(3) Optical rotation-- Specific optical rotation is a useful physical measurement in the field of carbohydrate chemistry. Since optical activity is a function of the groups surrounding an asymmetric carbon atom, a change in optical activity is a reflection of a change in the structure of the molecule. Jeanes and co-workers developed a rough correlation between $[\alpha]_D^{25}$ (in formamide) and the content of (1 ——→3) linkages present in the dextran. Another useful method was developed by Reeves (1951) based on the optical rotation of dextrans of their cuprammonium complex. Brosche (1964) used this technique to confirm that side chains were linked to the main chain in the 1,3 position.

E.2. Possible Branch mechanism: Branch factor

There is still no solid consensus on what is the most probable branching mechanism of this enzyme. When dextransucrase was first identified, Hehre and other early workers assumed that there was a branch factor, or another enzyme for branch formation, as in the case of Q enzyme on amylose (Bourne and Barker, 1948). This two enzyme mixture idea conformed to the results of Bailey, et al., (1955, 1957), who found a linear type of polymer produced by a partially purified enzyme solution of Birmingham strain which had been

aged for two months at 0°C. They concluded that one of the enzymes was destroyed. They also found that dextrans from growing cultures on a magnesium deficient medium tend to be more linear. Bovey (1959) reinforced this proposal. He used light scattering measurements to monitor the dextran formation during the synthesis, and obtained some data on molecular weight and molecular shape. He also gave some theoretical interpretations to the observation, claiming that the short branches, consisting of a single glucose unit, might be formed by the direct transfer of glucosyl groups from sucrose to the C3 hydroxyl group of a $\alpha(1 \longrightarrow 6)$ linked dextran. The branches of greater length were the result of the transfer of the cleaved dextran segment to a C3 hydroxyl group of a glucose unit of another dextran chain. In other words, there was a branching enzyme analogous to muscle gylcogen branching enzyme or to potato Q enzyme. However, dextran branching enzymes have never been observed or isolated, and their existence is still in question.

E.3. Branch formation by dextran as acceptor

Ebert and Brosche (1967) proposed that branch formation was caused by free dextran molecules acting as acceptors, and thus releasing the dextran molecule being synthesized from the dextran-dextransucrase complex. Therefore the enzyme was freed and formed a brank linkage. They tested

their hypothesis by adding ^{3}H-labeled low molecular weight dextran to a reacting system and found the ^{3}H-label was incorporated into a high molecular weight dextran. They also calculated that the average number of acceptor molecules incorporated into native dextrain was about unity, as predicted by this proposed mechanism. The fact that a dextran molecule can contain a certain number of branches was explained by the consecutive branching reactions with the same acceptor molecule under consideration.

Robyt and Taniguchi (1976) did more thorough experiments to intensify this acceptor proposal. Purified dextransucrase was insolubilized on Bio-Gel P-2 beads (BGD), and then the enzyme was tagged with ^{14}C-dextran by incubating with ^{14}C-sucrose. When labeled BGD was incubated with low molecular weight non-radiative dextran, all the BGD bound label was released. The released ^{14}C-dextran was shown branched and acetolysis revealed a new bond of α-(1$\longrightarrow$ 3) linkage was formed. A mechanism for this was proposed, in which a C3 hydroxyl group on an acceptor dextran acted as a nucleophile on C1 of the reducing end of a dextranosyl-dextransucrase complex. Thus dextran was displaced from the enzyme, and formed an α-(1$\longrightarrow$ 3) branch linkage. The proposed mechanism is shown in figure 8. It is currently believed to be the most possible mechanism for branch formation.

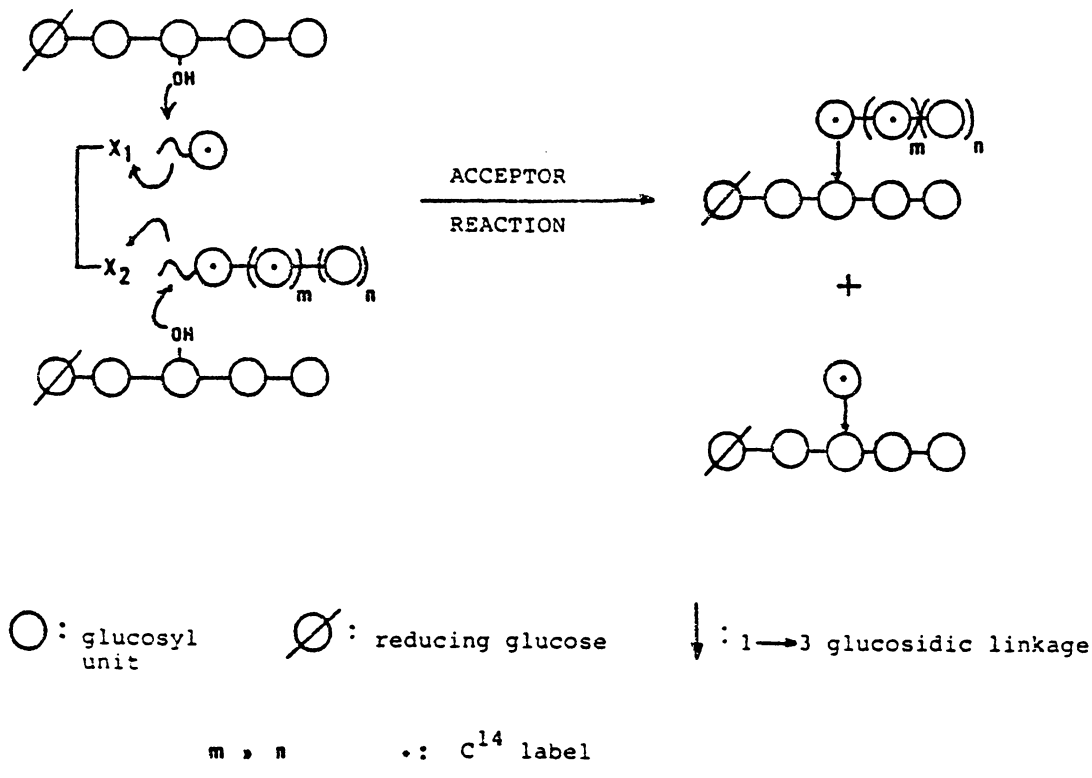


Fig. 8. This figure is the proposed biosynthesis of branch linkages by acceptor reactions with dextrans by Robyt and Taniguchi, reproduced after their article in "Archives of Biochemistry and Biophysics", volume 174 (1976).

To achieve multiple branches on a linear backbone, the displaced acceptor has to diffuse back, and goes through acceptor reactions separately.

E. 4. Some questions associated with the proposed branch mechanism

There are some shortcomings about this proposed branch mechanism. Shortcomings not explained and answers cannot be found in the literature. First is the unexplained source of the short chain dextran which will act as an acceptor and which results in a branch point. In a natural environment, there is no guarantee that a short dextran will be present in the vicinity of the dextransucrase; even if there are some cleaved dextran segments, the facts that dextransucrases of different strains will synthesize fairly uniform structural dextrans is hard to account for. Second, according to this mechanism, the synthesis of branching linkages is ascribed to a separate acceptor reaction in which the dextran chains are transferred from the enzyme to preformed oligosaccharide or dextrans molecules. Repeated transfer of chains to the same molecule would thus yield a multiple branched dextran, for example, 5% α1 $\rightarrow$3 branch linkages for NRRL B512 dextran. There must be some Christmas-tree structures in the dextran, but all the studies on the dextran structure excluded this possibility. So naturally, a question is raised: is it possible that dextransucrase mandates the branch linkage foundation as one of its regulating functions through its conformation?

F. C13 NMR Spectroscopy for Polysaccharide Structure

F.1. Basic C13 NMR spectroscopy principles

The energy gained as a result of the adsorption of electromagnetic radiation may bring about increased vibration or rotation of the atoms, or it may raise electrons to higher energy levels. Like electrons, the nuclei of certain atoms such as H^1, C^{13}, F^{19} are considered to "spin". The spinning of these charged particles, i.e. the circulation of charges, generates a magnetic moment along the axis of spin, so that nuclei act like tiny bar magnets. If a nucleus is placed under an external magnetic field, its magnetic moment, according to quantum mechanics, can be aligned in either of two ways: "with" or "against" the external field. Alignment with the field is more stable, and energy must be absorbed to "flip" the tiny magnet over to the less stable alignment, against the field. How much energy is needed to flip the nucleus depends on the strength of the external field:

$$\nu = \frac{\gamma H_o}{2\pi} \qquad \text{(Eq. 12)}$$

where ν = frequency, in Hz

H_o = strength of magnetic field, in gauss

γ = a nuclear constant, the gyromagnetic ratio

In practice, we would place a substance in a magnetic field,

keep the radiation frequency constant, and vary the strength of the magnetic field. At some value of the field strength the energy required to flip the nucleus matches the energy of radiation, absorption occurs, and a signal is observed. Such a spectrum is called a nuclear resonance (NMR) spectrum.

The frequency at which a nucleus absorbes depends on the magnetic field which that nucleus feels, and this effective field strength is not exactly the same as the applied field strength. The effective field strength for each nucleus depends on the environment of that nucleus, especially the electron density at the nucleus and the presence of other, nearby nuclei. Each set of equivalent nuclei will have a slightly different environment from every other set of nuclei, and hence will require a slightly different applied field strength to produce the same effective field strength. The result is a spectrum showing many absorption peaks which yield much information about molecular structure: (a) the number of signals tells how many different kinds of nuclei are in the molecule; (b) the position of signals tells something about the electronic environment of each set of nuclei; (c) the intensity of signals tells how many nuclei of each kind are present. Stereo-specific information can be obtained by looking at the chemical shifts and splitting patterns. Also, because nuclear transitions have longer decay times, relaxation times can be easily measured.

The common NMR spectrum uses tetramethylsilane (TMS) as a reference, and the scale is given in δ units (ppm) downfield from TMS in positive numbers, and upfield from TMS in negative numbers.

C^{13} is not an abundant isotope and it represents only 1.11% of the carbon in a natural system. Its magnetic moment is smaller than that of H^1, thus the NMR spectrum for C^{13} is roughly 10^4 times more difficult to detect than H^1. Nevertheless, with the development of the Fourier transform technique and modern electronic instruments, it has become possible to detect C^{13} routinely in compounds of carbon in natural abundance at concentrations as low as 10^{-2}M. Coupling to H^1 makes C^{13} spectra very complex, but they can be removed by broad-band irradiation at the H^1 resonance frequency (noise decoupling), or by a H^1-C^{13} cross relaxation mechanism known as the nuclear overhauser effect. C^{13} NMR has become an everyday tool in the hands of practicing chemists. A large amount of data on an enormous variety of compounds is available, and can be effectively used as a means of identification of molecular structure.

F.2. C^{13} NMR on dextran

It is convenient to reiterate the major features and relationships normally encountered for polysaccharide structures.

Each saccharide residue is normally linked through the

reducing group (anomeric position) to the hydroxy group of another residue. Each residue contains a number of hydroxy groups but only one anomeric position. When a residue is linked only through the anomeric position, this residue is a non-reducing terminal residue. When a residue is linked through both a hydroxy position and an anomeric position, it is one of the linear chain extending units. In the same manner, should a residue be linked through both the anomeric position and two hydroxy positions, a branch point residue results (please refer to fig. 9 for the NRRL B512 dextran structure at the branch point).

In general, any polysaccharide contains a single reducing end group and b+1 terminal residues, when b represents the number of branch-point residues. Therefore, a large polysaccharide effectively contains no reducing end group and an equal number of branch-point residues to terminal non-reducing residues.

Seymour, et al., (1976; 1979) did a thorough NMR study on C13 in the dextran structures. An important contribution for the analysis of 25 MHz spectra was the realization that elevated temperatures greatly aid spectra acquisition and interpretation. The original approach to polysaccharide C13 NMR spectral analysis consisted of making a minumum number of hypotheses about expected structure-to-spectra relationships. The first was that each polymer could be

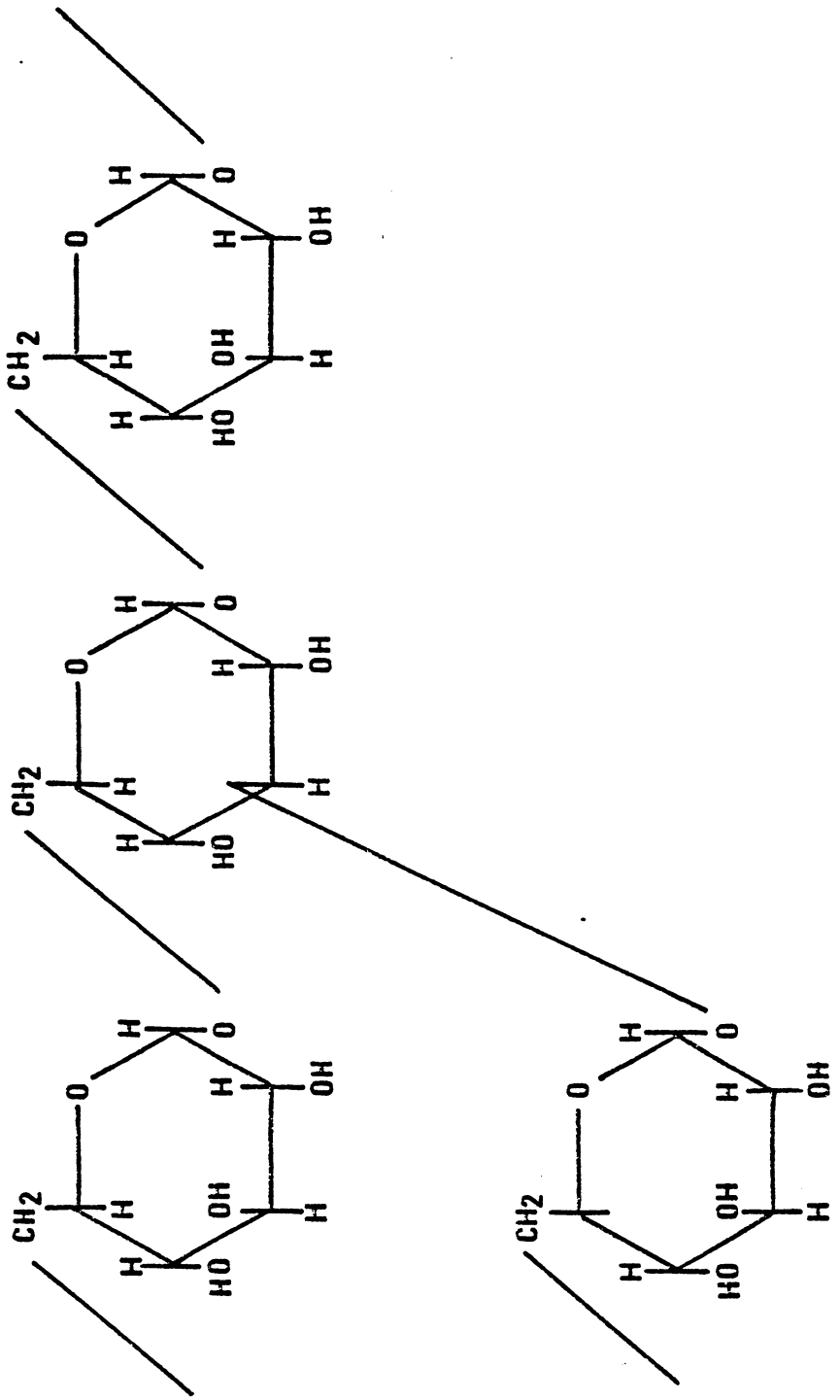


Figure 9. Basic dextran structure for the NRRL B-512 dextran at the branch point.

considered as an assembly of independent saccharide monomers. The second was that these hypothetical saccharide monomers would be O-alkylated (O-methylated) in the same position as the actual saccharide linked residues. With comparison to conventional fragmentation g.l.c.-m.s., permethylation fragmentation g.l.c.-m.s., HPLC of acetolysis products, Seymour and his collaborators could interrelate C13 NMR spectra to structural analysis of dextran.

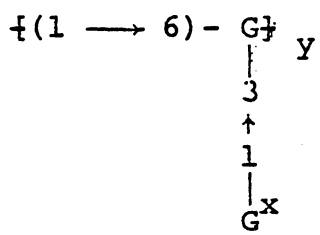
Linear dextran has a simple spectrum (67.8, 71.5, 72.0, 73.2, 75.2 and 99.6 ppm) closely approximating the saccharide resonances of a 1.6-di-O-substituted α-D-glucopyranoside monomer. Structural variants of linear dextran occur that are due to branched α-D-glucopyranosyl residues; branched dextrans exhibit resonances identical to those of linear dextran, but contain additional resonances proportional in intensity to the degree of branching. The chemical shifts of these branching resonances are dependent on the type, but not on the degree, of branching of the dextran.

Based on the O-substituted α-D-glucopyranoside's spectrum, only four spectral regions are of interest: the 60-70 ppm region, containing both free and linked C6 resonance, the 70-75 ppm region containing the free C2, C3 and C4 resonance and the pyranoside ring C5 resonance, the 75-85 ppm region containing linked C2, C3 and C4 resonance and the 95-103 ppm region containing linked anomeric (C1) resonance.

The 75-85 ppm region presents another straightforward situation, as each branch type results in a specific branching residue and the linked carbon resonance is now displaced about 10 ppm downfield from the 70-75 ppm region. At 90°C, α(1⟶ 2)-, α(1⟶ 3), α(1⟶ 4)- branch linkages resulted in diagnostic resonance respectively at approximately 78, 80, 82 ppm. It was also concluded that the intensity of all resonances present was to a first approximation, proportional to the number of atoms of the carbon position present. Observation from C13 NMR spectroscopy added tenuous support to the hypothesis that the (1⟶ 6) linked α-D-glucopyranosyl residues of dextran might form a backbone that was comb-like rather than detritic due to the fact that (1) value of relaxation suggested all the (1 ⟶ 6)-linked residues were in a backbone chain, (2) the narrow peak width of the C13 resonances of dextrans were similar to those of comb-like synthetic amylose, and differed from the broader resonances of dentritic glycogen.

Among published dextran structural analysis by C13 NMR spectroscopy, one has α1⟶3 branch linkages and could serve as a good reference for the chemical shifts assignment and judgement on the structural variations of the linear NRRL B-512 dextran under study is the dextran B742 fraction S. The data is made available by Seymour and coworkers (1979a and c). Combined g.l.c.-m.s. analysis of the per-O-acetyl-aldononitriles from a hydrolysate of permethylated dextran

B-742 fraction S established that the molar ratio of its methylated D-glucose components was 2,3,4,6-tetra:2,3,4-tri: 2,4-di=10:1:11. Structurally, ∿ 96% of this dextran fraction consists of a disaccharide residue as the repeating unit. The remaining 4% consists of α-D-(1⟶ 6)-linked residues depicted.



Dextran B-742 fraction S has no residues linked exclusively α-D-(1 ⟶6). The C13 NMR spectrum of this B-742 S dextran at 90°C reveals 11 resonances as predicted. This is shown in table 2 and figure 10. By the carbon-13 spin-lattice relaxation studies, all the peaks have been assigned. The chemical shifts and the corresponding carbon positions are assigned as follows: resonance 1 to xC-1 (where x refers to terminal residue resonances, and y refers to branch-point), resonance 2 to yC-1. Resonance 3 has been assigned to yC3; the branch point α(1⟶ 3) linkage. Resonance 4 and 7 are assigned to the C-2, C-4 and C-5 of y residue. Resonances 5,6 and 9 are assigned to the x-residue (C-2 through C-5) and resonance 10 represents yC-6. Resonance 8 is assumed to be a composite x- and y- residue resonance.

The diagnostic peak at resonance 3 (82.89 ppm) that corresponds to the 1,3,6-tri-O-substituted α-D-glucosyl

Table 2. ^{13}C NMR chemical shifts for the S fraction of dextran produced by Leuconostoc mesenteroides (NRRL) B-742. (Reproduced after Seymour, Knapp and Bishop, Carbohydrate Research, Vol. 72, 1979).

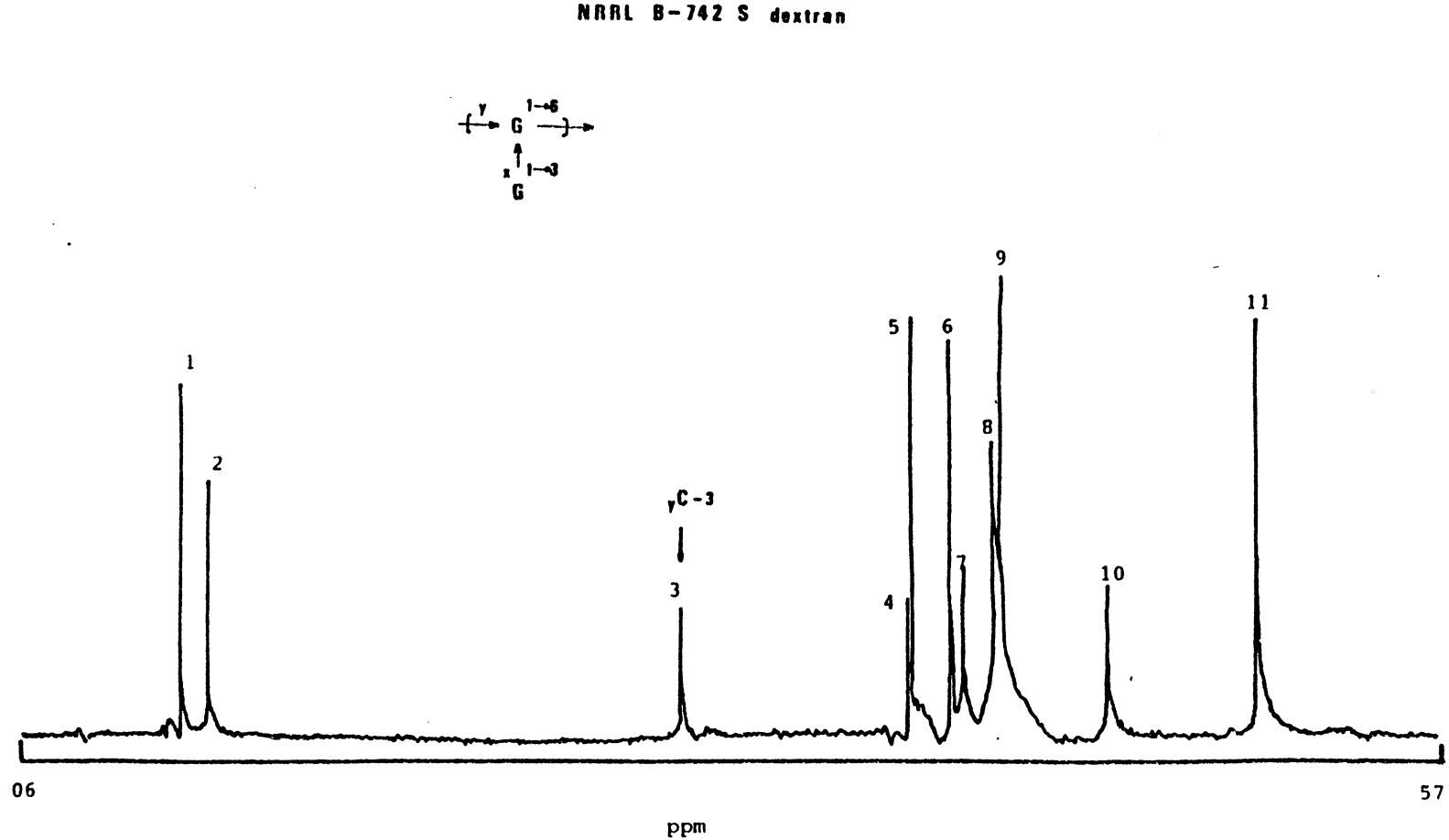
Peak number

Peak Number	1	2	3	4	5
Chemical shifts δ (ppm)	100.81	99.56	82.89	75.09	74.97

6	7	8	9	10
73.57	73.14	71.74	71.41	67.67

11
62.46

Figure 10. ^{13}C-NMR spectrum of the dextran from NRRL B-742 S at 90°C. Reproduced after Seymour, Knapp and Bishop, Carbohydrate Research, vol. 72, 1979.



residue shows the validity of the C13 NMR spectroscopy on the branch feature of the backbone dextran. However, the relationship between ^{13}C NMR spectroscopy and polysaccharide structure is as yet only partially developed, and needs a semi-empirical approach to the subject.

III. EXPERIMENTAL METHODS AND MATERIALS

The three phases of the experimental work on which this dissertation is based were as follows: (1) the production and purification of dextransucrase, (2) the study of shear inactivation of the enzyme preparation by a simple shear field, and (3) the characterization of the synthesized polymer products.

A. Cultivation of Leuconostoc Mesenteroides

A.1. Medium:

A. Jeanes, in Methods in Carbohydrate Research edited by R. L. Whisterl, recommends the following media for maintenance of stock culture and growth of Leuconostoc mesenteriodes, and they were adopted for this project.

a) Medium A -- This medium was used for maintenance of stock cultures. Composition per 100 ml of distilled water was: 10 ml of liver extract, 0.5 g of yeast extract, 1.0 g of trypton, 0.2 g of dipotassium hydrogen phosphate, and 0.5 g of glucose. The final pH must be adjusted to about 7.4 with 10 N sodium hydroxide; a few liver particles were placed in each 16-x 25 rimless test tube; the tubes were filled about one third full of the medium solution, plugged with cotton, and sterilized at 121°C for 15 minutes.

b) Medium B -- A sugar agar was used for initiating dextran synthesis (enzyme induced by 2% sucrose) or

for selecting of good dextran synthesis from a sluggish culture. Composition per 100 ml of distilled water was as follows: 2.0 g of sucrose, 0.5 g of yeast extract, 0.25 g of trypton, 0.25 g of dipotassium hydrogen phosphate, and 2.0 g of agar. All the constituents except sucrose were combined in water at 90% of the final volume; the pH was adjusted to about 74 with 10 N sodium hydroxide, and the mixture was heated in a flow system to dissolve the agar. The sucrose, dissolved in the rest of the water, was then added, and about 7 ml of the hot solution was delivered into 16-x 125 mm test tubes. The tubes were plugged with cotton and sterilized at 121°C for 15 minutes.

(c) Medium C -- A precaution medium was used when an old culture failed to yield good dextran synthesis. It had the same composition as Medium A except that D-glucose was replaced by sucrose (10%); agar was present, and liver particles were omitted.

(d) Medium D -- This medium was used for the build up of innoculum and for dextran production. It contained per 100 ml of distilled water: 2.0 g of sucrose, 0.5 g of yeast extract, 0.25 g of trypton, and 0.5 g of dipotassium hydrogen phosphate.

(e) Medium E -- This medium was used for dextransucrase production. The composition per 100 ml of distilled water was as follows: 2.0 g of sucrose, 0.5 g of yeast extract, 2.0 g of dipotassium hydrogen phosphate,

0.02 g of magnesium sulfate heptahydrate, and each of 0.001g of NaCl, $FeSO_4 7H_2O$, and $MnSO_4 H_2$. The addition of $CaCl_2$ to the production medium, 0.05 g, was made as suggested by Robyt and Walseth (1979) for an increased dextransucrase production level.

The phosphate and the $CaCl_2$ were sterilized separately and were added septically to the cool, sterile solution of the other ingredients. The initial pH of the sterile mixture was in the range of 7.2 to 7.4. To avoid any complication of the sterilization procedure, an equal amount of ferric citrate may be used as a substitute for $FeSO_4 7H_2O$.

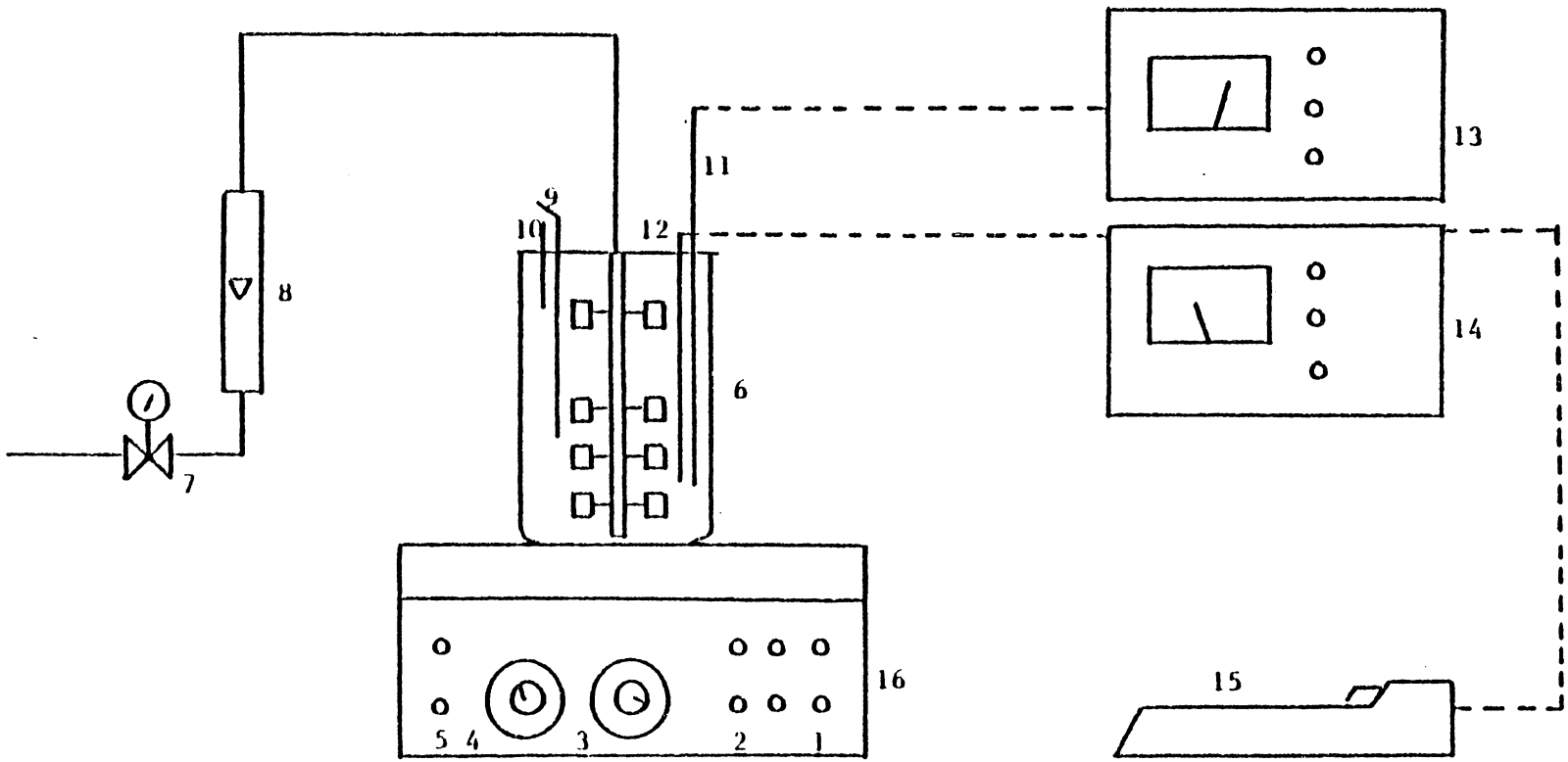
A.2. Fermentor and auxiliary equipment

In order to produce a large amount of dextransucrase, stirred tank fermentor cultures were used. Both a 14 liter fermentor and a 2 liter fermentor were available. Because of the fact that nascent dextransucrase is not stable for long storage times even at low temperatures, and the fact that the subsequent purification process was too cumbersome to perform on a working volume of 10 liters, I chose a 2 liter fermentor. It was advantageous to have control actions on the culture parameters such as temperature, aeration, and pH in order to maximize the bacterial culture density. The fermentation equipment is shown schematically in figure 11.

The New Brunswick Scientific Multigen model F-2000

1. Power On/Off switch
2. Heat On/Off switch
3. Temperature control
4. Agitation speed control
5. Agitation On/Off switch
6. New Brunswick 2 liter fermentor
7. Air inlet valve
8. Flow meter
9. Medium feed port and innoculum port
10. Vertical exhaust tube
11. pH electrode
12. Dissolved oxygen probe
13. pH meter
14. Dissolved oxygen meter
15. Strip chart recorder
16. New Brunswick Multigen fermentor console

Figure 11. Schematic diagram of the fermentor equipment for growth culture.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2 liter fermentor was assembled according to the manufacturer's manual. The fermentor vessel was constructed of Pyrex glass, and fittings were provided for innoculation, chemical addition, aeration, baffling, heating, cold water circulation, temperature measurement, and sample taking. The vessel was secured on the Multigen fermentor console, which included the control mechanism for temperature, agitation speed, and power control. An inlet air line with valve, pressure gauge, and flow meter was constructed and connected to the compressed air line in the laboratory. The inlet air went through a dried, sterile air filter with a glass wool filter just in front of the air inlet port at the top of the agitation shaft. A similar air filter was put on the exhaust tube for outgoing air. An Ingold pH electrode was positioned through the cap of the fermentor vessel, together with a lead-silver galvanic dissolved oxygen probe constructed in the laboratory (Borkowski and Johnson, 1967). The pH electrode and D.O. probe were connected through insulated cables to a New Brunswick Scientific model pH-22 pH controller, which was used only as a pH meter, and a New Brunswick Scientific model DO-40 D.O. controller that acted as a D.O. meter. A Cole-Parmer two-channel strip chart recorder was wired through the auxiliary recorder terminal of the D.O. controller. A 2 liter Fernbach flask, which contained the final sterile medium E, was capped

by a rubber stopper with a long stainless steel tube and a quick connector on it. All the lines and connections were constructed in such a fashion that sterile conditions for pure culture maintenance was achieved.

A.3. Inoculum preparation and the growth culture procedure

The inoculum cultures were prepared in two stages. The first stage buildup was accomplished by inoculating a shake flask containing 5 ml of sterile Medium D with a loop from a stock slant of L.mesenteriodes NRRL B-512. (ATCC, 10830) and by incubating it in a New Brunswick Scientific incubator shaker at 25°C for 12 hours.

The second stage inoculum buildup was competed by transferring 1 ml of the first stage inoculum to a sterile 250 ml Erlenmeyer flask containing 50 ml of sterile Medium E. This was incubated in the shaker for 12 hours at 25°C. The culture appeared turbid with a yellow color. Too long an incubation time resulted in a white, cloudy color; the culture, when checked under a microscope, was found to contain much cell debris.

The fermentor was prepared by first cleaning and drying the fermentor vessel, checking the glass wool filters and all parts to insure closure, and then placing the vessel assembly in the autoclave for sterilization. After the vessel assembly cooled down to room temperature, all the lines were connected using aseptic techniques.

The production medium (Medium E) of 1.2 liter volume was prepared and steam sterilized. When it had cooled, the medium was fed into the fermentor vessel. The medium had a pH of about 5.0, and 10 N NaOH was added through a syringe to adjust it to 7.4. The temperature controller was set at 25°C, agitation at 300 rpm, and aeration rate at 1 liter per minute. The fermentor was inoculated by pouring 50 ml of the second stage inoculum culture through a sterile funnel into the fermentor. After inoculation, the fermentation was allowed to proceed for almost 10 hours. Every hour, fermentor parameters were recorded; these data are shown in table 3. At the very beginning and one-half way through the fermentation, samples were drawn from the vessel, and microscopic wet mounts were made to verify that no contamination had occurred.

The end of the fermentation was signaled by an increase in the dissolved oxygen level toward the saturation level and a rapid drop in pH of the culture fluid. When the growth culture fermenation was stopped, all the utility lines and fittings were disconnected and the fermentor vessel was immersed in an ethanol-ice water (70:30 v/v) bath to halt further growth.

A.4. Harvest and storage

After the enzyme-containing fermentation broth chilled to approximately 10°C, the broth fluid was evenly distributed

Table 3. Fermentation data on growth culture for dextransucrase

Time	pH	D.O.	Temperature	RPM	Remarks
1:20 p.m.	7.4	80%	25°C	200	innoculate
2:25	7.35	74	25	300	
3:20	7.35	72	25	300	
4:20	7.35	68	25	300	
5:05	7.3	61.8	25	300	
7:10	7.25	8	25	400	aeration rate up to 1.5 liters/min.
7:15	7.15	33	25	400	some antifoam
7:30	7.1	16	25	400	aeration rate up to 2.0 liters/min.
8:20	6.9	5	25	500	aeration rate up to 2.5 liters/min.
9:20	6.7	40	25	500	
9:40	6.65	40	25	500	stop fermentation

in six 250 ml Nalgene centrifuge bottles. The bottles were placed in a Sorvall RC-5 model refrigerated centrifuge in which the temperature was maintained between 5 and 10°C, and centrifuged at 12,000 rpm for 20 minutes. The dextran and microbial cells formed a soft, yellow pellet at the bottom of the centrifuge bottle. The clear supernatant was measured and decanted into a single, sterile Fernbach flask. Then, appropriate amounts of 30 nM NaN_3 were added so that the final volume contained 3 mM sodium azide as a bacteriocide. The supernatant was then stored at 4°C, and a small aliquot was perserved for protein and reducing sugar assay. The centrifuged cell pellets were destroyed.

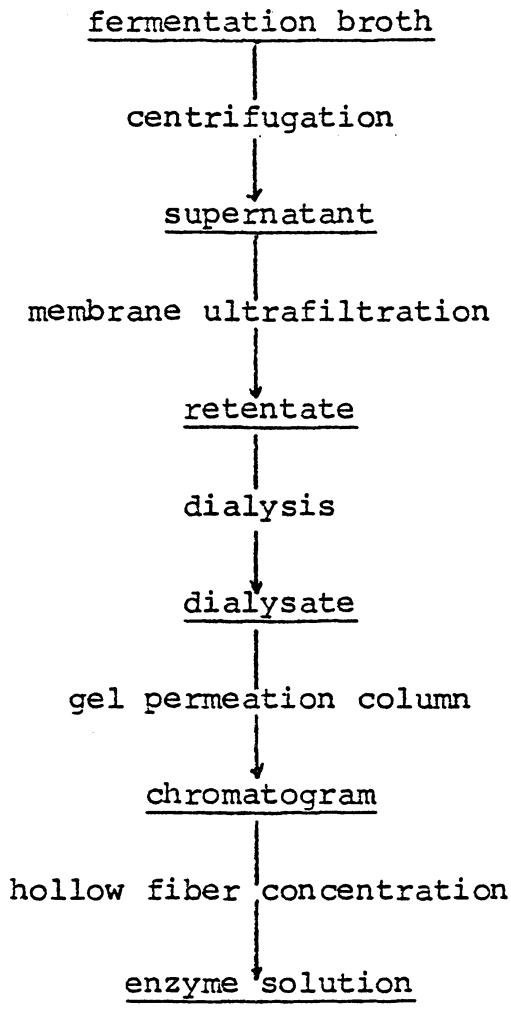
B. Purification of Dextransucrase

B.1. Ultrafiltration and dialysis

From this stage on, a series of purification and concentration strategies were used to obtain a pure and concentrated dextransucrase solution. The general purification scheme is shown in table 4.

A Millipore Pellicon Cassette system was used for the ultrafiltration and dialysis of the supernatant solution at 4°C. This system consisted of several layers of packets made of cellulose acetate membranes with a nominal molecular weight cut-off of 10,000. These membranes were arranged in sandwich fashion between two solid end pieces. The

Table 4. Purification Scheme for Dextransucrase Solution

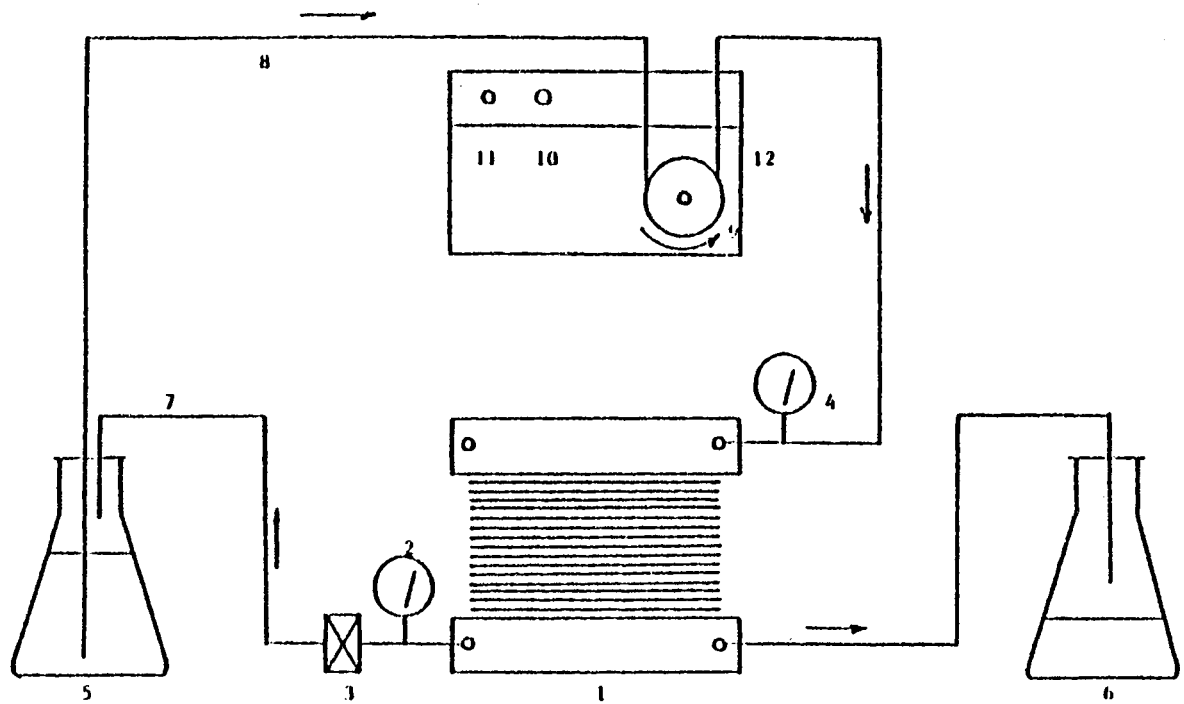


system is shown schematically in figure 12. In line with the most common application for ultrafiltration, the upper right port was used as the sample inlet, the lower left port as retentate outlet, the lower right port as the filtrate outlet, and the fourth port was plugged. A Millipore variable speed perestatic pump forced the supernatant feed through Tygon tubing and recirculated the retentate. Pressure gauges were installed at the sample inlet and the retentate outlet line to monitor the pressure gradient through the cassette cell. I concentrated the retentate volume to 1/6 of the original supernatant volume by setting the sample inlet pressure at 22-24 psig, the retentate outlet pressure at 16-18 psig, the flow rate ratio at 1.5, and in recirculation mode. The retentate appeared brown and oily. Samples were taken from the filtrate and retentate fraction and were analyzed for dextransucrase activity and protein content.

The cassette system was set up again at 4°C to dialyze the retentate against acetate buffer. A slight modification on the line connections enabled the cassette system to be operated under the counter current flow mode. A fresh acetate buffer (approximately 2 liters) at pH of 5.2 ± 0.1 with 3 mM NaN_3 in it was fed into the system through the upper right port inlet, and was passed countercurrently against the enzyme concentrate--the retentate--to replace the fermatation broth and residual salts and

Figure 12. Schematic diagram of the membrane ultrafiltration system.

1. Millipore Pellicon Cassette system
2. Pressure gauge
3. Back pressure valve
4. Pressure gauge
5. Sample reservoir
6. Filtrate
7. Recirculating retentate
8. Tygon 1/4 x 3/8 tubing
9. Cole-Parmer Model No. 7017 pump head
10. Speed control
11. Forward/Reverse switch
12. Millipore Variable Speed Tubing Pump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sugar with buffer. The two streams were pumped separately by a Masterflex pump with a double pump head. The resulting dialysate appeared clear and was stored at 4°C.

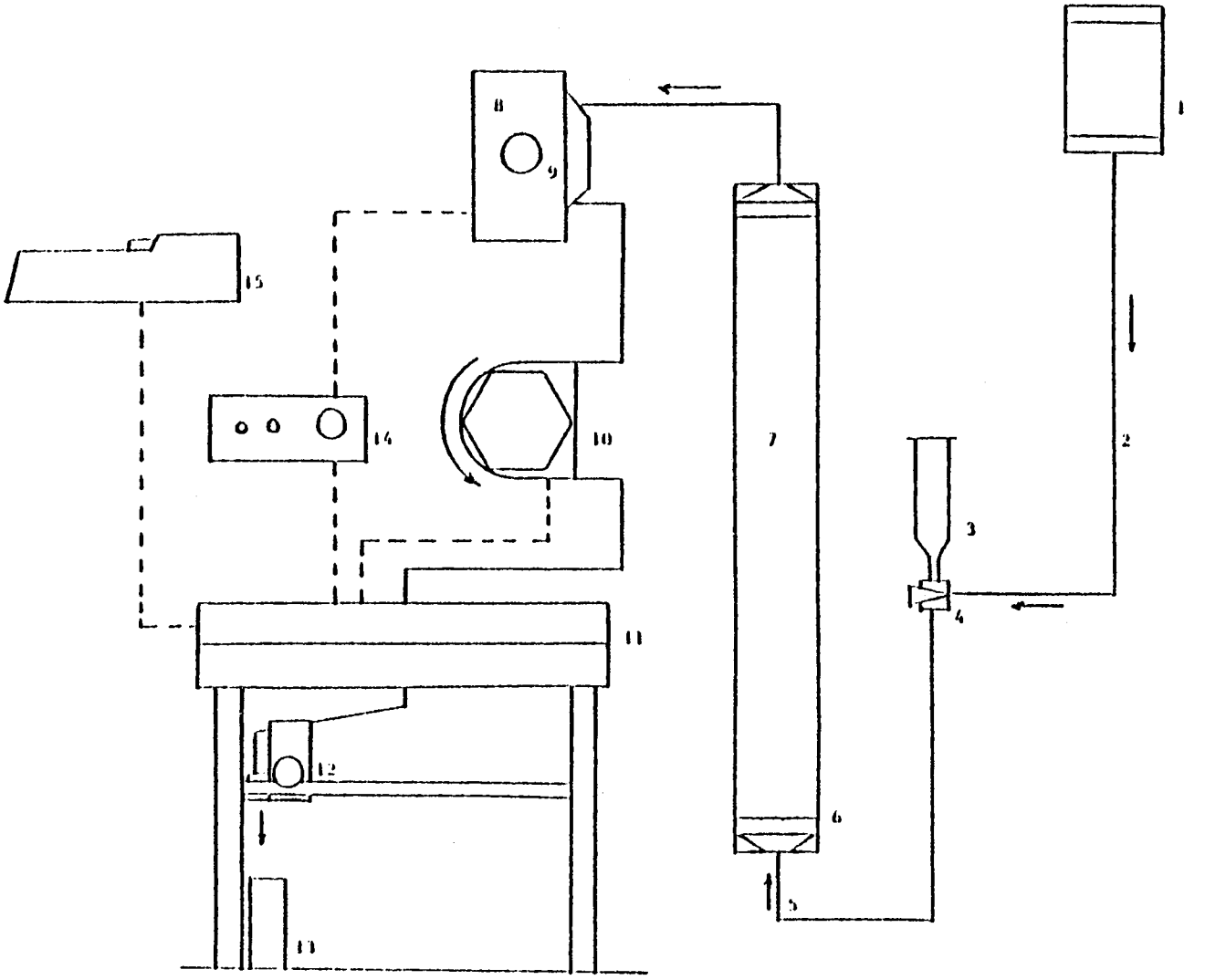
B.2. Gel permeation chromatography

The final purification step was the separation of dextransucrase from the other high-molecular weight material which had been retained in the ultrafiltration process. Generally, this material might include a variety of enzymes produced during the fermentation; their presence was highly undesirable. One of the most powerful tools for separating proteins on the basis of molecular size is gel permeation chromatography, or gel filtration, which proved to be quite successful for the purification of dextransucrase. This technique, reported by Robyt and Walseth (1979), was used as a guide for the present purification.

The whole gel permeation chromatography system is shown in figure 13. The separating system was composed of a LKB 2137 26 mm diameter chromatography column and accessories, LKB Ultrogel AcA22, LKB 2111 MultiRac fraction collector, LKB level sensor, LKB 2120 perpex pump, LKB 2138 Uvicord S monitor device and LKB 2210 potentiometric recorder. A three-way toggle valve and 50 ml syringe serves as a sample port. All the lines were constructed of 1/16" O.D. Teflon tubing with adequate fittings and joints. The column was assembled according to the manual and secured in vertical

Figure 13. Schematic diagram of gel permeation chromatography system

1. Buffer reservoir
2. Teflon tubing
3. Sample injection port
4. Three-way toggle valve
5. Plunger
6. Filter
7. LKB AcA 22 column
8. LKB 2138 Uvicord S
9. Lamp
10. LKB 2120 Varioperpex II pump
11. LKB 2111 MultiRac fraction collector
12. Drop counter head and motor assembly
13. Test tube rack
14. LKB Level sensor
15. LKB 2210 Potentiometric Recorder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position by clamps to the monkey bars. The gel, which was in a slurry form when purchased, was soaked in acetate buffer with 3 mM NaN_3, deaerated, and packed in the column by gravity. Special attention was paid to avoid trapping air bubbles and drying the gel. This AcA22 gel contained 2% polyacrylamide, 4% agarose and had an effective fractionation range of 100,000 to 1,200,000. When packed the column measured 37 cm long. The gel bed was allowed to equilibrate for approximately 24 hours by pumping clean acetate buffer through the column with the LKB Perpex pump.

Prior to sample purification, the packed bed was tested for the uniformity of elute flow by the application of 3.5 ml solution of blue dextran. Blue dextran was chosen as the marker because of its nomial molecular weight (greater than 2×10^6) that would preclude it from entering the matrix pores of the gel, and would keep it in the interparticle space.

The most important device in this system was the MultiRac fraction collector, which contained a programmable microcomputer that was interfaced with a pump, level sensor, monitor, and recorder. Parameter values were entered via the push buttons on the front panel and were displayed on the front panel display. The collection mode was either the time mode, drop mode or pump mode. The level sensor, attached between the monitor signal and the MultiRac, indicated

to the fraction collector whether the output signal from the Uvicord exceeded the value chosen, i.e., if a peak is eluting or not. After they were all installed and connected, calibration of the equipment and evaluations of operation parameters such as delay time and level sensor setting were performed using blue dextran. The Uvicord was equipped with a 280 nm ultraviolet lamp and corresponding filter. The Perpex pump was set at speed dial 5, and scale factor X10, which gave a volumetric flow rate of the buffer of 50 ml/hour. The main program for the fraction collector was:

B2
Pump mode 278
Scale 20
Delay 114
Waste 999
Wait 1

which simply gave the command that a waste fraction had the volume of 17.98 ml and a peak fraction would be collected, 5 ml of each in test tubes loaded in the test tube rack under the delivery head. Level sensor was set as:

BELOW
WASTE
Level: 85

and the recorder was set at 0-100 mV range with recorder paper moving at 5 mm/min.

The dialysate was injected in ten 15 ml batches and then was eluted from the column using the acetate buffer. All gel chromatographic work was conducted at room temperature. The sample was charged to the column through the sample port with the administration of the Perpex pump. This was done by changing the WAIT time to 20 and switching the three-way valve. Care was taken to prevent air bubbles in the sample line. It took 5 hours and 15 minutes to finish a run.

The chromatogram was shown on the recorder chart along the volume axis, with event-markers for each test tube; two peaks were revealed. All fractions were covered and stored at 4°C.

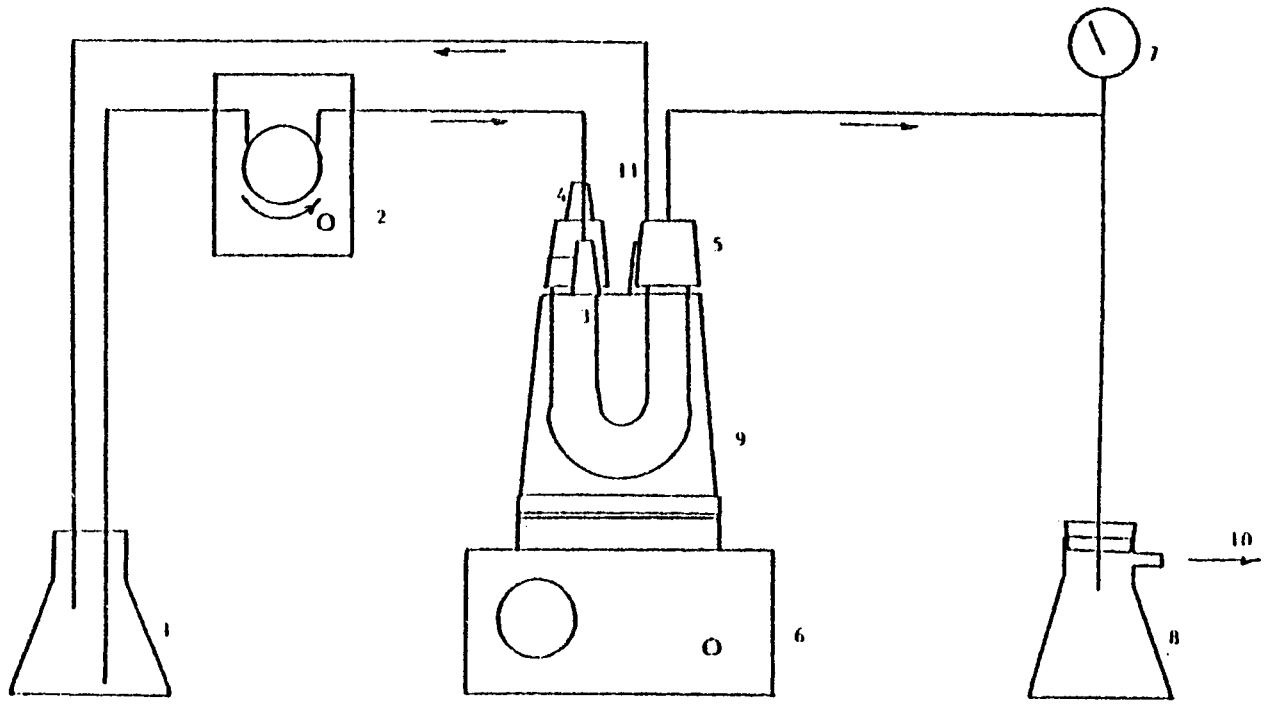
Later biochemical assays identified the first peak as a dextransucrase containing fraction, and the second peak was an extraneous protein. It is worth noting that according to Robyt and Walseth, dextranase treatment prior to gel permeation chromatography on the enzyme concentrate will help separate dextransucrase from levansucrase, a contaminating enzyme of small quantity elaborated by the microorganism. We had tried once, but the eluting pattern did not change on this AcA22 column, so the dextranase treatment was not performed. This repeatedly slow-moving task consumed most of the time of the whole purification scheme. All the contents of the first peak were pooled and ready for the subsequent concentration operation.

B.3. Membrane concentration

After gel permeation chromatography, it was inevitable that we would gain some volume. Due to the fact that a pure and concentrated enzyme preparation was needed to synthesize enough dextran in a reasonably short period of time, a hollow fiber membrane was used to concentrate the enzyme solution. A Bio-Fiber 80 was set up as shown in figure 14. The hollow fiber unit was made of an isotropic membrane of cellulose acetate C with a nominal molecular weight cut-off at 30,000 and a total internal fiber surface area of 1000 cm^2. Fiber bundles were held between two fiber ports, and the jacket had a volume of 100 ml and two jacket ports. The enzyme solution to be concentrated was recirculating in the jacket and the sample reservoir through a MasterFlex tubing pumpt. One fiber port was capped with a rubber cap, and the other, connected to the water asperator, served as the vacuum source. A vacuum gauge was installed on this line because it was suggested in the manual that the pressure difference across the fiber should be less than 1 atm (15 psi). An integrity test was performed before each run. A magnetic stirrer was placed beneath this hollow fiber unit which had a cap for a stir bar, to help remove the external built-up solute from the surface of the fiber bundle. In about 1 houre, the enzyme solution was concentrated down to 3/5 of its original volume. After the experiment, the hollow fiber unit was

Figure 14. Schematic diagram of hollow fiber membrane concentration system

1. Enzyme Solution to be concentrated
2. Masterflex pump with Model No. 7017 pump head
3. Jacket port as inlet for feed
4. Capped fiber port
5. Fiber port for drainage
6. Magnetic stirrer
7. Vacuum gauge
8. Filtrate in vacuum trap
9. Bio-Fiber 80 Beaker hollow fiber unit
10. Water asperator
11. Tygon 1/4 x 3/8 tubing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back washed with distilled water at a rather fast flow rate and stored in 2% formaldehyde solution. Two critical things to be noticed are: (1) do not allow fibers to dry out, and, (2) avoid breaking the fibers.

B.4. Lowry Procedure

In order to assess the purification work, quantitative measurement of the protein content of each fraction and the original broth is very important. There are several colorimetric assays for quantitating protein. One of the oldest is the biuret reaction, where a color-producing reaction occurs between alkaline copper ions and at least two sequential peptide bonds. The advantages of this biuret assay are that the color does not vary with the species of protein and that few other biochemicals interfere. Lowry et al., (1951) improved the biuret assay by utilizing the Folin-Ciocalteu phenol reagent. The phenol reagent reacts with the biuret-protein complex, producing a brighter color; hence, the Lowry assay can be 10 to 100 fold more sensitive than the biuret. The procedure used in this research was the Hartree's Lowry protein assay. Reagent solution stocks of A, B and C were prepared according to the recipe. A stock of Bovine Serum Albumin at the concentration of 1 mg/ml was also prepared, which was used to make a set of tubes containing 0, 5, 10, 20, to 100 micrograms per tube in a volume of 1 ml per tube as standard. The procedure

was as follows:

(1) In a 1 ml portion sample, 0.9 ml of Reagent A was added. The combined solution was heated to 50°C in a water bath for 10 minutes. Then it was cooled down to room temperature.

(2) 0.1 ml Reagent B was added to the above solution and mixed; it was allowed to sit at least 10 minutes.

(3) 3 ml Reagent C was added fast and vortexted immediately. Since the phenol reagent in alkaline solution was not stable, it was imperative to mix immediately.

(4) The whole assayed solution was reheated to 50°C for 10 minutes, then it was cooled down to room temperature.

(5) Absorbance at 650 nm was read in 1 cm glass cuvette on a Varian model 635 spectrophotometer.

When the absorbances were taken and plotted on graph paper, a straight line should be seen from Abs=0.0 for 0μg/ml as the standard. A least square calculator program was used to find the slope and intercept. The result is shown in Figure 18 in the next chapter.

B.5. Dextransucrase Activity Assay

In general form, dextransucrase catalyzes the following reaction:

$$n\ \text{Sucrose} \longrightarrow (\text{Glucose})_n + n\ \text{Fructose}$$

Measurement of the fructose liberated under conditions providing a zero-order reaction was the basis for estimating dextransucrase activity, which was the major index of purification work and also the basis of all the subsequent kinetic experimental data. The procedure was adopted from Keopsell (1951) and Tsuchiya et al. (1952) with some modification. The dextransucrase unit was defined as the amount of enzyme in 1 ml of the reaction mixture which would liberate 1 micro mole of fructose per minute at 25°C. The fructose amount was determined by Somogyi and Nelson reducing sugar assay (Somogyi, 1945; Nelson, 1944). They found that fructose was oxidized by a low alkaline copper reagent and then the product was combined with Nelson chromagen to form a green color which was read as absorbance in the spectrophotometer at 500 or 560 nm. A fructose stock at a concentration of 100 microgram of fructose per ml of distilled water was prepared. Then a set of tubes, each containing 0, 10, 20, to 100 microgram fructose in final volume of 1 ml as standards was made; duplet was needed. The reaction solution which was the freshly made 10 ml of 0.2 M sucrose in acetate buffer (pH = 5.2 $\pm$ 0.1) was distributed in 50 ml Erlenmeyer flasks. Adequate amounts of enzyme solution (to the right dilution factors) were mixed and then incubated in a Lab-Line shaker with a temperature-

controlled water bath set at 25°C for 30 minutes. A boiled enzyme solution, boiled at 96°C for 15 minutes, served as control. The reducing sugar assay procedure was as follows:

(1) The enzyme catalyzed reactions were stopped by delivering a 1 ml portion to the hot Somogyi copper reagents in glass beads covered test tubes which had been set in a hot water bath for 5 minutes.

(2) The Somogyi copper reagents were also added to the aliquots of fructose standards.

(3) These tubes were incubated 10 minutes in a 100°C boiling water bath. Then they were removed and cooled down to room temperature.

(4) Nelson chromagen in 1 ml portions was then added to all the tubes. The combined solutions were thoroughly mixed.

(5) When the foam was subdued, absorbances were read at 500 nm or 560 nm at the lower concentration end.

Fructose standard should be a straight line from Abs = 0.0 for 0µg/ml to Abs = 1.34 for 100 µg/ml, the standard curve is shown in Figure 19 in the next chapter. A least square calculator program was used to find intercept and slope. Due to the fact that the dextransucrase activity

is based on the products of a reaction under certain conditions, consistency of conditions and time elapsed for reaction are critical for the soundness of the data. It was found that 0.1 ml of enzyme solution being delivered into 10 ml sucrose in buffer and 1 ml of the reaction mixture taken to be assayed for the reducing sugar content after 30 minutes incubation would give us readings in the range of fructose standards. Since the boiled enzyme solutions always gave Abs = 0.35, it showed the validity of the proper control. A sample calculation for enzyme activity from the absorbance data is shown in Appendix 1.

To sum up, all the equipment needed for this enzyme activity assay are listed below.

(1) Clean 50 ml Erlenmeyer flasks marked with a sign pen.

(2) Small test tubes and test tube racks

(3) Pipetteman auto pipette and adequate tips

(4) Vortex mixer

(5) Distilled water jug

(6) Acetate buffer pH = 5.2

(7) 10 ml pipette

(8) 0.2 M sucrose in acetate buffer, must be freshly made

(9) Hot water heater

(10) Timer

(11) Thermometer

(12) Glass marbles

(13) Inactivated (boiled) enzyme solution stock

(14) Somogyi copper reagent

(15) Nelson chromagen

(16) Spectrophotometer and clean glass cuvettes

B.6. Contaminating enzyme tests

The presence of any other enzyme capable of yielding reducing sugar from sucrose will of course render the assay inaccurate. It is necessary to test the invertase or levansucrase presence in the enzyme preparation. Methods of differentiating various sucrose derived products were described in Hehre's paper (1946) and Forsyth's paper (1950).

The following descriptions of the tests conducted on the pooled first peak fractions from the GPC column were given for protease, a class of enzymes which breaks down the protein molecule; levansucrase, an enzyme that will hydrolyze sucrose to levan (a polyfructose) and glucose; invertase, an enzyme which hydrolyzes sucrose to glucose and fructose; and dextranase, an enzyme capable of degrading dextran to disaccharide maltose.

(a) Protease activity: In a 10 ml assay test tube, 0.5 g Azocall was suspended in 5 ml of acetate buffer. A sample of 0.5 ml was added, and the solution was incubated at 37°C for 3 hours. The appearance of a red solution was

a positive indicator for protease activity.

Invertase and levansucrase activity: In a large test tube 0.5 ml of sample to be assayed was mixed with 20 ml of a 0.1 M raffinose solution. After incubation at 25°C for 16 hours, the solution was assayed for reducing sugar contents.

Dextranase activity: In a large test tube, 10 ml of 1% dextran (M.W. 80,000) solution was mixed with 0.5 ml sample, incubated at 25°C for 16 hours, and assayed for reducing sugars. As each step of the purification scheme proceeded, the results of the analytical assays on the protein concentration, dextransucrase activity and the volume of the enzyme-containing fractions were recorded in book-keeper fashion. The whole purification work on dextransucrase preparation is summarized in tables 5 and 6 in the next chapter.

Methods for reagent preparation are given in Recipes, Materials and Suppliers - section E of this chapter.

C. Shear Inactivation Study

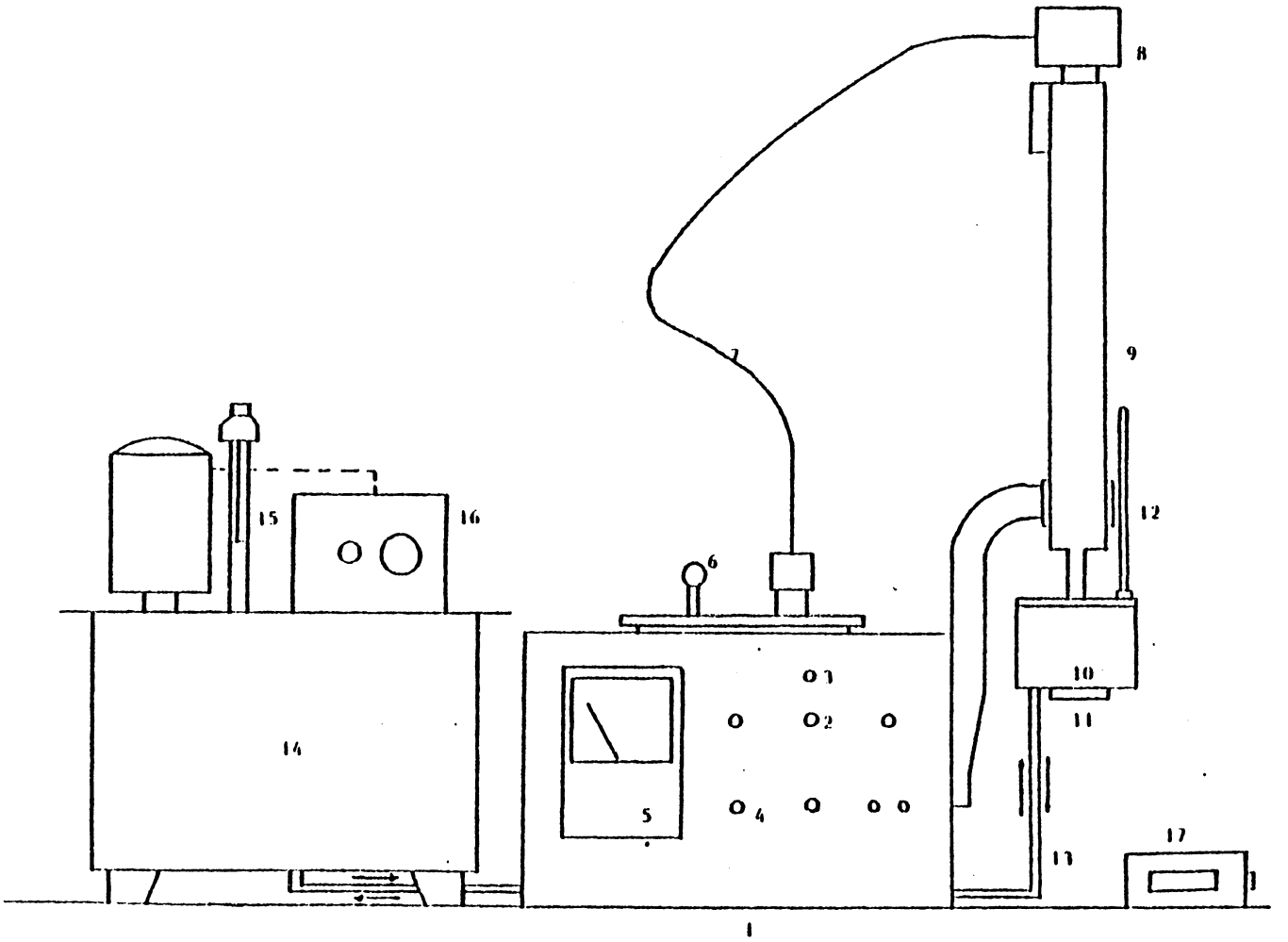
With the dextransucrase enzyme solution purified and concentrated to a value of 0.95 U/ml to 1.59 U/ml from one batch to another batch, I proceeded to the kinetic experiments to study the shear inactivation on the enzyme solutions and the substrate binding effect on enzyme structure under shear.

C.1. Viscometer and its auxiliary equipment

A Haake Rotovisco model RV3 viscometer was used to generate a uniform shear field. The whole system is shown in figure 15. It should be noted that the viscometer was designed to measure the viscosity of substances under a certain range of shear rates and under a constant temperature. I used it here as a device just to provide a uniform shear field. The enzyme solution was introduced into the gap between the two coaxial concentric cylinders of the Couette sensor system--one was the rotating bob, the other the stationary cup--by pipette; then the measuring sensor system was secured by the locking cap at the bottom of the temperature assembly. The bob rotates at different fixed speeds, selected by the gear shift lever on top of the cabinet. The metal control cabinet contains the propulsion motor, gear box, indicating scale, control switches, and recorder terminals. The drive mechanism is a synchron motor with a 10-speed gear system. The gear was engaged by pushing the lever to the right into the appropriate gate. When the lever is pushed to the left, it is always neutral, so it can switch gears while the motor is running. By changing a speed factor from 1 to 162, the viscometer gives ten different rotating speeds of the rotating bob from 583.2 rpm to 3.6 rpm respectively. It is usually set at a speed factor of 3, which corresponds to 194 rpm and a shear

Figure 15. Schematic diagram of the viscometer and its auxiliary equipment.

1. Haake Rotovisco RV3 viscometer
2. Main On/Off switch
3. Signal lamp
4. Electrical zero adjustment knob
5. Indicator meter
6. Gear shift lever
7. Flexible connecting cable
8. Reducing gear box
9. Measuring head
10. Temperature control assembly
11. Locking cap for measuring system
12. Thermometer
13. Circulating oil
14. Haake bath and circulator
15. Thermometer and set point knob
16. Electronic temperature controller
17. Timer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rate $\dot{\gamma}$ of 1046 sec^{-1} for the NV sensor system.

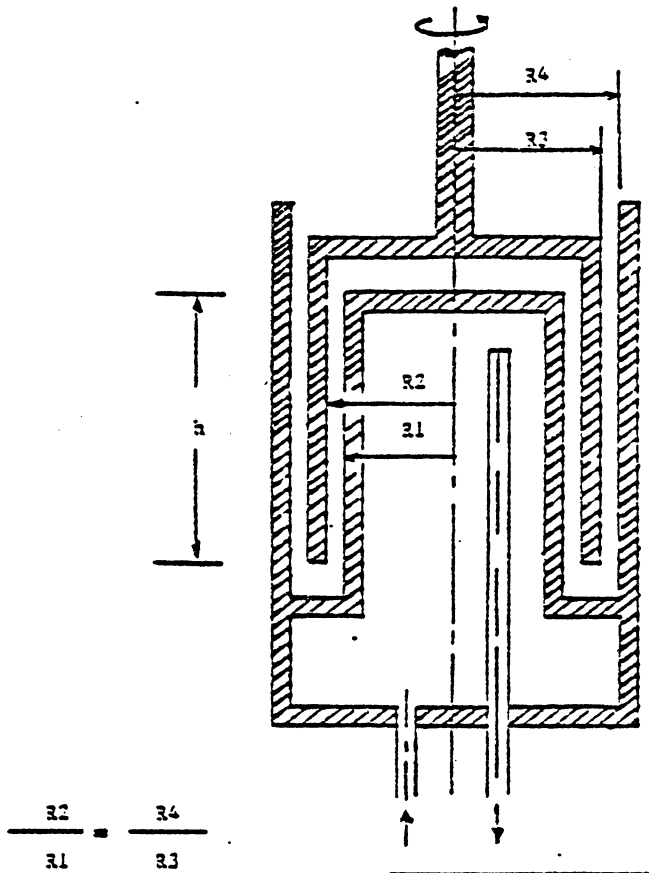
To hold the enzyme solution at a constant temperature during shear, the temperature control assembly was connected to a Haake oil bath and circulator, which as a built-in thermometer and set point knob. Also an electronic temperature controller was installed to regulate the circulating oil temperature.

The bob and cup Couette system applied was the NV sensor system as the design figure show in figure 16. It holds a maximum sample volume of 8 ml and maintains the radii ratio at 0.98, and it provides a quite homogeneous simple shearing flow throughout the loaded enzyme solution.

When all the mechanical parts were in place, it was necessary to tune the machine and set the temperature controller setting at least 30 minutes prior to beginning any shear action on the enzyme solution. In order to measure the exposue time to shear, a timer was set near the viscometer.

C.2. Shear inactivation studies

To understand what shear force will do on enzyme molecules, and study the shear inaction on a dextransucrase preparation, a batch reactor with gentle but good mixing was used to generate non-shear enzymatic reaction data which would serve as the basis of comparison with sheared enzyme reactions. The New Brunswick Scientific Model F-1000 1 liter fermentor vessel and its accessory parts were used



$$\frac{R2}{R1} = \frac{R4}{R3}$$

SENSOR SYSTEM	NV
ROTOR (308)	
radius R1 (mm) R2 ; R3	17.65 ; 20.1
height (mm)	60
STATOR (CUP)	
radius Ra (mm) R1 ; R4	17.3 ; 20.5
RADII RATIO R1/Ra	0.98
SAMPLE VOLUME V (cm^3)	5
TEMPERATURE: max. (°C)	130
min. (°C)	-30

Figure 16. Design figure for the Couette viscometer NV sensor system.

as the batch reactor as shown in figure 17. The batch reactor's temperature was well controlled at 25°C by the control mechanism in the fermentor console through the action of cold tap water in a cooling water jacket and the electrical heating element. Agitation at 150 rpm by the turbine blades on the magnetic drive shaft gave good mixing.

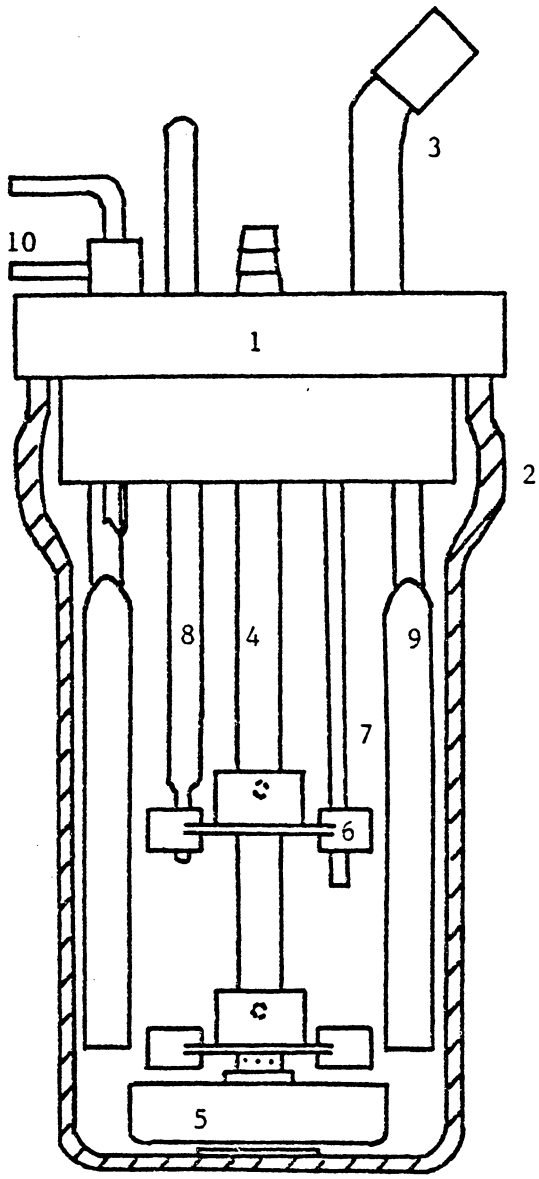
The substrate was a sucrose solution in acetate buffer (pH 5.2) at a concentration of 0.2 M and final volume of 400 ml which was freshly made prior to the kinetic experiment and was fed into the batch reactor. The temperature of this sucrose solution was closely watched and reached 25°C for at least 10 minutes before the enzyme solution was fed into the batch reactor.

The pooled first peak fractions which had a dextransucrase activity of 0.95 U/ml analyzed by Somogyi and Nelson reducing sugar assay and a protein concentration of 0.0125 mg/ml, was the material for this inactivation study. A proper amount of enzyme solution was taken out of the stock which had been stored in the refrigerator at 4°C and had been allowed to equilibrate at room temperature for at least 30 minutes. The non-sheared enzyme reaction and subsequent two sheared enzyme reactions were all conducted on the same day.

For the non-sheared enzyme reaction, a volume of 4 ml of enzyme solution was delivered to the batch reactor through

Figure 17. Diagram of the batch reactor

1. Cap
2. Pyrex glass vessel
3. Sample port
4. Impeller shaft
5. Magnet assembly
6. Impeller
7. Thermocouple well
8. Electrical heating element
9. Baffles
10. Cooling water jacket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its sample port by a pipette, and seven sets of samples of the reaction mixture were taken along the reaction course to monitor the reducing content of the batch reactor.

The procedure for obtaining data along the enzymatic reaction was the following:

(1) All the necessary equipment needed for Somogyi-Nelson reducing sugar assay as stated in the previous section was set up.

(2) Fructose standards solutions were combined with Somogyi copper reagent in test tubes.

(3) All the test tubes were marked with numbers which corresponded to the reaction mixtures at 0, 2, 5, 10, 15, 30 and 45 minutes after the enzyme solution was mixed with sucrose solution. A duplicate of sample was used.

(4) A work sheet was scheduled such that each sample of 1 ml volume of the reaction mixture, taken by the Pipetteman automatic pipette, might be delivered immediately to the corresponding test tube containing 1 ml copper reagent which had been placed in a hot water bath for 5 minutes. The combined solution was then thoroughly mixed by vortex and covered with a glass marble. It was placed back into the hot water bath after mixing, boiled for another 10 minutes, and was taken out of the water bath. All these maneuvers

up to the boiling step took less than 1 minute. Skill acquired through practice made this possible.

(5) After 45 minutes of the reaction, the last set of samples was taken and all the oxidized solutions were cooled down to room temperature and then the Nelson chromagen was added to it.

(6) The absorbance of the standards and samples was read on the spectrophotometer and the results were calculated.

For the sheared enzyme reaction, the viscometer was tuned at 25°C for at least 30 minutes. The speed factor was chosen at 3 which would give a rotating speed of 194 rpm on the rotating bob of the NV system. It also meant that a shear rate of 1046 cm^{-1} was obtained. A 5 ml portion of enzyme solution was loaded into the gap between the cup and bob, and simultaneously the shift lever was pushed into the gate and the timer was set to measure the exposure of time for shear. For a shearing time t = 900 second, the enzyme solution would be given a shear strain($\dot{\gamma}t$) of 9.42×10^5. After this exposure time to shear, the viscometer was stopped, and the sheared enzyme solution was quickly unloaded. A portion of 4 ml of this sheared enzyme solution was delivered to the batch reactor with the mechanical feature and all other parameters such as temperature, agitation speed, sucrose concentration, final volume kept

the same as those of the non-sheared enzyme reaction. The procedure was repeated for shearing time t = 3000 seconds on dextransucrase solution.

This shear inactivation study was conducted in the manner that first sheared the enzyme solution, then monitored its action in the batch reactor. It was found that shear action caused a partial permanent loss of the catalytic power of the enzyme molecules. The result is shown in figure 23 in the next chapter.

There are two critical problems associated with this research that haunted the researcher's mind. The first is the total lack of understanding of the structure of dextransucrase; the second is the inadequate analytical ability to verify and substantiate any structural changes accompanying the dextransucrase molecules after shear action.

I tried to resolve this pair of tacky problems by measuring the UV spectra of the sheared enzyme solution immediately after shear. It is worth mentioning that the UV spectra is the only option remaining available to the researcher. Other more sophisticated methods such as optical rotary dispersion and circular dichorism, light scattering measurement were all not available.

A stock of enzyme solution which was concentrated after the membrane concentration step and which resulted in a dextransucrase activity of 1.59 U/ml and a protein

concentration of 0.02 mg/ml was the subject material for this study and all the later experiments. An appropriate amount of this enzyme solution was transfered into an Erlenmeyer flask to equilibrate with the water bath temperature of the Lab-Line shaker at 25°C for at least 30 minutes. The viscometer was tuned at 25°C and a speed factor of 3 was chosen, but the shearing time was changed from 0 to 300, 900, 1200, and 1500 seconds respectively. A portion of the 5 ml of the enzyme solution was loaded into the NV system each time. The UV spectra of the sheared enzyme solution was taken in a 1 cm quartz cuvette by Varian, model 635 spectrophotometer along 230, 240, 250, 260, 270 and 280 nm against the distilled water as the blank. The sheared enzyme solutions were preserved in separate vials and stored in the refrigerator for next experiment.

The data are shown in figure 24 of the next chapter and the possible interpretations are discussed.

C.3. Remaining enzyme activity vs. shear strain

It was the researcher's intention to generate a correlation curve on dextransucrase that would relate the remaining activity to shear strain. Furthermore, the researcher wanted to compare the relationship between the cases of dextransucrase under shear in the presence of substrate and in the absence of substrate in the hope of finding support for any conformational changes or protection

effect of the dextransucrase molecules on the substrate binding.

For the correlation curve of the dextransucrase solution in the absence of substrate, all the sheared enzyme solutions which had been subjected to the shear at different periods of shearing time in the previous experiment of UV spectra measurement were the material of study. Sixteen 50 ml Erlenmeyer flasks were used as the tiny batch reactors. To each Erlenmeyer flask, a 8 ml portion of freshly made 0.2 M sucrose solution in acetate buffer was allocated. All the Erlenmeyer flasks were marked with numbers corresponding to non-shear, 300, 900, 1200, and 1500 seconds shear and boiled enzyme. Then they were placed in the Lab-Line shaker with its water bath temperature controlled at 25°C and the reciprocal motor dial set at a speed factor of 4.5.

For each value of shear strain, two reactions were run and each reaction generated two data values. A 0.1 ml portion of the enzyme solution was delivered to the appropriate Erlenmeyer flask and the reaction mixture was incubated for 30 minutes. Then a 0.5 ml portion of the reaction mixture was drawn for reducing sugar assay following the standard procedure as stated in section B.5 of this chapter. The sample calculation for this remaining percentage of enzyme activity is presented in Appendix 2, and

the resulting correlation curve is shown in figure 26 in the next chapter.

For the correlation curve of dextransucrase in the presence of substrate, the kinetic data were taken in such a fashion that the enzymatic reaction and the shear action were taking place in the viscometer simultaneously. A portion of 8 ml 0.2 M sucrose in an acetate buffer solution was made and then mixed with 0.1 ml of enzyme stock solution in an Erlenmeyer flask. The mixture was mixed by hand shaking and then quickly loaded to the NV system of the viscometer which had been tuned at 25°C for at least 30 minutes. The speed factor was chosen as 2,3,6,9,8, and 27 each time and it gave a rotating speed of 292, 194, 97, 65, 32, and 21 rpm respectively. The reaction mixtures were incubated and sheared for 30 minutes, then were quickly unloaded, and a 0.5 ml portion was taken for the reducing sugar assay. A zero shear or non-shear basis was done by the reaction taking place in the viscometer with the rotor standing still. Another non-shear reaction was run in an Erlenmeyer flask on the shaker to justify any variation due to the external mass transfer effect for this dextransucrase reaction. The result obtained corresponding to shear strain values of 2.86×10^6, 1.88×10^6, 9.42×10^5, 6.28×10^5, 3.14×10^5 and 2.09×10^5 is shown in figure 27, next chapter. It is surprising that the presence of substrate provides

protection against the shear inactivation.

In order to assure this result, another set of shearing and reacting experiments was conducted under the same experimental conditions but with a heavy load of enzyme. In each of the experiments, 4 ml of enzyme solution was mixed with 4 ml of 0.2 M sucrose in an acetate buffer and the subjected to shear. The reducing sugar contents of the reaction mixtures were determined with a 1/50 dilution factor. The result is shown in figure 28, next chapter.

D. Structural Analysis of Dextrans Synthesized from the Sheared Dextransucrase

It is generally believed that enzyme molecules are very stiff. When the enzyme solution is subjected to hydrodynamic shear, the result is a sharp distribution of the molecular population of the enzymes, i.e., there is nothing between inactivated enzymes (denatured completely) on the one hand, and active enzymes (as active as non-sheared ones) on the other. It was the researcher's purpose to test this consensus by incubating the sheared dextransucrase with sucrose and to verify any structural variations of the synthesized polymer dextrans. If there were some variations in the synthesized dextrans, then I could prove the existence of a sub-denaturation state. That is, among the sheared enzyme populations, there could be some impaired ones that still could carry out a

catalyzed reaction, but might do so with less fidelity. If there were no structural variation in the products, then this research offered a proof of the general thought by eliminating the possible negative.

Another fringe benefit from the outcome of this experiment was a possible clue to the branch mechanism of dextransucrase. If dextransucrase imposed a regulating function through its conformation, and the branch mechanism might be related to the conformation of the enzyme as opposed to being the action of short dextran chains, the structural variation should be specifically branch linkages.

D.1. Ethanol precipitation procedure

To produce enough quantity of dextran, the batch reactor as shown in figure 17 was used again for the enzymatic synthesis of dextran. A 200 ml portion of 0.2 M sucrose solution in an acetate buffer was mixed with 8 ml of the stock enzyme solution having a dextransucrase activity of 1.59 U/ml and then the reaction was carried out at 25°C for 5 hours. At the end of the reaction, the reaction mixture looked turbid with the typical dextran solution opalescent character. The following ethanol precipitation procedure was recommended by A. Jeanes in the same paper which presented the fermentation medium for Leuconostoc mesenteriodes (please refer to section A.1, but the procecure was for the purification of dextran out of growing

culture, so it was adopted but simplified by the researcher. The reaction mixture was poured out of the reactor vessel into a 500 ml Erlenmeyer flask and immediately the flask was set into a boiling water bath for 10 minutes to stop the enzymatic reaction. Then the contents were cooled down to room temperature and mixed vigorously with a stirring glass rod into a beaker containing 1000 ml absolute ethanol. White precipitate of dextran settled out of the ethanol-water solution; it was a gummy, tacky mass. A Millipore filtration unit was set up and loaded with a Whatman No. 3 filter paper with a high retention property. The filter paper had been cut into a circular form with a diameter of 1 5/8" to fit into the filtration set. The whole filtration unit was connected to the water aspirator which provided a vacuum source, and ethanol solution was filtered through. The dextran precipitate was washed with some quantity of absolute ethanol on the filter paper. Then the filter paper with dextran precipitate on it was put on an aluminum pan and placed in a vacuum dessicator. The pan and filter paper had been weighed on the Mettler balance before the precipitation procedure and the weight had been recorded.

From the same stock enzyme solution, a portion of 8 ml dextransucrase was drawn and subjected to shear ($\dot{\gamma}$ = 1046 sec^{-1}) by viscometer for 300 seconds. It was transferred

into the bath reactor for the synthesis of dextran, and reaction conditions were kept the same except that reaction time was longer to compensate for the less catalytic power. Another batch of reaction was carried out for the dextransucrase being subjected to shear for 1200 seconds. The ethanol precipitation procedures were repeated for the dextrans synthesized from the sheared enzymes in the batch reactor.

For the sheared enzyme reaction, the dextran precipitate was so fine that a centrifugation step was needed to agglomerate the particles. The clear ethanol solution was decanted, and then the aggregate of dextran was resuspended in absolute ethanol solution and filtered. A summary of this experiment is in table 7 next chapter.

D.2. Molecular weight distribution of the dextran precipitates

The purpose was to see what difference or variation could be seen about the molecular weight distributions among these dextran precipitates from the previous ethanol precipitation procedure.

The gel chromatography column and all the auxiliary equipment was used as shown in figure 1^3, section B2. The only modification was that the long column of AcA 22 was substituted for two short column in series head to tail. The first column was packed with gel AcA 34, with gel bed height of 21.5 cm which had an effective fractionation range

from 20,000 to 350,000. The second column was packed with AcA 22 but only 24 cm long. The two columns had been equilibrated with distilled water by pump with distilled water through the columns for at least 24 hours. The Uvicord monitor had been changed to a 208 nm lamp and filter. Prior to the administration of the samples, 250 mg of the blue dextrans (nominal M.W. 2.0×10^6) was dissolved in 25 ml distilled water and charged 3 ml portion to the columns for the calibration of the columns. The calibration curve of the blue dextran is shown in figure 29 of the next chapter. The program used on the MultiRac was:

B2

Pump 287

Delay 59

Waste 999

Scale 20

Wait 1

and the level sensor set at:

Below

35

Waste

Then, 50 mg of each dextran precipitate was weighed on a Mettler balance and dissolved in 5 ml distilled water. Then the dextran solutions were charged to the columns. The elution patterns that were recorded on the KLB 2210 Potentiometric recorder are shown in figure 30.

D.3. Intrinsic viscosity measurement

A very sensitive and convenient method to characterize a polymer is to measure the intrinsic viscosity. Taken out from the vacuum dessicator, 80 mg of each dextran precipitate was weighed and dissolved in distilled water. Then the dextran solutions were brought up to the final volume of 10 ml in volumetric flasks. These dextran solutions were sent to the polymer chemistry laboratory for viscosity measurements on Ubbelohde Viscometer at 0.8 g/dl, 0.667 g/dl, 0.5 g/dl, and 0.4 g/dl concentrations through successive dilutions and at the temperature of 25°C. The data on efflux time and intrinsic viscosity are tabulated on table 8 and in figure 31.

D.4. C 13 NMR spectra

In order to solidly verify whether any structural variation occurred on the dextrans synthesized by dextransucrase which had been subjected to shear action for a certain period of time, the C 13 NMR spectra of these dextran precipitates were taken. The limited quantity of the dextran samples precluded any other chemical analyses. The powerful technique of C 13 NMR spectrum provided information at the molecular level which yielded a clear answer to the structural properties of dextrans--if the dextrans were more branched, the C 13 NMR would also clearly reveal it.

The sample preparation procedure for these dextran precipitates was pretty straightforward; 200 mg of each dextran precipitate was weighed on the Mettler balance and carefully fed into the sample tube through a Nalgene small funnel. Each of these 10 mm diameter sample tubes had been thoroughly cleaned by rinsing with acetone and drying in the overn, and then each had been properly marked. After the dextran samples were transferred, 6 ml of D_2O was pipetted into each sample tube and the dextrans were dissolved in D_2O by capping the sample tubes and by vigorously shaking them.

The three prepared samples were then sent to the Analytical Laboratory in the Chemistry Department. The spectra were obtained on the Geol model FX-200 spectrometer equipped with a Teac N808 system in the Fourier-transform-data-processing mode. The temperature of the samples was controlled at 90°C as suggested by Seymour (1979a). The chemical shifts were expressed in p.p.m. relative to external reference of the tetramethylsilane. The resultant C13 NMR spectra of these dextrans precipitates are shown in figure 33, 34, and 35 of the next chapter. Also the computer printouts of the chemical shifts and intensitites of the absorption peaks are listed on tables 10, 11, 12.

E. Recipes, Materials and Suppliers

E.1. Buffer

All the reactions and assays were carried out in 0.02 M

acetate buffer with 3 mM NaN_3 in it. 2.72 g of $NaCH_3COO \cdot 3H_2O$ was weighed and dissolved in distilled water; then the dissolved solution was brought up to a final volume of 1 liter. The pH was adjusted to a value of 5.2 $\pm$ 0.1 by droplets of glacial acetic acid measured by Radiometer PHM63 digital pH meter and Ingold pH electrode. Enough quantity of acetate buffer could be made all at once, autoclaved and stored in a carboy. It could last several months without microbial contamination. However, it should be filtered through a Whatman No. 3 filter paper before use.

E.2. Lowry reagent A

2.0 g of Rochelle salt (sodium potassium tartrate) and 100 g of $NaCO_3$ were weighed, and dissolved in 500 ml 1N NaOH solution. Then the mixture was brought up to a final volume of 1 liter in a volumetric flask by addition of distilled water.

E.3. Lowry reagent B

2.0 g of Rochelle salt and 1.0 g of $CuSO_4$ $5H_2O$ were dissolved in 90 ml of distilled water, then the mixture was added to 10 ml 1 N NaOH. Both reagent A and reagent B are very stable. It stored in polyethene bottles, they remain good up to 6 months.

E.4. Lowry reagent C

One volume of Folin-ciocalton reagent was diluted with fifteen volumes of distilled water. This solution, prepared

daily should have an acidity of 0.15-0.18 N when titrated to pH 10 with 1 N NaOH. If the acidity exeeds 0.18 N, it should be adjusted with NaOH.

E.5. Low alkaline copper reagent

1.2 g of Rochelle salt and 2.4 g of anhydrous Na_2CO_3 were weighed and dissolved in 25 ml portion of distilled water. $NaHCO_3$ was weighed and added to the above solution. Then 0.4 g of $CuSO_4 \cdot 5H_2O$ was weighed and dissolved in a minimum amount of distilled water, and added to the above solution. Another solution was made by dissolving 40.8 g $Na_2SO_4 \cdot 10\ H_2O$ in 50 ml distilled water, then boiled to expel air. All solutions were combined later to make up 100 ml and left to stand for at least 3 days. This copper reagent should be filtered before use.

E.6. Nelson chromagen

2.5 g of ammonium molybdate was weighed and dissolved in 45 ml of distilled water. Then 2.1 ml of 96% H_2SO_4 was pipetted and added to the above solution. 0.3 g of $NaHAsO_4 \cdot 7H_2O$ was dissolved in 2.5 ml portion of distilled water and added to the above. The whole mixture was incubated at 37°C for 24 hours. This chromagen should be stored in an amber bottle and exposure to light should be avoided.

E.7. Materials and Supplies

Material	Source
Water	Distilled in Bellco still, deionized with Bantam demineralizer.
Yeast extract	Difco Laboratories
Trypton	Difco Laboratories
K_2HPO_4	Fisher Scientific Co. Lot No. 762140
Glucose	Fisher Scientific Co. Lot No. 756214
Sucrose	Fisher Scientific Co. Lot No. 792275
$MgSO_4 \cdot 7H_2O$	Baker Chemical Co.
NaCl	Fisher Scientific Co. Lot No. 766293
Ferric citrate	Fisher Scientific Co. Lot No. 761940
$MnSO_4 \cdot H_2O$	Fisher Scientific Co. Lot No. 705110
10 N NaOH solution	Fisher Scientific Co. Lot No. 781706
Antifoam	Arthur H. Thomas Co. Catalog No. 1130-D12
NaN_3	Fisher Scientific Co. Lot No. 763165
$NaCH_3COO \cdot 3H_2O$	Fisher Scientific Co. Lot No. 763723
Glacial acetic acid	Baker Chemical Co. 3-9507
Blue dextran average N.W. 2,000,000	Sigma Chemical Co. Mp. D-5751

Bovine Serum Albumin	Sigma Chemical Co. No. A-4378
Glass cuvette	Fisher Scientific Co.
Fructose	Fisher Scientific Co. Lot No. 774299
Azocall	Calbiochem Co. Lot.No. 510082
Rochelle salt crystal	Fisher Scientific Co. Lot No. 762477
$CuSO_4 \cdot 5H_2O$	Fisher Scientific Co. Lot No. 745735
Quartz cuvette	Arthur H. Thomas Co.
Folin-ciocaltour 2N	Fisher Scientific Co. Lot No. 795431
Na_2CO_3	Fisher Scientific Co. Lot No. 793181
$NaHCO_3$	Fisher Scientific Co. Lot No. 761015
Na_2SO_4	Fisher Scientific Co. Lot No. 791181
Ammonium molybdate	Baker Chemical Co.
96% H_2SO_4	Fisher Scientific Co. Lot No. 783075
$NaHAsO_4 \cdot 7H_2O$	Fisher Scientific Co. Lot No. 753530
D_2O	Pfaltz and Bauer Co.

IV. RESULTS AND DISCUSSIONS

A. Accuracy of the Assays

It is necessary to discuss briefly the accuracy of the assays before presenting other results.

A.1. Lowry protein assay

The standard curve for the Lowry protein assay which is the absorbance value at 650 nm versus microgram Bovine Serum Albumin (BSA) in 1 ml of acetate buffer is shown in Figure 18. The absorbance value should be 0.0 at 0 microgram BSA and 0.34 at 100 micrograms BSA, and should present a straight line after a least square data fitting procedure. An error of $\pm$ 0.02 absorbance units, which corresponds to $\pm$ 5 micrograms of protein, is the maximum deviation. It is worth mentioning that during the assay, all the fractions constituting the first peak of the chromatogram that contained a high concentration of dextransucrase, showed a white precipitate when Lowry reagent A was added, but the precipitate disappeared when reagent C was added. This probably can be explained as a salt-out phenomenon.

A.2. Somogyi-Nelson reducing sugar assay

The standard curve for the Somogyi-Nelson reducing sugar assay after a least square data fitting procedure is shown in Figure 19. It is a straight line from the range of 0 to 100 micrograms fructose. For each kinetic measurement or dextransucrase activity assay, a standard curve was

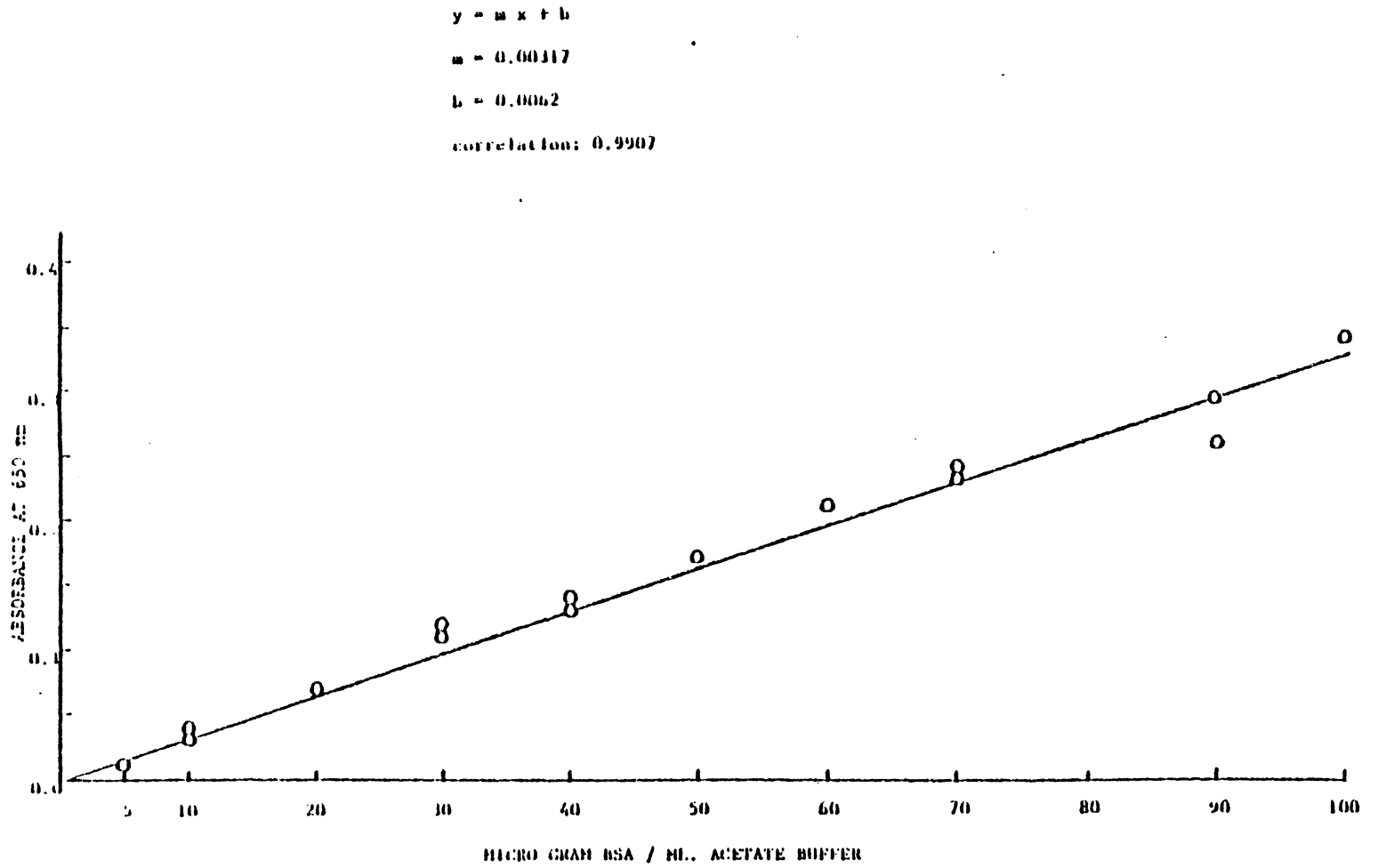

Figure 18. Standard curve for Lowry protein assay.

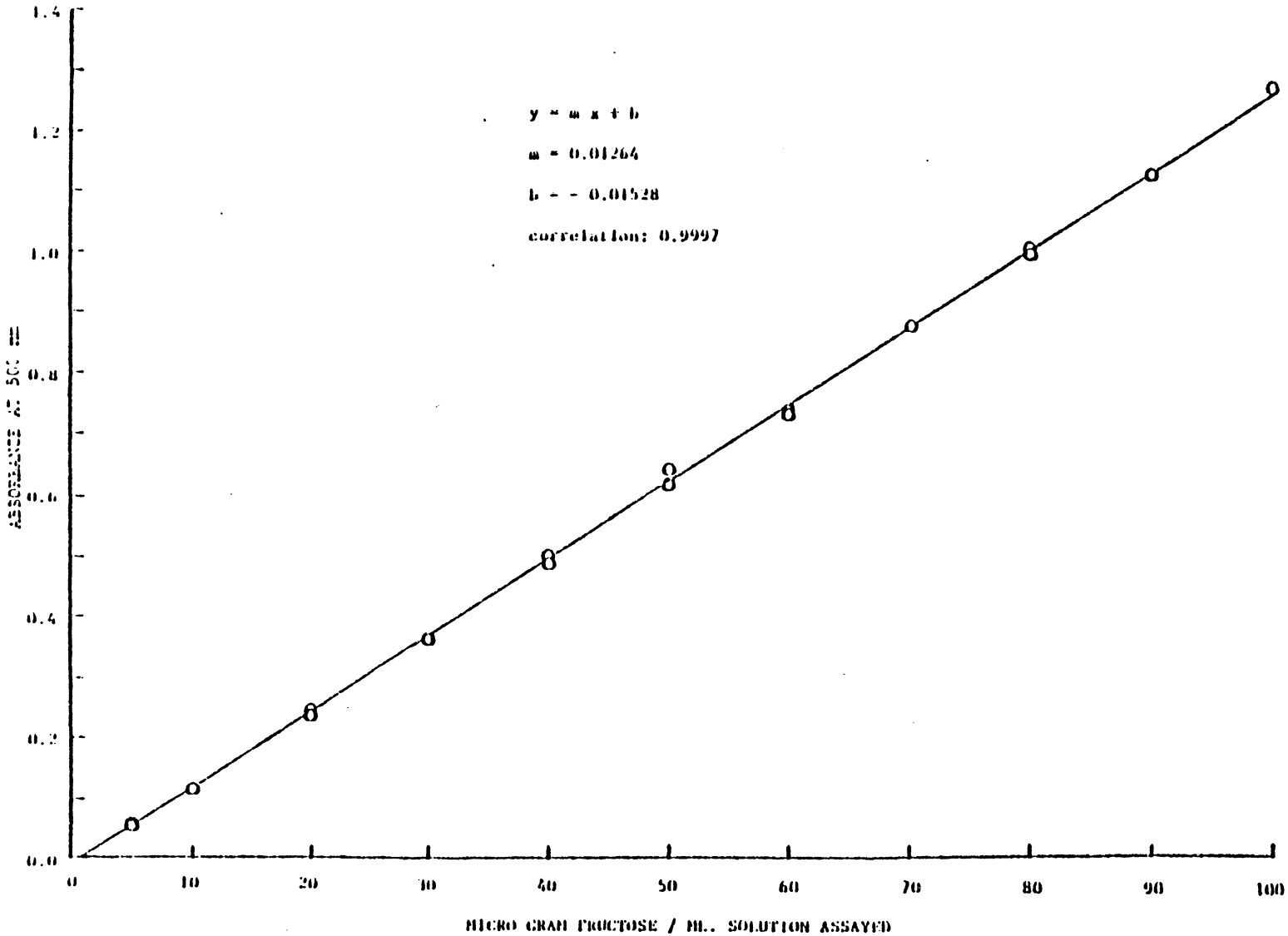

Figure 19. Standard curve for Somogyi-Nelson reducing sugar assay.

constructed and compared to the others. The reproducibility of the standard curve serves as a good indication of the soundness of the data obtained from this assay. The Somogyi-Nelson reducing sugar assay is very sensitive, and its accuracy is within ± 5 micrograms fructose/ml. Two points about this assay should be noted. First, it was found that the sucrose in an acetate buffer solution for the shear inactivation study should always be fresh, because sucrose tends to hydrolyze. If the sucrose solution is stored for a long time, it contains a substantial amount of fructose and glucose, both of which possess reducing power which will interfere with the assay. Second, it was noticed that after adding the Nelson chromagen reagent to the assayed solution, the color did not change for 48 hours and if stored in a refrigerator, precipitate formed, and good mixing was necessary if a measurement was to be carried out.

B. Purification Work of Dextransucrase Preparation

B.1 Enzyme production and purification

The 1.2 liter fermentor production stage for dextransucrase produced 1180 ml of an enzyme containing broth after the bacterial cells were removed by centrifugation. Concentration by ultrafiltration and subsequent dialysis with acetate buffer yielded a dialysate of 260 ml volume. This concentration step resulted in a 78% reduction in volume and an approximately 40% drop in protein content.

For the repeated batches of gel permeation chromatography operation, the chromatogram as monitored by Uvicord at 280 nm is shown in Figure 20. The abscissa is the elution volume which tells how much volume is collected in each fraction. The results of the dextransucrase activity and protein assays on each fraction are shown in figure 21, and a complete summary of the results of the enzyme production and purification is presented in Table 5.

Fractions #3, 4, 5, 6, 7 and 8 constitute the first peak which had dextransucrase activity, and fractions #13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, and 24 constitute the second peak, which was found to contain much extraneous protein, but virtually no dextransucrase activity. One unit of dextransucrase activity is defined as the amount of enzyme that liberates 1 micromole reducing sugar taken as fructose per minute under zero order reaction condition at 25°C. Specific activity can be taken as the index of enzyme purity, which is tabulated in the column of units/mg protein in Table 5. From the specific activity values, the purification factors can easily be calculated and are tabulated in the last column of the same table. The center fraction of the first peak reaches 79-fold purification relative to the original fermentation broth supernatant.

Prior to the dextransucrase purification work on the packed AcA22 gel column, the void volume of this column has

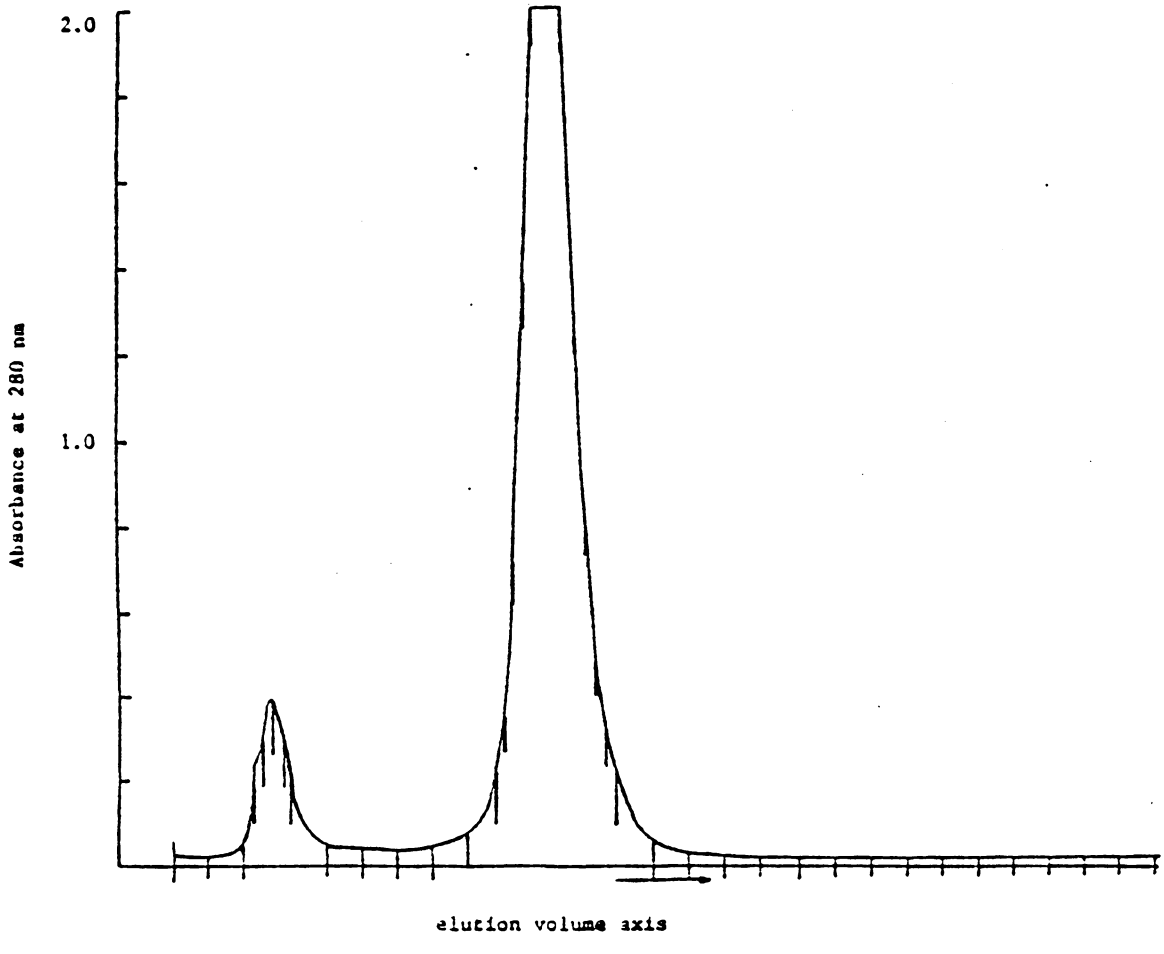

Figure 20. Chromatogram of gel permeation column

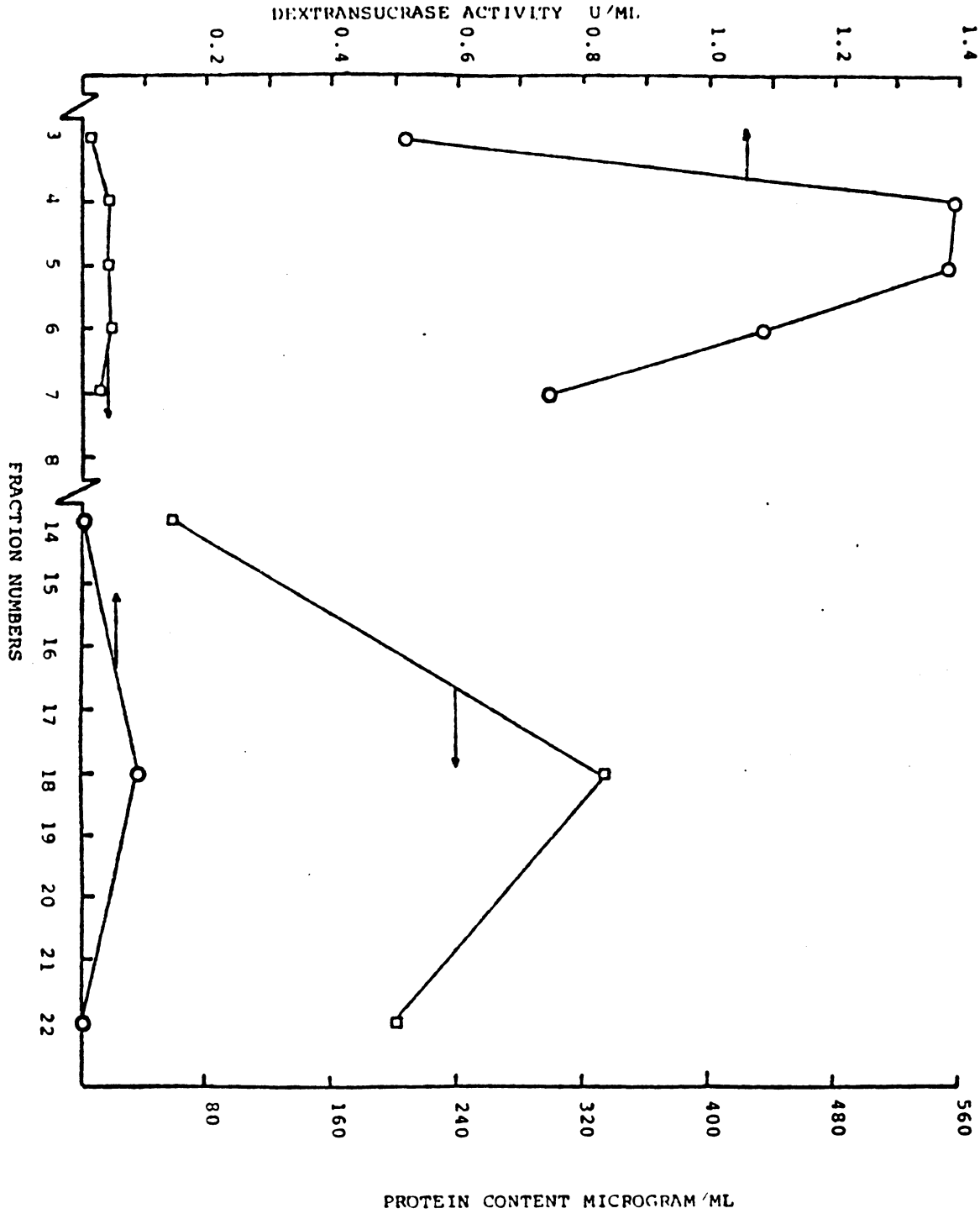

Figure 21. Dextransucrase activity and protein assays on the chromatogram

Table 5. Purification work on dextransucrase preparation

Procedure	Fraction No.	Volume (ml.)	Units / ml	Total units	Protein mg/ ml	Units / mg prot.	Yield %	Purification factor	Remarks
Original Fermentation Supernatant		1180	1.503	1773.5	1.436	1.0467	100	1	
Filtration Retentate		260	3.178	826.3	0.886	3.587	46.6	3.42	
AcA 22 gel column chromatogram	3	10	0.517	5.17	0.0065	79.54	0.3	75.98	
	4	5	1.39	6.95	0.0167	83.23	0.4	79.52	
	5	5	1.38	6.90	0.0175	78.86	0.4	75.34	
	6	5	1.09	5.43	0.0170	63.94	0.3	61.09	
	7	5	0.75	3.75	0.0120	62.42	0.2	59.63	
	8	3.5	0.99	3.46	0.0104	94.90	0.2	90.66	
	14	5	0	0	0.0573	0	0.		
	18	5	0.098	0.49	0.339	0.289		0.276	
	22	5	0	0	0.206	0	0.		

been determined to be 55.3 ml by blue dextran solution (M.W. of 2 x 10^6) taken as the effluent volume corresponding to the half-height of the leading edge of the solute peak. The elute volume of dextransucrase on the column was about 60 ml. Hence dextransucrase was found near the void volume of this AcA22 gel column and may have an effective size larger than 1.2 x 10^6 (the upper limit of the gel's fractionation range) as opposed to the 280,000 expected of the nascent enzyme. This is most likely because the dextranase treatment on the enzyme solution before charging to the gel permeation column as conducted by Robyt and Walseth (1979), was not performed in this work, and dextransucrase has often been reported to be closely associated with dextran molecules. This enzyme-dextran complex would have a rather high effective molecular weight and could be the reason that the enzyme was found near the void. A similar elution pattern was obtained by P. Reihly (1980) using a AcA34 gel column and applying a parallel purification scheme. The pooled fractions which comprise the first peak had a collective volume of 300 ml and a dextransucrase activity of 0.95 U/ml. After performing a hollow fiber membrane concentration on the pooled enzyme solution, the volume was reduced to 180 ml and enzyme activity value increased to 1.59 U/ml.

B.2. Contaminating enzyme tests

Tests were carried out on the pooled fractions to detect any contaminating enzyme activity as suggested by Robyt and Walseth (1979). The results are listed in Table 6.

Table 6

RESULTS OF ENZYME PURITY TESTS

Possible Contaminating Enzymes	Results
Protease	Negative
Dextranase	Negative
Levansucrase	less than 2% of the reducing sugar liberated

There was no indication of protease or dextranase activity, even after prolonged incubation (3 and 16 hours, respectively). Analysis of raffinose solution which had been incubated with the enzyme preparation for 16 hours did suggest the presence of levansucrase or invertase, but at a very low level. Raffinose is a tri-saccharide with a structure of O-α-D-galactopyranosyl-(1→ 6)-o-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside. The products from levansucrase action would present levan (β-2,6-fructan) and melibiose (O-6-α-D-galactopyranosyl-D-glucose) while products from invertase action would present fructose and melibiose. Either one would be detected as an increase of reducing

sugar released: No attempt was made to determine which particular enzyme was present. However, the presence of the undesired enzyme did cause some unexpected results in the shear inactivation study and from the C 13 NMR spectrum of the synthesized products, it is concluded that the contaminating enzyme is levansucrase. Based on the increase of the reducing sugar content of the test experiment, it is calculated that levansucrase liberated 187 μg reducing sugar/ml-hr contributing less than 2% of the reducing power of the dextransucrase activity of the enzyme preparation, which is within the error range of the Somogyi-Nelson reducing sugar assay (± 5%). So it renders no significant interference to the initial reaction rate measurements. The presence of a small quantity of levansucrase in the purified dextransucrase preparation should bear some special notice and its signficance will be discussed in section F of this chapter.

C. Shear Inactivation Study

C.1. Non-sheared enzymatic reaction

In order to observe how the enzyme molecules behave if subjected to a shear field, I ran a non-sheared enzymatic reaction which serves as the basis of comparison for the sheared enzyme reactions. The purified dextransucrase solution that assayed 0.95 U/ml was reacted with 0.2 M

sucrose solution in a batch reactor, and the reducing content of the reaction mixture was monitored. The results are shown in Figure 22. As stated in the literature, it was found that the reducing content increases linearly with time in the early stages. This agrees with the known kinetics of dextransucrase at the sucrose concentration, i.e. 200 mM, at which the reaction is zero order. The reducing content tapers off in the later stages along the reaction coordinate. It was reported that when the dextranosyl chains become longer, the rate of reaction of dextransucrase with sucrose slowed down (Ebert and Schenk, 1968; McCabe and Smith, 1973). This was explained by the assumption that small polymer chains in the reaction complex (ESPn) grow more rapidly than do larger ones. However, the author feels that this is better explained by the external masss transfer effects. For instance, the substrate sucrose must diffuse into the dextranosyl boundary layer, which remains attached to dextransucrase, in order to adsorbe on the enzyme molecule for the insertion polymerization to occur. In other words, the author proposes that there are two distinct regions along the enzymatic reaction as seen in Figure 22. One is the reaction controlled region that plays the dominant role at the beginning of the reaction, the ohter is the diffusion controlled region that is more important as the polymerization reaction goes on.

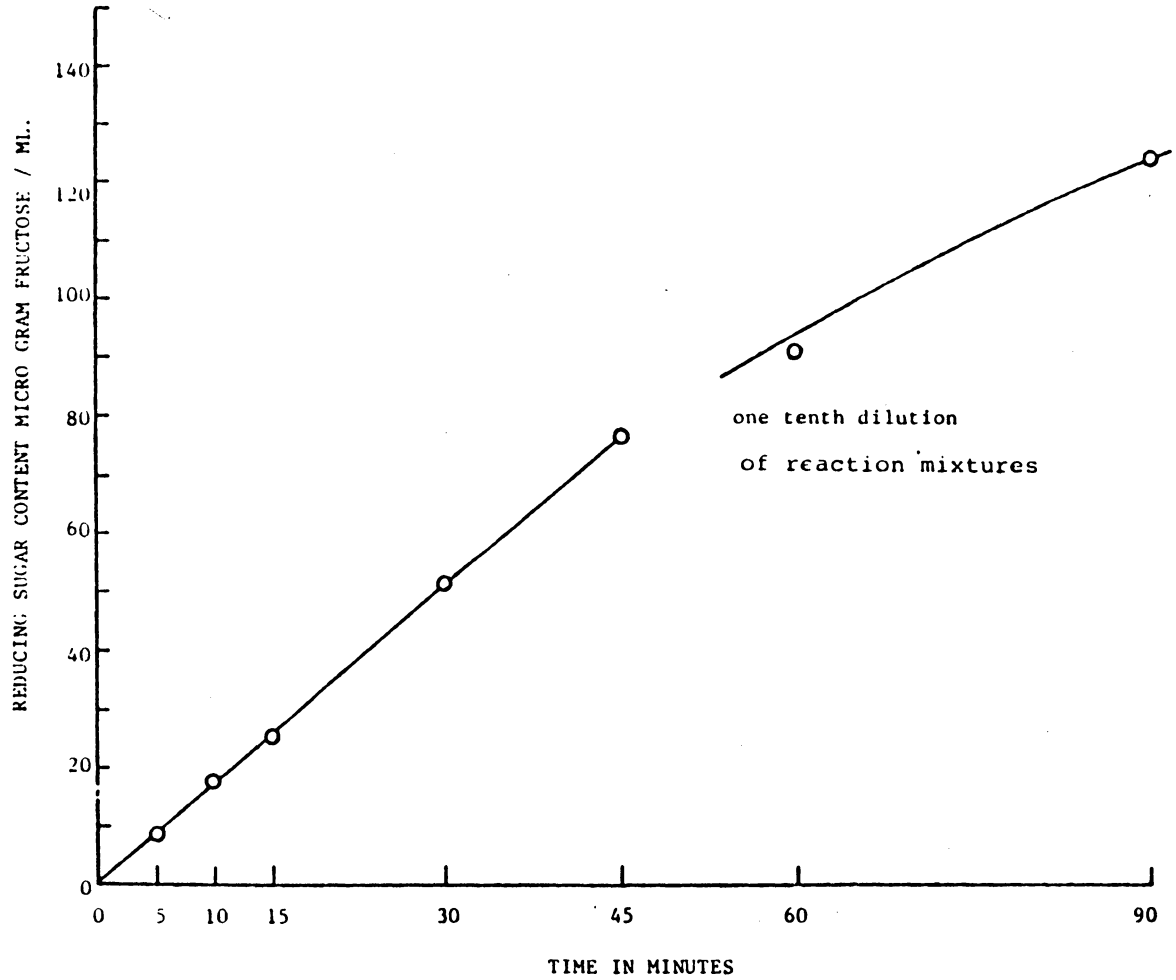

Figure 22. Non-sheared enzymatic reaction of dextransucrase

One might argue that the tapering off of the reducing content is the result of fructose accumulation such that the end product inhibits the reaction rate. A simple estimation of the fructose concentration will reveal that in 60 minutes, dextransucrase will liberate 41.04 mg fructose in the whole batch reactor corresponding to a fructose concentration of 5×10^{-4}M. Tsuchiya et al.(1955) studied the fructose inhibition effect and found that fructose in equimolar amounts with sucrose only decreases the reaction rate by approximately 20 percent. The effect of fructose is apparently not a sound explanation.

This proposal is supported by the observation that the reducing content of a dextransucrase reaction can reach only one third of the value it would have if the reaction was taking place in the thin annular space between the stagnant bob and cup of the viscometer rather than in a 50 ml Erlenmeyer flask that is shaking vigorously in a shaker. Further confirmation of this external mass transfer effect is the author's data of the shear inactivation study of a reacting system which is subjected to a shear field simultaneously. The results are shown in Figure 28. The rate of reaction increases 24% over the non-sheared rate if the rotor of the viscometer is moving at such a speed that it will reduce the diffusional resistance. So the limiting mass transfer phenomenon takes preference over shear inactivation in this case.

C.2. Enzymatic reactions of shear inactivated enzymes

The enzyme solutions which had a dextransucrase activity of 0.95 U/ml and a protein concentration of 0.0125 mg/ml, were sheared in a Couvette type of viscometer at a shear rate of 1046 sec^{-1} for 900 seconds and 3000 seconds, then were transferred into a batch reactor at the same temperature (25°C) to react with sucrose solution at a concentration of 0.2M. A non-sheared enzyme reaction was also performed to ensure the grounds of comparison. The reducing contents of the reaction mixture were monitored up to 45 minutes. These results are shown in Figure 23. It should be pointed out that the data taken 2 minutes after the dextransucrase was added to the batch reactor showed some deviation from the linearity that was expected as in Figure 22. The reason for this low reducing sugar content might be that dextransucrase is not yet well mixed in the reactor, and samples might be drawn from a point in the reaction mixture where dextransucrase was not present.

As shown in Figure 23, shear inactivation of dextransucrase causes a partial loss of activities, as illustrated by the slopes of the curves. Too long a shear action will tend to inactivate the enzyme totally as shown by the 3000 seconds shear data. It is thought that the shear field causes the molecular distortions that result in breaking molecular bonds, hence the loss of catalytic activity. At

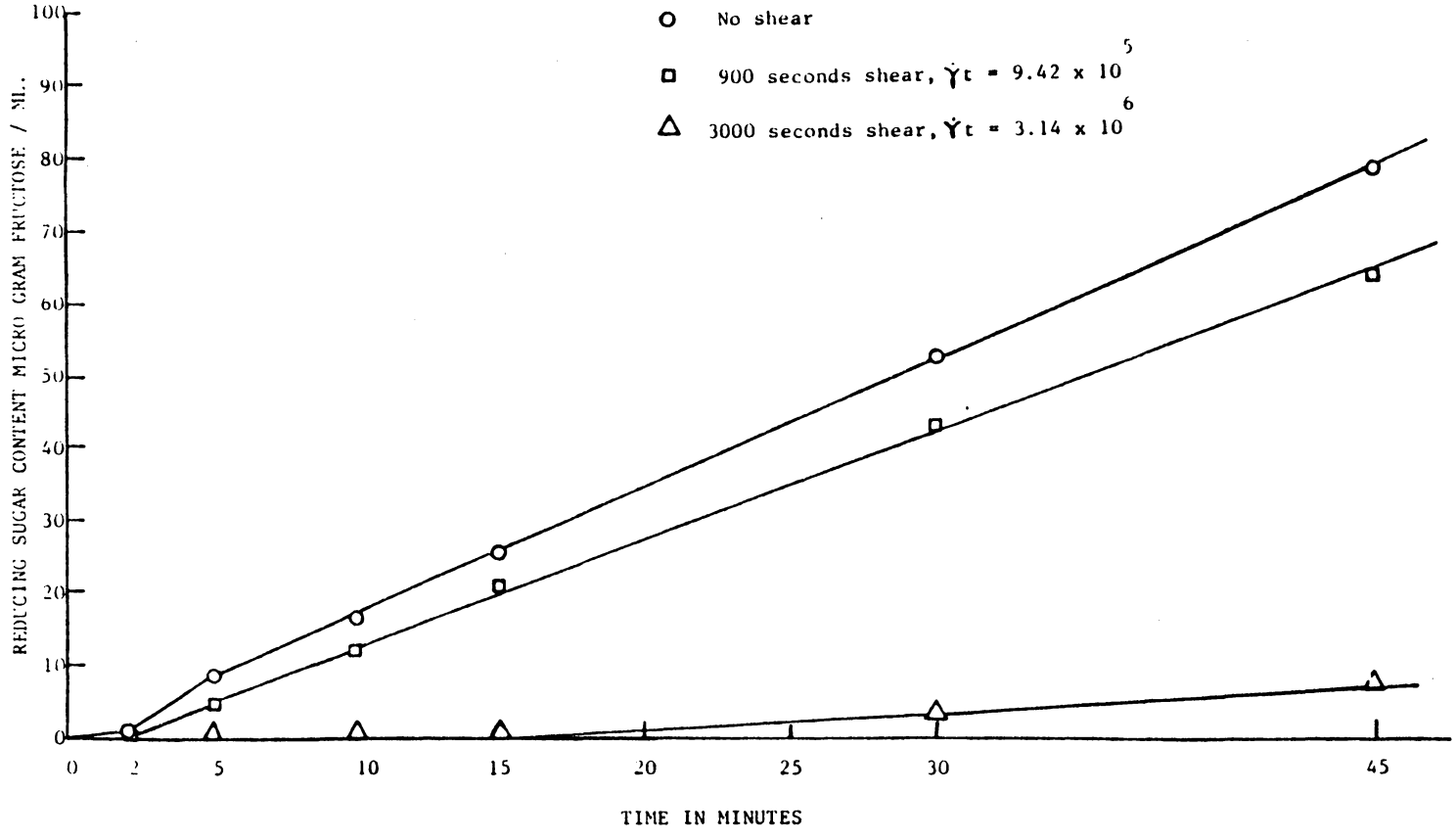

Figure 23. Shear effect on dextransucrase activity

certain shear forces (or shear rate), a longer shear action will cause a larger molecular population to cross the bond breaking energy barrier, and this results in inactivation. The limiting case will be total inactivation or total denaturation by shear.

C.3. UV spectra of the sheared dextransucrase

To verify any structural changes in the enzyme molecule by the applied shear forces, the measurement of UV spectra of the enzyme solutions which had a protein concentration of 0.02 mg/ml was conducted immediately after shear ended. The results are shown in Figure 24. As stated in the previous chapter, the critical problem that I was faced with was the lack of adequate analytical ability to quantify and study the structural changes of enzyme molecules accompanying the shear action. UV spectra remains the only option for me. Ultraviolet absorption by peptides is caused by electronic transition in the protein molecule. Although the data is not quantitative enough to explain how the enzyme molecule deforms, it serves as a good indication that the sheared enzyme molecules have different structures. By looking at the data, one may argue that there is not much difference between the absorbance values of the different sheared enzyme solutions and the non-sheared one. It is true that the difference of absorbance is small, from 0.4 absorbance unit to 0.2 absorbance unit, and the error bar will

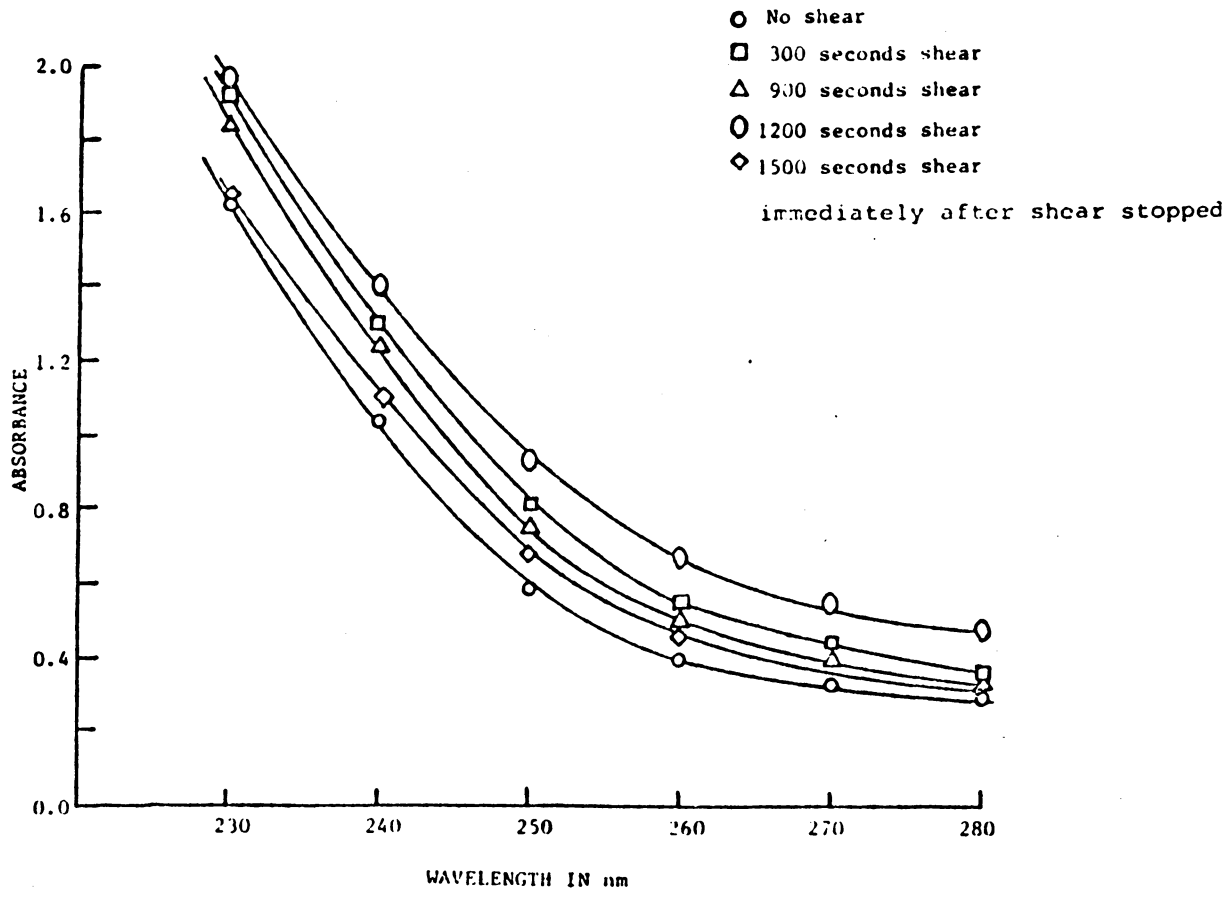

Figure 24. Ultraviolet absorption spectrum of dextransucrase immediately after shear ended.

cover a ± 0.02 unit range. Another argument is the spectrum curves that represent enzyme solutions subjected to shear for different periods of shearing time all show higher absorbance values than the non-sheared one, but the position from the non-sheared enzyme spectrum did not match the shear history that dextransucrase has been through. These arguments are hard to answer, especially since the researcher does not know anything about the native structure of the dextransucrase molecule, nor is there any information about it in the literature. But by referring to some published data on the UV spectra of protein solution addressing the same question of shear induced structural change in enzyme molecules, the author thinks this data should not be discredited. One of them is the ultraviolet absorption spectra of lactic dehyrogenase samples at the concentration of 0.080 mg/ml with various shear histories as reproduced in Figure 25 (Tirrell and Middleman, 1978). The maximum difference among them is less than 0.3 absorbance units, and the disposition is not necessarily proportional to the shear history.

D. Comparison of Intactivations Between the Presence and Absence of Substrate

D.1. Correlation curve of the percentage of remaining activity vs the shear strain

A correlation curve of the percentage of the remaining

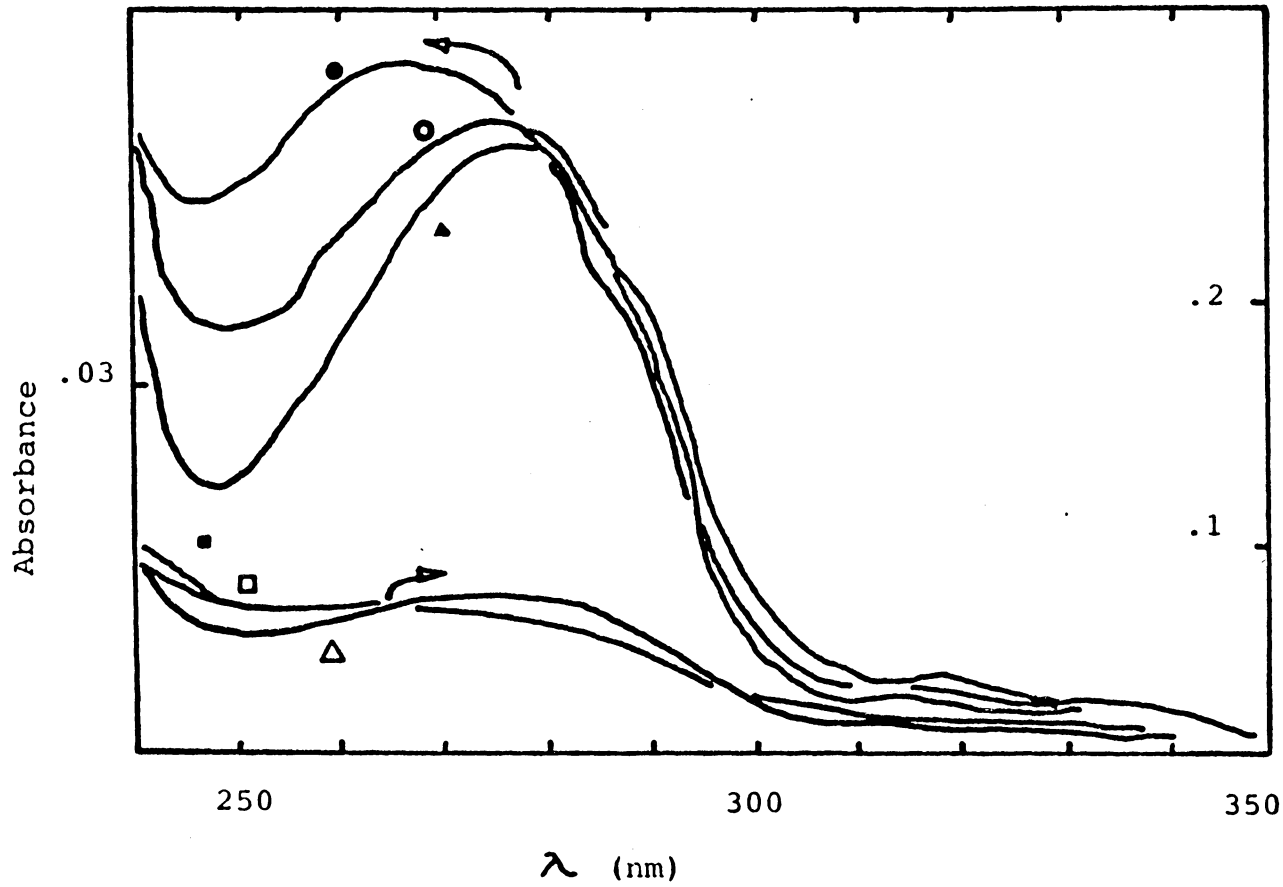

Figure 25. Ultraviolet absorption spectrum of lactic dehydrogenase with various shear histories. Reproduced after Tirrell and Middleman, Biotechnology and Bioengineering, vol. 20, 1978, p. 609.

catalytic activity and the dimensionless group, shear strain, was obtained. This correlation serves to check whether such a correlation exists as stated in the literature. Figure 26 shows this correlation based on data on the loss of enzyme activity after subjecting to shear for different periods of time. The reference is the non-sheared enzyme reaction. A log-log scale is used, and the general trend of this curve resembles what is reported in the literature, although numerical values of different enzymes are different. It should be noted that when shear strain is less than 10^5, there is little inactivation.

Another point worth noting regarding the inactivation in two enzyme preparations, is the discrepancy between what is revealed in Figure 23 and the correlation curve in Figure 26. The first enzyme solution is more dilute, which assayed at 0.0125 mg/ml protein and a dextransucrase activity of 0.95 mg/ml protein and a dextransucrase activity of 0195 U/ml. At $\gamma t = 9.42 \times 10^5$, it has 80% of its remaining activity as illustrated by its slope in Figure 23. The second enzyme solution was prepared by an additional membrane concentration procedure and it had 0.02 mg/ml protein concentration and a dextransucrase activity of 1.59 U/ml. At the same shear strain, dextransucase retains only 36% of the original activity on Figure 26. The author thinks the reason for this discrepency is the difference of enzyme

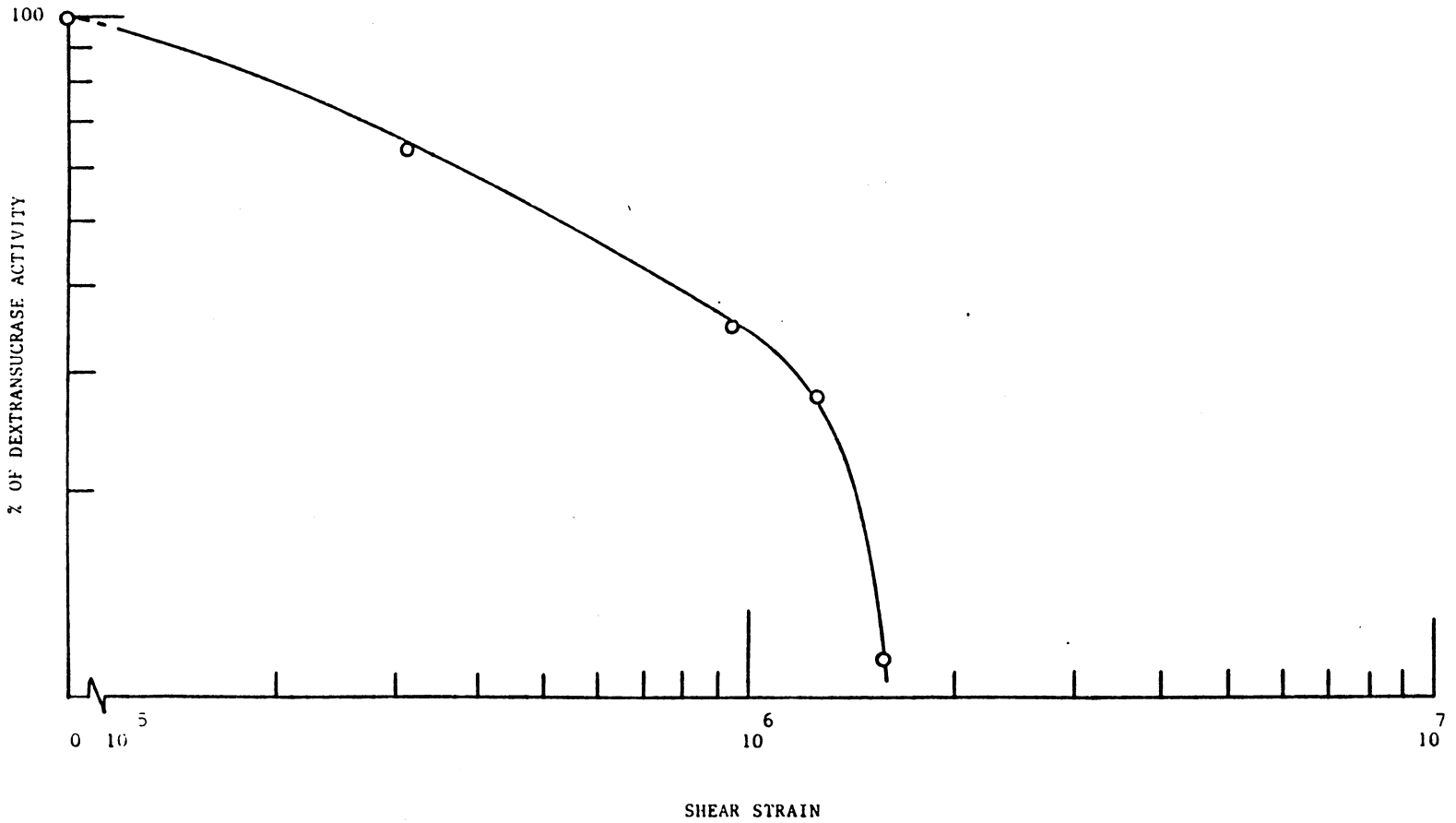

Figure 26. Correlation curve of the percentage remaining activity versus shear strain in the absence of sucrose.

concentrations. Though at this level of dilute enzyme solution, interactions between the enzyme macromolecules such that entanglements were experienced, is unlikely, but the fact that an enzyme solution of higher concentration is more sensitive to shear inactivation is conceivable. Though there is not much concern about the effect of enzyme concentrations in the literature on enzyme inactivation, the author thinks it is worth noting in the future.

D.2. Protective effect against shear inactivation on substrate binding.

Probably the most interesting result of this research is the correlation between the percentage of remaining activity of the same dextransucrase solution versus the shear strain, but the data are taken on the reacting system in the presence of sucrose while simultaneously applying the shear force on it. The results are shown in Figure 27. The non-uniformity of the data may come from the uncontrollable state of mixing of the small portion of enzyme, which is 0.1 ml, in an 8 ml volume of sucrose solution. To magnify and ensure this observation, another reacting system was tested with a heavier load of enzyme. The results are shown in Figure 28. Both correlation curves for the remaining dextransucrase activities in the presence of sucrose are plotted against the non-sheared enzyme reaction taking place in the viscometer as the reference. The increase of activity in Figure 38 at the shear strain less than 10^6 is explained

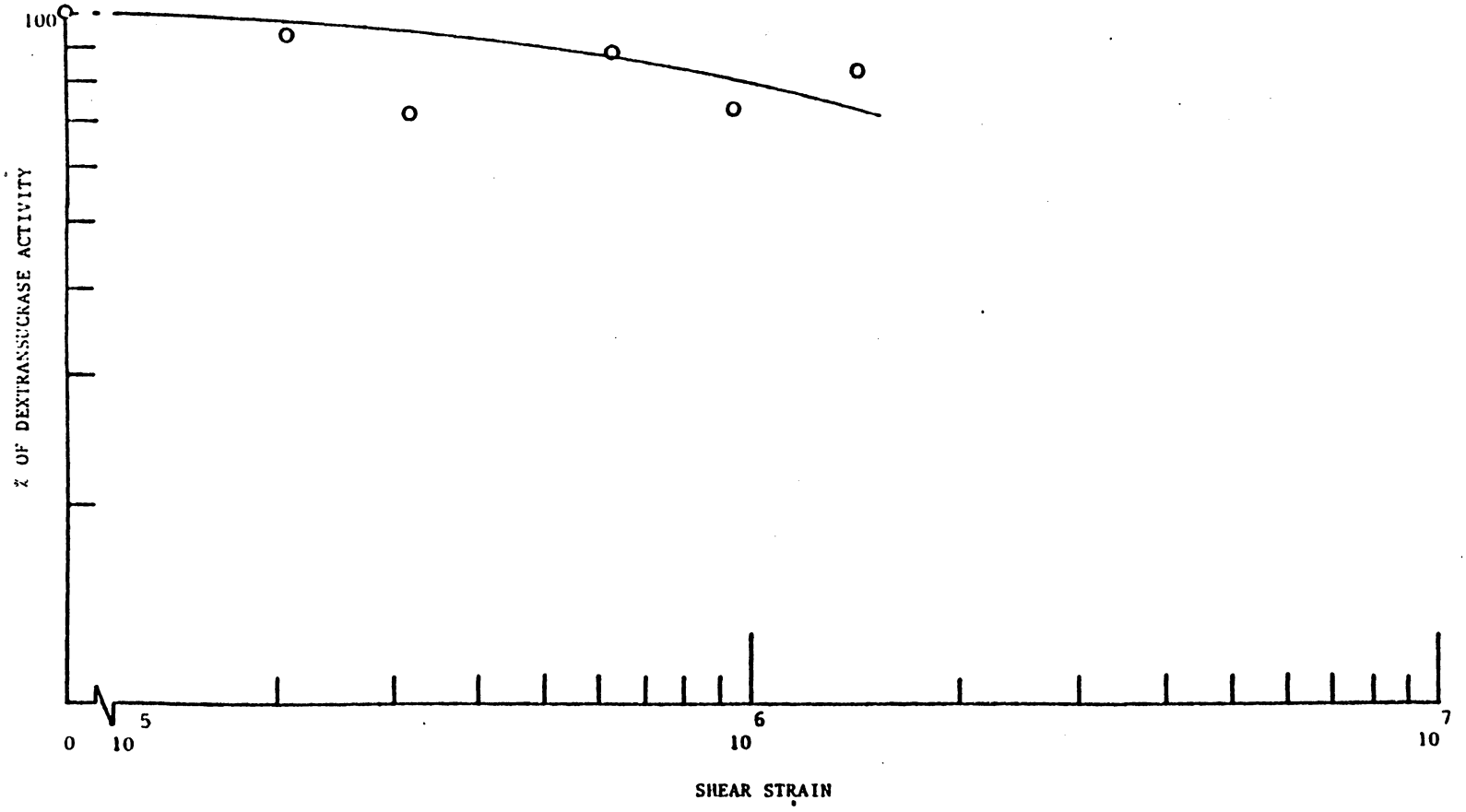

Figure 27. Correlation curve of the percentage remaining activity versus shear strain in the presence of sucrose.

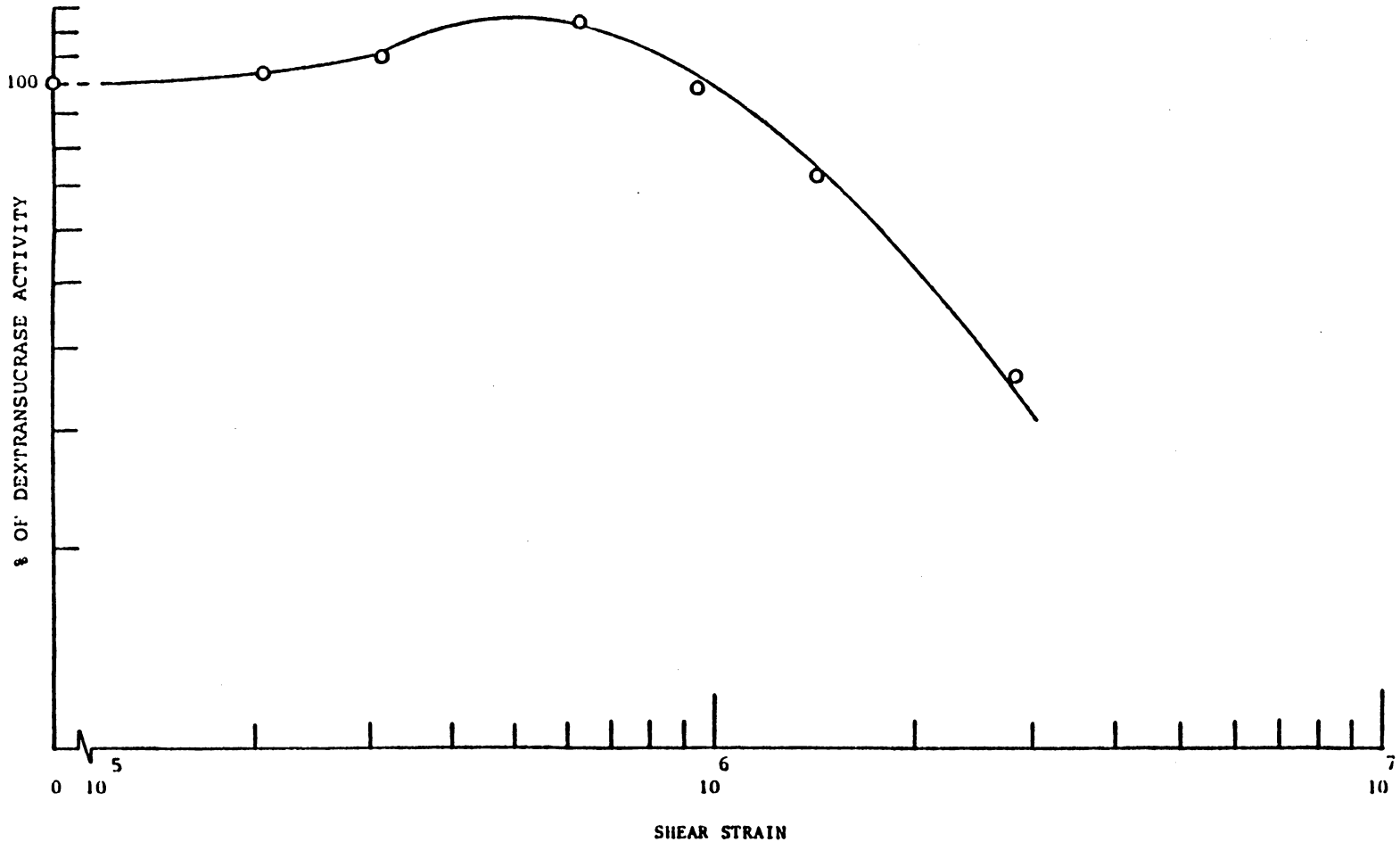

Figure 28. Correlation curve of percentage remaining activity versus shear strain in the presence of sucrose with heavier load of enzyme.

by the idea that external mass transfer process plays a more important role than shear inactivation.

A comparison of the results displayed on Figures 26, 27, 28 leads us to an observation: shear inactivation on dextransucrase is less severe in the presence of sucrose than in the absence of sucrose.

As a matter of fact, the results from the reacting and shearing systems are quite contrary to what has been stated in the literature, and what one would expect. Tirrell (1977) in his Ph.D. thesis compared the percent urease activity after shearing as a function of shear strain in the presence and in the absence of urea. The presence of urea caused more inactivation, though he did not offer any explanation for this finding. As stated earlier, since the growing dextran is an integral part of dextransucrase, and the whole complex will have a large molecular weight at the very beginning of the reaction, one would expect more severe effects by shear on the enzyme because shear action is sensitive to the molecular weight of the subject. (Harrington and Zimm, 1965) Yet the restults in Figure 27 and 28 show much flatter curves, which indicate less severe shear inactivations. To explain this observation, the most reasonable account is the protective effect of substrate binding. There are several reports in the literature about the enzyme conformation changes upon substrate binding; examples are lactic dehydrogenase with pyruvate binding

and NADH binding. There are also reports about the protective effect of substrate presence against shear inactivation (Vorob'ev and Kukhavea, 1965). Myosin solutions, subjected to shearing in a Couette device, in the presence of ATP, showed increasing rate of ATP cleavage (up to 120% increase over zero shear rates with increasing shear rate up to 4500 second^{-1}, but if myosin is sheared in the absence of ATP it loses activity. Similar results were reported for actomyosin.

With this protective effect on sucrose binding in mind, the results strongly endorse Ebert and Schenk's hypothesis on dextransucrase insertion mechanism (1968). They hypothesized that during most of the time, the reaction complex is in the form of sucrose-dextransucrase-dextran, and there are many coordination bonds between the enzyme and the sucrose molecule as well as the growing dextran chain as shown in Figure 7 in Chapter II. The interaction between them forms on internal frame-like structure which tends to stabilize the whole complex against deformation upon exposure to an external shear field. The explanation gets additional support from the results on the high pressure enzyme kinetics of dextransucrase (Greulich and Ludwig, 1977; 1978). They found that the free enzyme was denatured under pressure, but the enzyme-substrate complex was stable. They also found large volume changes on sub-

strate binding and this contributed to the substrate induced conformational changes of the dextransucrase complex.

E. Dextran Synthesized from Sheared Dextransucrase and Structural Analysis on It

I analyzed the structures of the dextran polymers produced by incubation with partially shear-inactivated dextransucrase in the hope of finding any variations, so that a clear answer could be obtained on the imposed question of any possibility of a sub-denature state among the molecular populations after subjecting them to an applied shear field. Another minor purpose was to try to find some clues to the branch mechanism of the dextransucrase.

E.1. Yield of high molecular weight dextran from ethanol precipitate procedure

The experiments of the dextrans synthesized by sheared dextransucrases are summarized on Table 7. For the 300 seconds shear action on dextransucrase, reaction time was 8 hours and two successive reactions were performed in order to have enough sample for the subsequent analysis by C 13 NMR spectrum. For the 1200 seconds shear action on dextransucrase, the catalytic activity retained by the enzyme was 27% of the non-sheared one, the reaction time was 21 hours and three successfive runs were performed. As stated in the previous chapter, the ethanol precipitate

Table 7. Reaction conditions for the enzymatic synthesis of dextran from dextransucrases of different shear histories.

Shear rate	Shear time	Catalytic power	Temperature	Enzyme load	Reaction time
1046 sec^{-1}	0 second	100%	25°C	8 ml	5 hrs.
1046 sec^{-1}	300 seconds	63.7%	25°C	8 ml	8 hrs.
1046 sec^{-1}	1200 seconds	27%	25°C	8 ml	21 hrs.

High Molecular Weight dextran quantity	% Yield on ethanol ppt.
0.43203 g	66.7% out of theoretical dextran synthesized
0.178275 g	27.5%
0.090333 g	13.8%

procedure was modified from what was developed by A. Jeanes(1965). The original procedure was used to isolate dextran from the cell-free culture fluid; dextran precipitated out at the ethanol concentration of 35%. Dr. Jeanes, then, completed the precipitation by increasing ethanol concentration gradually to 42-45%. The molecular weight of the dextran precipitate was estimated to be 130×10^6 by the light-scattering technique. The procedure I used was a one-step procedure which precipitated out crude dextran from the reaction mixture at the ethanol concentration of 83%, together with a subsequent filtration procedure. From the literature on molecular weight distribution of dextran by the ethanol or methanol precipitation method (Tsuchiya, et al., 1955), 50% ethanol solution should precipitate dextran of molecular weight of 50,000. So at 83% ethanol concentration, any dextran with molecular weight 50,000 or larger will definitely precipitate out and be present in the yield calculation of high molecular weight dextran on ethanol precipitate. The observation of the reduction of % yield on ethanol precipiation is an interesting one. Does shear inactivation of the dextransucrase cause the enzyme to synthesize a larger amount of oligosaccharide or a small molecular weight dextran (< 50,000) instead of a high molecular one? One may argue that the reduction of high molecular dextran

is the consequence of a change in the enzyme to substrate ratio. The assumption is that an active enzyme concentration is represented by the catalytic power of the enzyme solution; this assumption proved to be true later in this study. The [E] active/[S] ratio reduced to 0.637 and 0.27 of the original value of the non-sheared enzyme reaction from one sheared enzyme reaction to another. But what is stated in the literature about the effect of enzyme to substrate ratio is opposite to this observation. Tsuchiya and co-workers (1955) found that at different sucrose concentrations, lower enzyme to substrate ratios would increase the yield of high molecular weight dextran by dextransucrase synthesis. So the factor of enzyme to substrate ratio is not the most logical explanation for the results, though a satisfying explanation of it is still not available at this stage.

E.2. Molecular weight distribution of the dextran precipitate

Because molecular weight is such an important parameter characteristic of the synthesized polymer, the molecular weight distributions of dextran precipitates were determined. The gel chromatography columns were composed of AcA 34 and AcA 22 which had the fractionation range of 20,000 to 350,000 and 100,000 to 1.2×10^6 respectively. Prior to the sample application, a calibration curve by

high molecular weight blue dextran solution (nominal M.W. 2×10^6) was obtained and is shown in Figure 29. Then the dextran precipitates were dissolved in distilled water, and were charged to the gel permeation column to observe their elution patterns. The results are shown in Fig. 30.

The dextran precipitate of non-sheared enzyme reaction does show a higher average molecular weight since the peak edge elutes earlier than the dextran precipitates from the sheared dextransucrase. These elution patterns do elicit more confidence in the yield measurements of the high molecular weight dextrans on the ethanol precipitation procedure presented in Table 7. As discussed in the previous section, the possible factor of changes of active enzyme to substrate ratios cannot account for this reduction of molecular weight of synthesized dextran. Another interesting observation is the broader distribution of the dextran from the non-sheared enzyme reaction compared to the more defined, sharper distribution of the dextrans from sheared dextransucrase. The forthcoming question will be: can I attribute this exclusively to the shear inactivation effect? or are other possible factors responsible for this observation? For example, what if the synthesized dextrans have different structures? I think it is more meaningful to expalin and discuss my findings about the molecular weight distribution, viscosity data and ^{13}C NMR spectra in conjunction with each other.

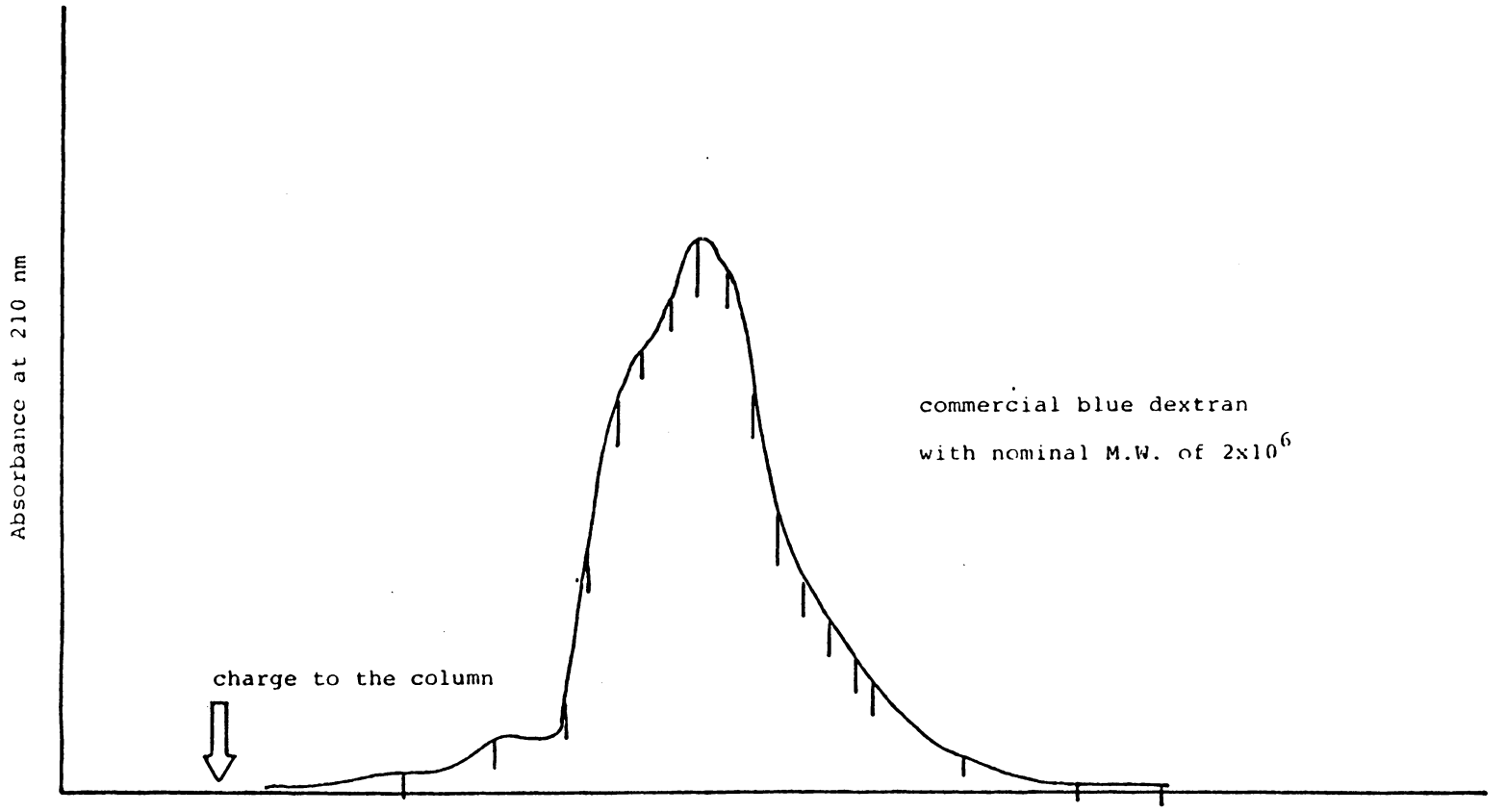

Figure 29. Calibration curve on gel permeation columns by high molecular wieght blue dextran.

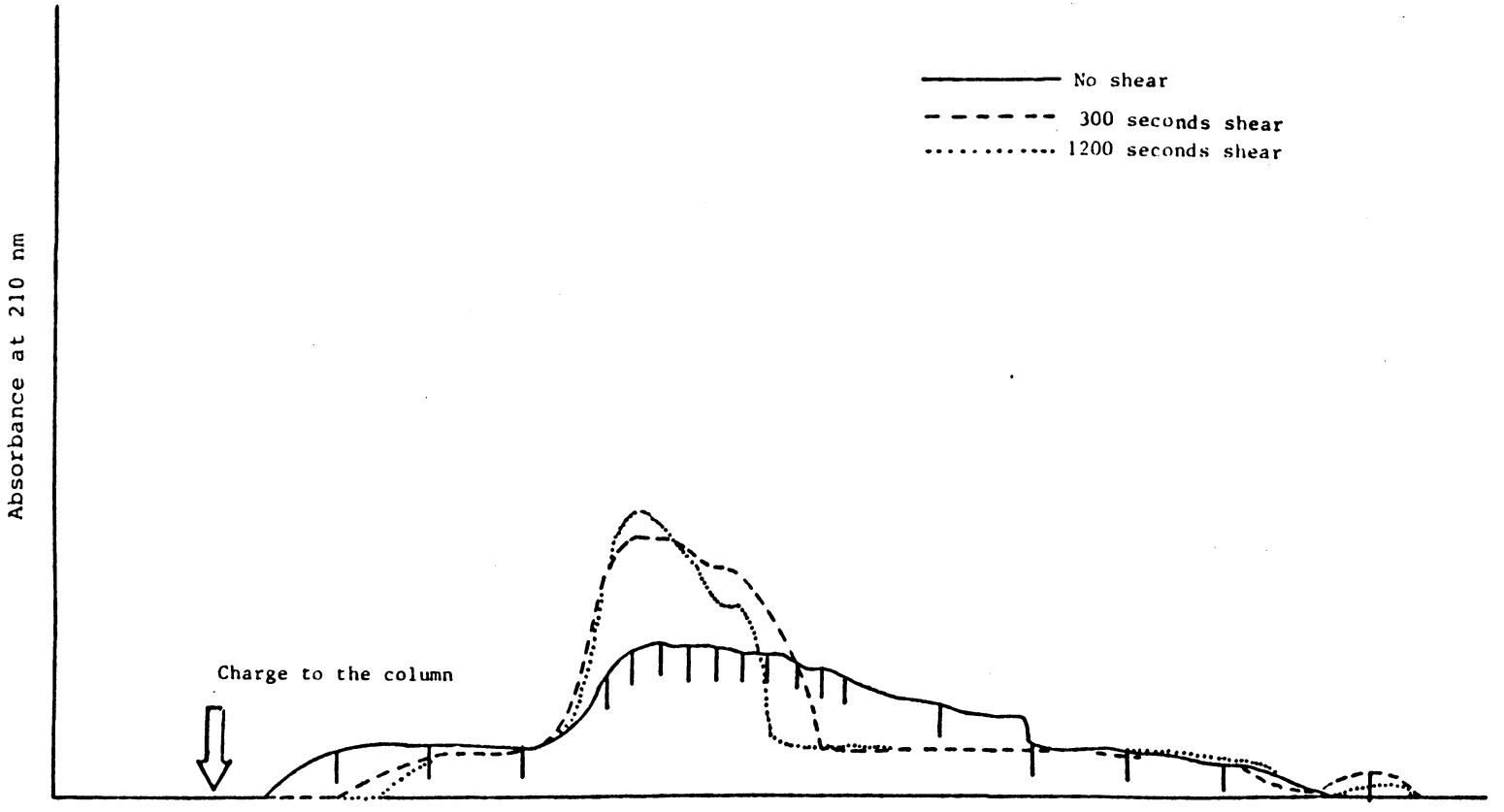

Figure 30. Gel permeation column elution patterns of the dextran precipitates synthesized by dextransucrases of different shear histories.

E.3. Viscosity data and C 13 NMR spectra

Intrinsic viscosity data was obtained on these dextran precipitates in distilled water, and the result is shown in Table 8 and Figure 31. The intrinsic viscosity [η] is the ratio of specific viscosity η_{sp} to the concentration c at infinite dilution:

$$[\eta] = \lim_{c \to o} \frac{\eta_{sp}}{c}$$

where $\eta_{sp} = (T - T_o)/T_o$

T: efflux time of the polymer solution

T_o: efflux time of the solvent

the linear plots follow fairly well what has been reported in the literature as the Huggins equation:

$$\eta_{sp}/c = [\eta] + k^1[\eta]^2 c$$

The intrinsic viscosity [η] for dextran synthesized from non-sheared enzyme is 0.238 dl/g, while [η] for sheared enzyme dextrans are 0.694 and 0.672 respectively.

The [η] of the native dextran from the non-sheared enzyme synthesis is rather low compared to what is reported in the literature. Values of the intrinsic viscosity of B-512 dextran (water, 25°C) ranged from 0.953 to 1.332 depending on the average molecular weight of the dextran (A. Jeanes, 1954). Wales et al. (1953) studied the intrinsic viscosity--molecular weight relationship of

Table 8. Intrinsic viscosity data of dextran precipitates

Content	Efflux time T	Average T	T_0	η_{sp}	η_{sp}/C
For the dextran precipitate from unsheared dextransucrase:					
solvent	111.4				
	111.2		111.3		
0.800 g/dl	465.5				
	463.5	464.5	111.3	3.1734	3.9668
0.667 g/dl	345.9				
	345.3	345.6	111.3	2.1051	3.1561
0.500 g/dl	248.5				
	248.5	248.5	111.3	1.2327	2.4654
0.400 g/dl	206.5				
	205.8	206.2	111.3	0.8527	2.1318
For dextran precipitate from dextransucrase under 300 seconds shear:					
solvent	111.4				
	111.2		111.3		
0.800 g/dl	259.8				
	259.6	259.7	111.3	1.3333	1.6666
0.667 g/dl	223.2				
	224.5	223.6	111.3	1.0090	1.5127
0.500 g/dl	183.6				
	183.0	183.3	111.3	0.6469	1.2938
0.400 g/dl	163.7				
	164.6				
	163.9	164.1	111.3	0.4744	1.1860
For dextran precipitate from dextransucrase under 1200 seconds shear:					
solvent	111.3				
	111.3		111.3		
0.800 g/dl	224.8				
	225.1	224.95	111.3	1.0211	1.2764
0.667 g/dl	196.8				
	197.0	196.9	111.3	0.7690	1.1529
0.500 g/dl	169.2				
	169.0	169.1	111.3	0.5193	1.0386
0.400 g/dl	154.7				
	154.9	154.8	111.3	0.3908	0.9770

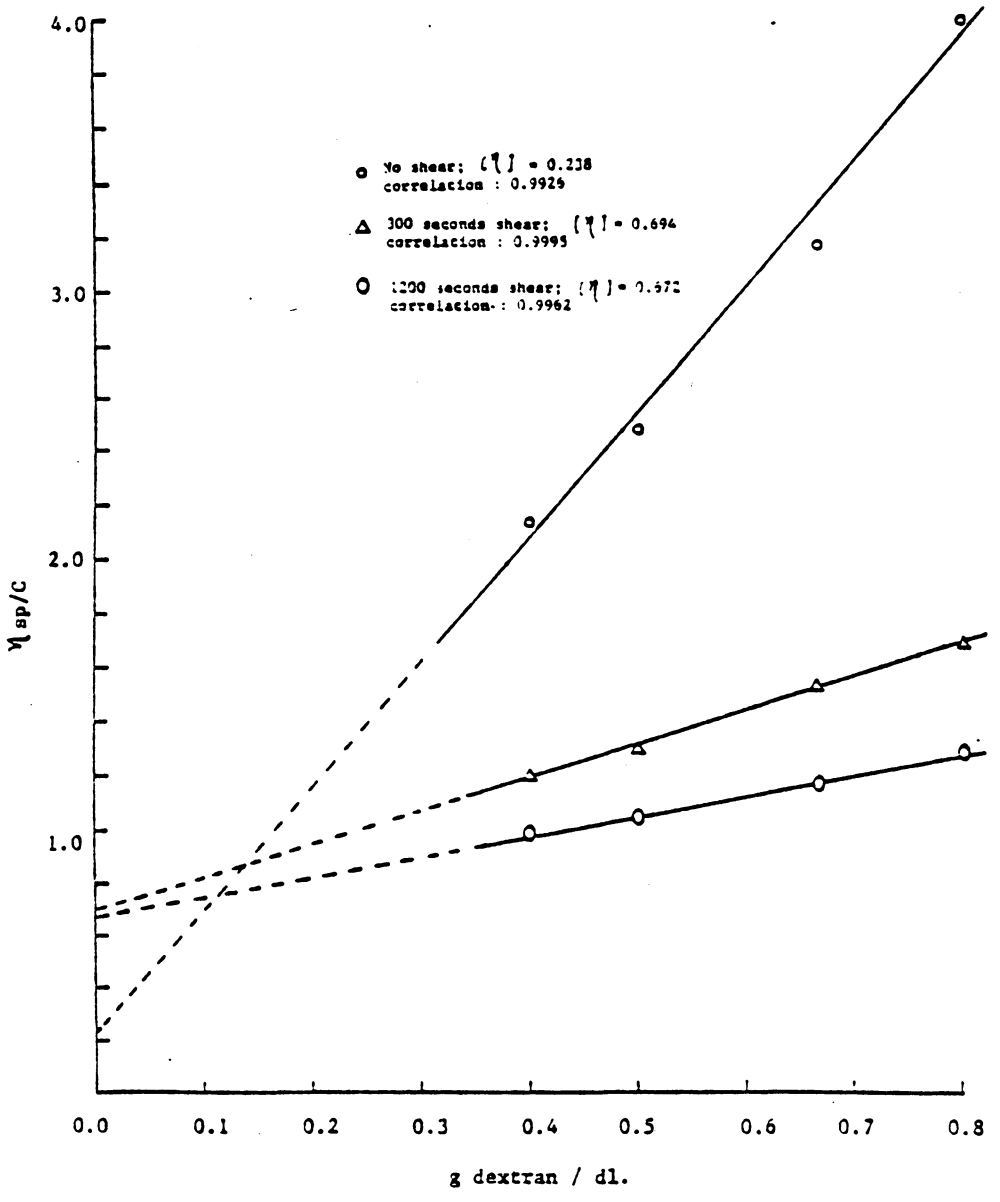

Figure 31. Intrinsic viscosity data of dextran precipitates synthesized by dextransucrases of different shear histories.

dextran B 512 intensively. Based on their data, the value 0.238 dℓ/g should correspond to an average molecular weight of 5.6×10^4, which is far less than what I observed and resolved on the gel permeation column. The observation that dextran precipitates from sheared enzyme synthesis have higher values of intrinsic viscosities (0.694 dℓ/g and 0.672 dℓ/g) though they have smaller molecular weights imposes another question; it it due to the structural variations? By invoking the Mark-Houwink relationship,

$$[\eta] = KM^a$$

one would try to justify the observation by attributing it to the changes in the exponent values, a, which implies a structural change of the polymer. Or is there any possible explanation? In order to find a more logical explanation, I think C 13 NMR spectra need to be brought up in order to yield solid information on the dextran precipitates' structure at the molecular level.

The ^{13}C NMR spectra of these dextran precipitates at 90°C were obtained, as shown in Figures 33, 34, and 35, and the computer print-outs of detailed descriptions of the position (ppm) and intensity of each resonance peak of the spectrum are listed in Tables 10, 11, and 12. In order to substantiate and verify the published resonance peaks of a linear B-512 dextran, and to ensure that the dextran precipitate from the non-sheared dextran-

sucrase synthesis has the same molecular structure as it should be, a commercial dextran of molecular weight 80,000 from the Sigma company was dissolved in D_2O to make a concentration of 50 mg/ml, and the C 13 NMR spectrum was obtained at 90°C by a different spectrometer. The result is shown in Table 9 and in Figure 32.

By examining the spectrum of the commercial B 512 linear dextran in Figure 32 and the spectrum of the dextran precipitate from non-sheared dextransucrase reaction in Figure 33, the striking similarity though not exactness between the two in the resonance peaks' position and the relative intensity, proves the validity of the C 13 NMR spectroscopy technique. The small differences in the δ (ppm) may be attributed to the differences of operation conditions by two different spectrometers.

Referring to the published data on dextran C 13 NMR spectrum the resonance peaks of the spectrum of dextran precipitate from non-sheared dextransucrase are analyzed and interpreted (Jennings, et al. 1974). The 99.309 ppm is assigned to carbon 1 of the hypothetical 1,6-di-O-alkylated glucopyranosyl residue which is the repeating unit of the linear backbone of this dextran polymer, 74.956 ppm peak to carbon 3, 72.970 ppm peak to carbon 2, 71,802 ppm peak to the carbon 4, 71.393 ppm peak to the carbon 5, and 67.539 ppm to the carbon 6 of the pyranoside ring. These

Table 9. C 13 NMR spectrum of the commercial dextran (m.w. 80,000). The spectrum was taken at 90°C in D_2O with a sample size of 50 mg dextran/ml D_2O. Tetra methyl silane was used as an external reference.

Total Peak Nos. 6

Number	Designation	Frequency (Hz)	PPM	Intensity
1	A	2896.1181	99.2902	0.4713
2	B	3508.7585	74.9308	0.4700
3	C	3558.3496	72.9589	0.4786
4	D	3588.8671	71.7455	0.4630
5	E	3598.0224	71.3815	0.5184
6	F	3693.3898	67.5895	0.3187

Peak A was assigned to C1 resonance of the 1,6-di-O-substituted-α-D-glucopyranosyl residue, Peak B was assigned to C3 resonance, Peak C to C2, Peak D to C4, Peak E to C5 and Peak F to C6.

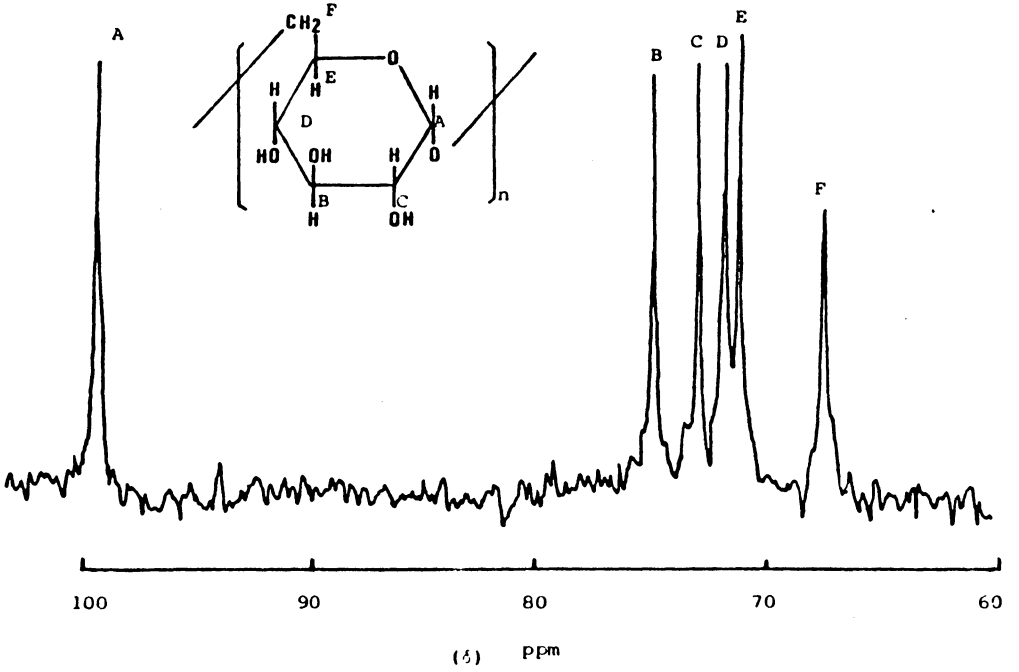

Figure 32. ^{13}C NMR spectrum of commercial dextran from Sigma Company in D_2O at 90°C.

Table 10. C13 NMR spectrum of the dextran precipitate from dextransucrase with no shear history in D_2O at 90°C.

Total Peak Nos. 9

Number	Designation	Frequency (Hz)	PPM	Intensity %
1	A	4983.59	99.309	1741
2	B	3761.50	74.956	1781
3	C	3661.86	72.970	1671
4	D	3603.24	71.802	1781
5	E	3582.73	71.393	1815
6	F	3389.30	67.539	1445
7	Im	2952.63	58.837	1044
8	U	2032.41	40.500	5989
9	Im	918.77	18.308	1232

The major resonance peaks designated as A, B, C, D, E and F are the characteristic peaks of linear dextran. U stands for an unknown peak, but it is not in the region of interest.

Im stands for impurity.

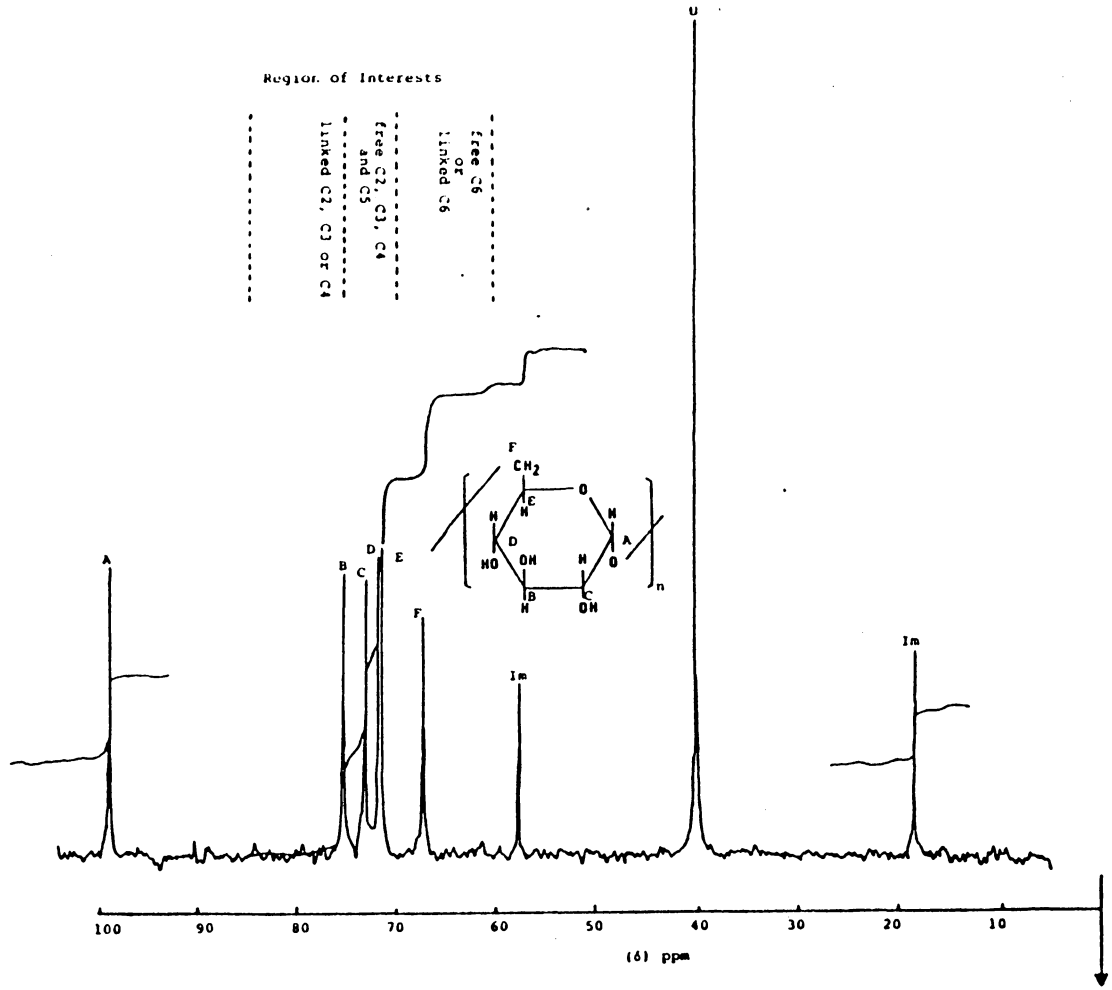

Figure 33. ^{13}C NMR spectrum of the dextran precipitate from dextransucrase with no shear history in D_2O at 90°C.

six typical resonance peaks of the linear dextran backbone made of α,-1→6 glucoside linkages then are labeled as peak A, B, C, D, E, and F on Figure 33. The extra peaks of 58.837, 40,500, and 18.308 ppm are hard to interpret. Because two peaks∿58 ppm and∿18 ppm remain in the following two spectra in Figure 34 and 35, they may be assigned as impurities which originated in the D_2O solvent and are designated as Im. The 40.500 ppm peak is an unknown peak designated as U. But these extra peaks are not meaningful in the carbohydrate spectrum, because as stated in section F of Chapter II, along the upfield of the spectrum, only four regions are of special interes: 1) the 60-70 ppm region, containing both free and linked C6 resonance, 2) the 70-75 ppm region containing the free C2, C3 and C4 resonance and the pyranoside ring C5 resonance, 3) the 75-85 ppm region containing linked C2, C3 and C4 resonance and, 4) the 95-103 ppm region containing linked anomeric (C1) resonance. Only the resonances in these four regions which are characteristic of polysaccharide resonance will yield significant information about the structural features of the dextran precipitates.

The analysis of this spectrum yielded a solid insurance that the dextran synthesized by the dextransucrase of my production and purification procedure would have the same structure as that stated in the literature, namely a high proportion of linear linkage in α1→6 linkages (95%) with

Table 11. C13 NMR spectrum of the dextran precipitate from dextransucrase with 300 seconds shear history in D_2O at 90°C.

Total Peak Nos. 18

Number	Designation	Frequency (Hz)	PPM	Intensity %
1	A	4980.65	99.250	9544
2	m	4429.69	88.271	898
3	L	3943.20	78.576	888
4	m	3793.74	75.598	816
5	B	3758.57	74.897	8937
6	m	3726.33	74.255	1112
7	m	3711.68	73.963	898
8	m	3679.44	73.320	1302
9	C	3658.92	72.912	9138
10	D	3600.31	71.744	9521
11	E	3579.80	71.335	9050
12	F	3386.37	67.480	7869
13	L	3251.56	64.794	971
14	L	3122.61	62.224	1198
15	Im	2949.70	58.779	6824
16	m	2272.72	45.288	800
17	m	1135.64	22.630	805
18	Im	915.84	19.250	6346

The major resonance peaks designated as A, B, C, D, E and F are the characteristic peaks of linear dextran. Those minor peaks are designated as m, their significance is minimum considering signal-to-noise ratio. Three minor peaks are designated as L, which may be assigned to resonance peaks of levan.

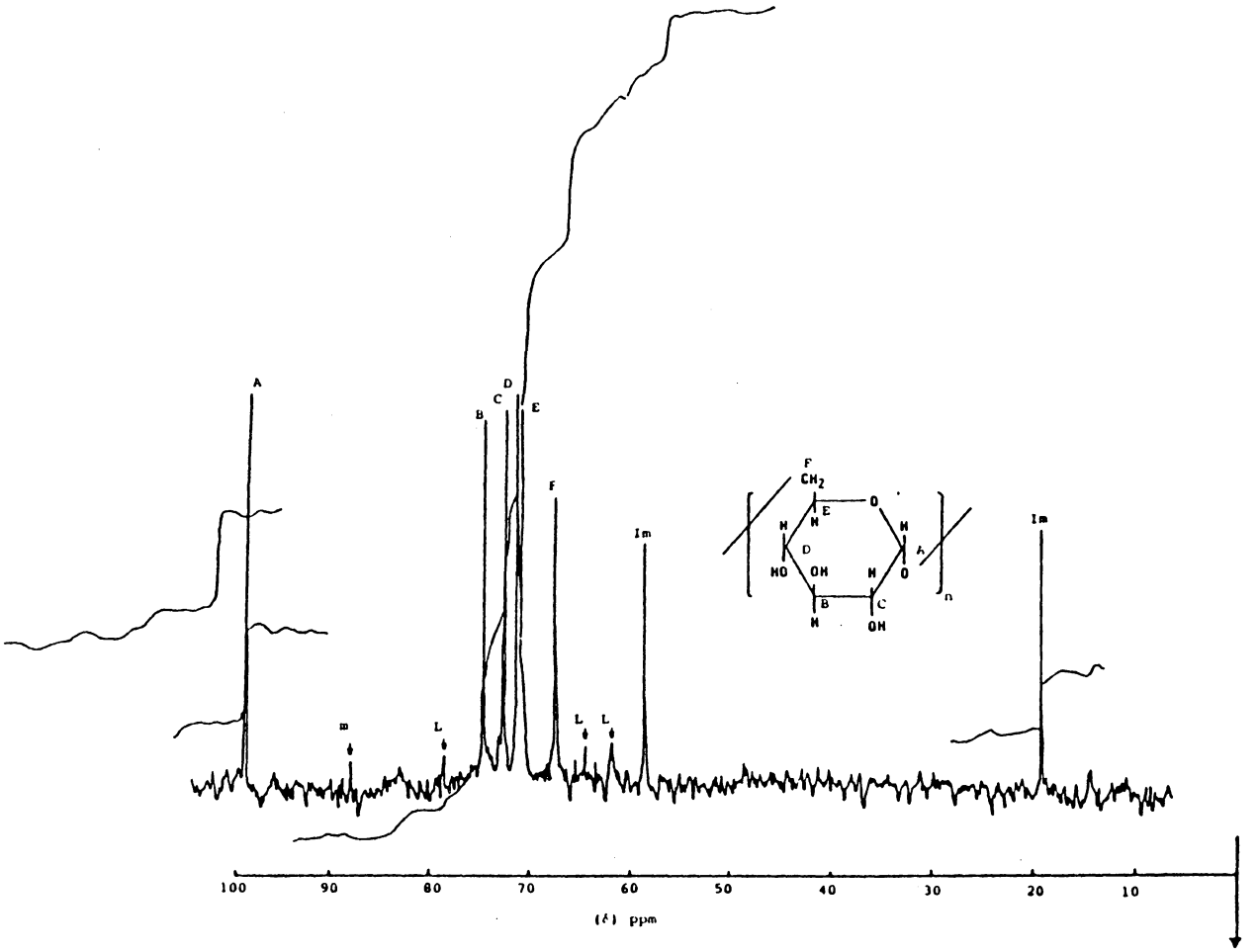

Figure 34. ^{13}C NMR spectrum of the dextran precipitate from dextransucrase with 300 seconds shear history in D_2O at 90°C.

Table 12. C 13 NMR spectrum of the dextran precipitate from dextransucrase with 1200 seconds shear history in D_2O at 90°C.

Total Peak Nos. 18

Number	Designation	Frequency(HZ)	PPM	Intensity%
1	L	5291.30	105.441	2577
2	A	4977.72	99.192	8220
3	m	4429.69	88.271	1081
4	L	4092.66	81.555	1648
5	L	3934.41	78.401	1666
6	L	3878.72	77.292	1115
7	m	3861.14	76.941	1524
8	B	3758.57	74.897	8126
9	m	3673.58	73.204	1545
10	C	3658.92	72.912	8477
11	D	3597.38	71.685	8202
12	E	3576.87	71.276	8405
13	F	3383.44	67.422	6681
14	L	3248.63	64.736	1604
15	L	3125.54	62.283	1986
16	L	3116.75	62.108	2012
17	Im	2949.78	58.779	6268
18	Im	912.91	18.191	6928

A, B, C, D, E, and F are the major resonance peaks of linear dextran.

L stands for levan resonance peak.

m stands for minor peaks.

Im stands for impurity.

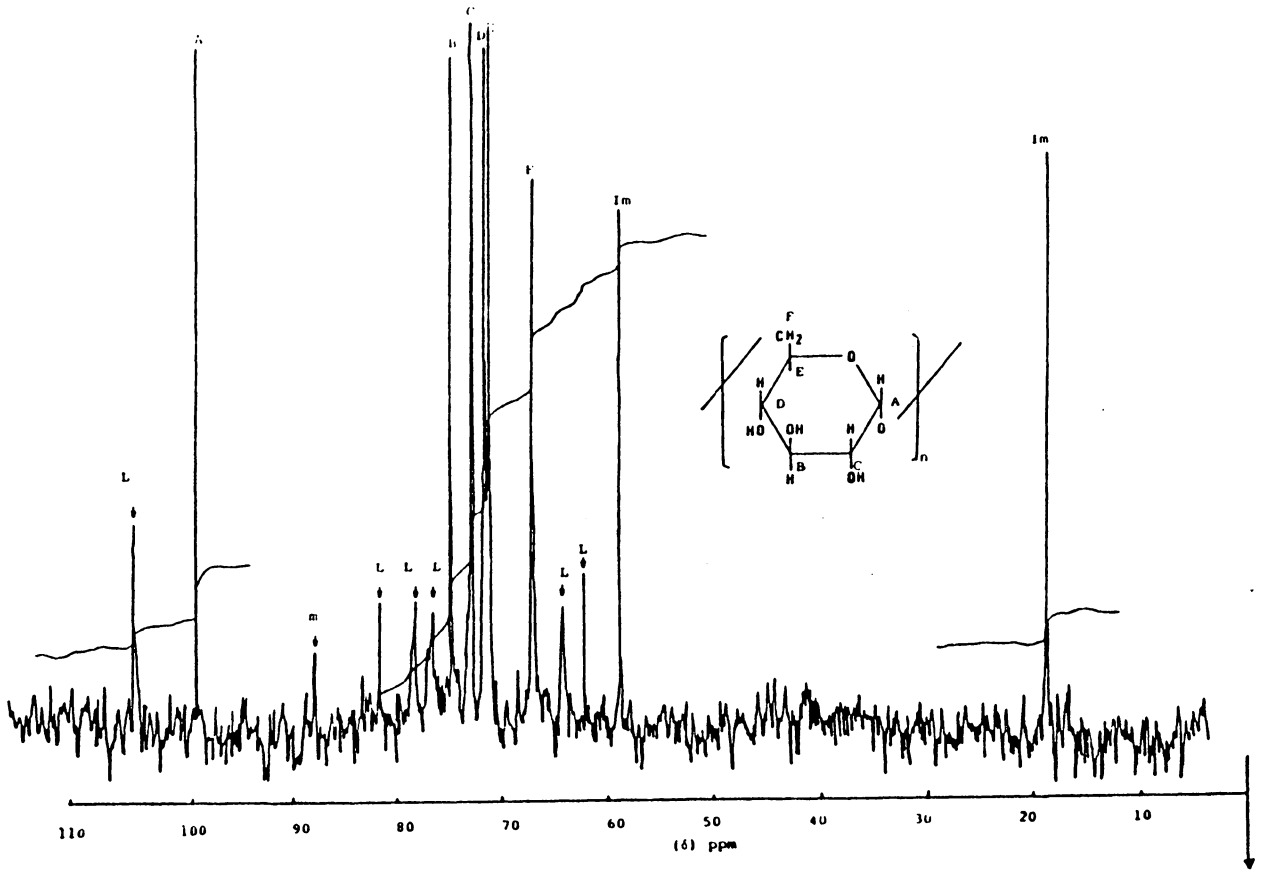

Figure 35. ^{13}C NMR spectrum of the dextran precipitate from dextransucrase with 1200 seconds shear history in D_2O at 90°C

a very small number of branch linkages (5%). Also it serves as the ground of comparison with the next two spectra to verify any structural changes.

The computer print-out (Table 11) of the spectrum of the dextran precipitate from dextransucrase with 300 seconds shear history reveals 18 peaks, but only 14 peaks are in the region of interest. From the intensities of the resonance peaks and the spectrum in Figure 34, only 6 peaks are major ones, marked as A, B, C, D, E, and F, with 4 identifiable minor peaks marked by an indicating arrow on the spectrum. The significance of these minor peaks is hard to judge. A lingering problem usually encountered by a C13 NMR spectroscopist is the noise-to-signal ratio and the significance of those minor peaks in the spectrum. Usually this problem is resolved by sound judgment based on experience and improvement of the spectrum acquisition techniques, such as longer acquisition time. At this point, my interpretation is focused on the major peaks. The six major peaks are virtually the same ones as the linear dextran's. So the resonance peaks of 99.250, 74.897, 72,912, 71.744, 71.335 and 67.488 are assigned to C1, C3, C2, C4, C5 and C6 respectively and the conclusion is that the dextran precipitate from this partially inactivated dextransucrase which has been subjected to shear action for 300 seconds has the same molecular structure as far as the C13 NMR scope can measure.

The resonance peaks of 58.779 and 19.250 are assigned to impurities.

The significance of the minor peaks will be resolved by examining the spectrum of the dextran precipitate from dextransucrase with 1200 second shear history, because if the shear action causes the dextransucrase to synthesize different dextrans, the extent of variation should be proportional to the shear strain. So the next spectrum of the dextran precipitate is to be examined and analyzed. It turns out that three of the minor peaks may be assigned to the resonance peaks of levan and are designated as L in Figure 34.

The computer printout again reveals 18 peaks, and the spectrum shows a dramatic change. At least 13 major peaks are seen in the region of interest and 2 major peaks at 58.779 and 18.191 are impurities. Careful analysis of the 13 major peaks on Figure 35 gives us the very same six major peaks with the highest intensities: 99.192, 74.897, 72.912, 71.685, 71,276 and 67.422 labelled as A, B, C, D, E, and F of the linear dextran spectrum. The most intriguing peaks are those of 81.555, 78.401, 77.292 and 76.941 ppm resonance positions, because they fall right into the region of 75 to 85 ppm which contains linked carbon, and for each of the linked carbon resonances, the existence of a branch type or a branch linkage is indicated. Though the whole pattern of the spectrum does suggest a structural

variation of the polymer, I am not able to confirm each resonance peak with the literature so as to determine what kind of linkage each one represents; and how much they represent related to the linear backbone . Special attention has been paid to the α1→3 branch linkage, because NRRL B 512 dextransucrase will synthesize dextran with 5% of this branch linkage. Compared to the C13 spectrum of NRRl B-742 S dextran (please refer to Fig. 10 and Table 2 in Chapter II), this result fails to convince me that the dextran precipitate is more branched in α1→3 position. One might argue that these resonance peaks of moderate intensities are the contribution of the molecular weight distribution changes in the dextran precipitate polymers as shown in Figure 30, or they may be an artifact of the denatured dextransucrase fragments. These arguments are judged to be over-cautious. Because C13 NMR spectrum is a powerful tool at the molecular level, it is very sensitive to the environment of the carbon atoms in the molecules, but it has a localized view. In other words, the spectrum can be considered to be practically independent of the nature of the adjacent linked residue, so the molecular weight will not exert any influence on the spectrum. As for the contribution of protein fragments, these resonance peaks are in a narrow region of the whole spectrum that are characteristic of the carbohydrate compounds. It is not likely to happen, because protein resonance position will

not appear in this region. By judging all these alternatives, another question is raised: is there any possibility of mixed linkages on branch linkages? A final breakthrough on the interpretation and analysis of the spectrum came with the help of Dr. Fred Seymour of South Dakota State University. The far end of the spectrum at the position of 105.441 ppm represents a β linkage anomeric carbon, which is rather surprising because dextran never has β linkages in its structure. By careful recoursing the resonance peaks of a β-linkage polymer--levan, which is composed of repeating fructose units in β-2,6 linkages, the conclusion is reached that this spectrum of dextran precipitates from dextransucrase which has been subjected to shear action for 1200 seconds, reveals a mixture of dextran and levan. The spectrum of levan of the same microorganism NRRL B-512 (Seymour, Knapp and Jeanes, 1979d) has been obtained and it consists of seven peaks at 105.69, 81,82, 78.76, 77.27, 64.97, 62.77, and 62.48 ppm position, which are almost identical to the major peaks in Figure 35 marked by indicating arrows and the letter L beside the A, B, C, D, E, F peaks of the linear dextran. Though I still have three unknown minor peaks designated as m in Table 12, the interpretation of the spectrum is made confidently. The significance of the presence of a small quantity of levan in the dextran precipitate will be discussed in the next section of this chapter.

The most important answer concerning any structural variations of the dextrans synthesized from partially inactivated dextransucrase has been obtained with the help of the C 13 NMR technique: there is no structural change among these dextrans.

With this clear conclusion in mind, I can go back to discuss the data on molecular weight distribution on Figure 30 and the data on intrinsic viscosity on Figure 31. Apparently the more narrow distribution of dextran precipitate from sheared enzymes than from non-sheared ones cannot be attributed to structural changes, but I am not sure whether it is exclusively due to shear inactivation. Recently published data on dextran production by purified dextransucrase revealed that the molecular weight of distribution of dextran became more refined and sharper as the function of reaction time, as opposed to the conclusion of Tsuchiya and coworkers (1955) based on ethanol precipitation technique. Monsan and Lopez (1980) obtained this observation by gel permeation chromatography on Ultrogel A2 column. The molecular weight distribution shown on Figure 30 may be a result of different reaction times, though a solid conclusion requires more study. The observation on the intrinsic viscosity data may be attributed to the variation of particle weights, especially since the dextran precipitates from sheared enzyme contain some quantity of another polymer--levan, with its molecular

weights reported in the range of 10^7 and 12% branch linkages (Feingold, 1957). This may be responsible for the increase of the intrinsic viscosities.

E.4. Summary of the findings from the characterization of dextran precipitates

At this point, I would like to summarize the findings from all these data and observations on dextran precipitates. It is clear that dextrans synthesized from the partially inactivated dextransucrase have the same molecular structure as the dextran from the purified, non-sheared dextransucrase, and their structure is the same compared to commercially available dextran. Upon this conclusion, the imposed question of any long range effect on the enzyme molecules after shearing would be given a negative answer. The results of this study also prove the hypothesis that after enzyme molecules are subjected to shear, the molecular population is eventually divided into two categories: inactive (denatured) and active, and the remaining catalytic power of the partially inactivated enzyme will represent the active enzyme concentration of the enzyme preparation.

The shear inactivation of dextransucrase will cause the enzyme to synthesize a smaller quantity of high molecular weight dextran; however, the observation on the molecular weight distribution and intrinsic viscosity needs further study to achieve any solid conclusion. This study

presents an impetus for future work on dextransucrase as well as the dextran properties from the enzymatic reaction.

F. The Significance of Levan Presence in Dextran Precipitate

The presence of the fructan, levan in one of the dextran precipitates detected by the powerful technique of C 13 NMR spectroscopy struck the researcher's mind unexpectedly, its appearance only in one of the spectra is another interesting finding worthy of discussion.

F.1. Levansucrase in dextransucrase production culture

The existence of a small quantity of undesirable contaminating enzymes, namely, levansucrase which was also excreted by the same organism NRRL B-512 in dextran producing culture had been reported (please refer to Section C, Chapter II, Literature Review) but it remained obscure, most probably due to the fact that the small quantity rendered it less significant. Levansucrase catalyses a similar polymerization reaction as dextransucrase.

Instead of polymerizing the glucosyl moiety into glucose polymer-dextran, it polymerizes fructose units into a fructose polymer--levan and liberates the free glucose molecules. Levan is a fructan consisting of a repeating fructose unit linked in 2,6 position by β linkages as a backbone and with the side chains having the same structure as the main chain connected through β 2 → 1 branch linkages.

Early workers on dextran production and its enzymatic synthesis had commented on some fructose containing polymers in the culture fluid but did not elaborate on it (Hehre, 1955). A. Jeanes (1965) reported that levan would precipitate from a reaction mixture at ethanol concentrations of 65% to 75%, as opposed to less soluble dextran which precipitated out at an ethanol concentration at 35%. The yield of levan was one fourteenth or one fifteenth of dextran. Robyt and Walseth (1979) did the most quantitative tests on all the possible contaminating enzymes. Their data showed 6% of the reducing power might be attributed to levansucrase. Others claimed the glucose level by levansucrase action was as low as 3% of the total reducing sugar (Reilly, 1977) or the "invertase-like" activity was as high as 25.2% of the total reducing sugar (Lawford, 1979). My data revealed 2% of the total reducing sugar might be the result of the levansucrase activity in the purified dextransucrase prepartion.

F.2. The significance of levan presence in dextran precipitate from shear inactivated enzyme preparation.

The most intriguing find is the levan presence in the dextran precipitate and it only appears notably in one of the dextran prepitates as demonstrated by C 13 NMR spectroscopy. The only logical explanation is that dextransucrase is more susceptible to shear inactivation while

levansucrase is much more persistent under the same shear condition. This claim can be justified by a quantitative analysis on the dextran precipitates' NMR spectrum. The contaminating enzyme test determined that levansucrase activity would contribute 187 µg reducing sugar per ml enzyme solution per hr. From the summary in Table 7, of the reaction conditions of these dextran precipitates, I conclude that levansucrase remains totally active as in the non-sheared enzyme solution after subjecting the enzyme solution to shear action for 300 seconds and 1200 seconds, while dextransucrase only retains 63.7% and 27% activity of its original catalytic power. The relative activity of the dextransucrase to the undesired levansucrase shifts in favor of the latter. At the end of the reactions, the theoretical levan quantities will be 748 mg for the enzyme reaction with no shear history, 11.96 mg for the enzyme reaction with 300 seconds shear history, 31.46 mg for the enzyme reaction with 1200 seconds shear history and consist of 1.7%, 6.7% and 34% respectively of the high molecular weight dextran precipitates assuming no loss during the ethanol precipitation procedure. This relative quantity of levans in the dextran precipitates corresponds to the C 13 NMR spectra pattern of the precipitates. Levan resonance peaks will not be resolved on the precipitate from the non-sheared enzyme reaction, but will be shown as minor peaks on the second spectrum. As a matter

of fact, the three minor peaks of 78.576 ppm, 64.794 ppm and 62.224 ppm in the second spectrum can be assigned to the levan resonance peaks. The theoretical quantity of levan in the dextran precipitate from the third enzyme reaction is 34% and is comparable to the peak intensities of the levan resonance peaks, because in polysaccharide's C 13 NMR spectrum, the peak intensity is in the first approximation proportional to the quantity present.

The rationale for the different sensititives to shear inactivation by dextransucrase and levansucrase is unknown at this stage. However, the most likely answer may be the difference of the molecular weight of these two enzymes. Dextransucrase from NRRL B-512 microorganism has a molecular weight of 280,000, while levansucrase from Aerobacter levanicum has a molecular weight of 22,000 as reported by Ebert and Stricker (1964), though the molecular weight of levansucrase from Leuconostoc mesenteriode is not known.

Another possible factor is the fact that levansucrase is present in such a low concentration that it is insensitive to the shear inactivation phenomenon.

V. CONCLUSIONS

Since this work began with the rather vague objective of simply trying to find out what long range effects, if any, are present after enzymes are subjected to a simple, defined shear field, it is not surprising that the information gathered during the course of investigation covers a wide range.

The purpose of this chapter is to enumerate concisely the most important discoveries of this study so that it may serve as the groundwork of future research.

1. A scheme of purifying the extracellular enzyme from the fermentation culture of microorganism has been carried out fairly successfully. The purification work consists of centrifugation of the culture broth, then a membrane device of ultrafiltration and dialysis, and finally a gel permeation chromatography to separate the extraneous proteins.

2. An experimental methodology of investigating the shear inactivation of enzymes in the absence of substrate and in the presence of substrate has been developed. The simple shear field was provided by a Couette system of a viscometer kept at a constant temperature.

3. After dextransucrase enzyme solutions have been subjected to shear action for different periods of time, the enzymatic activities of the enzyme solutions may be

studied by kinetic data on a batch reactor. It was found that the enzyme lost part of its catalytic activity, due to shear. Longer shear action totally denatures the enzyme molecules and results in virtually total loss of enzymatic activity. Shear inactivation study performed on two different enzyme concentrations also indicated that the shear inactivation phenomenon is a function of enzyme concentration present. The more concentrated enzyme solution will have a more severe effect than the dilute solution upon shear.

4. Structural change in dextransucrase is seen by UV spectra to accompany shear inactivation, though the interpretation of the data is tenuous due to a lack of understanding of the native structure of the dextransucrase molecule.

5. A correlation curve of the remaining dextransucrase activity versus shear strain in the absence of substrate is obtained when plotted on a log-log scale, and, with non-sheared enzyme as the reference point, the shape of the curve resembles other published work on different enzyme systems. From the curve, it is seen that when shear strain ($\dot{\gamma}t$) is less than 10^5, inactivation is minimal.

6. The remaining activity versus shear strain curves were obtained on dextransucrase but in the presence of substrate at two different enzyme levels. Both curves show much less shear inactivation effect. The threshold

for inactivation was extended to a shear stain of 10^6.

7. By comparing the shear inactivation effect on the same dextransucrase soluiton with and without the presence of sucrose, the conclusion is reached that for dextransucrase, there is a protective effect on substrate binding against an external shear field. This results supports Ebert and Schenk's hypothesis on the enzymatic mechanism of dextransucrase, where a whole complex structure held together by many coordination bonds between the constituents.

8. The dextransucrase solutions with different shear histories were incubated with sucrose in batch reactors to synthesize dextrans. The synthesized dextrans then were precipitated out by an 83% ethanol-water mixture, and the yield of high molecular weight dextran (MW larger than 50,000) was reduced substantially due to shear inactivation.

9. When the molecular structure of these dextran precipitates was studied by C 13 NMR spectroscopy technique at 90°C, it was found that these dextran precipitates have the same molecular structure as commercial linear dextran manufactured by the enzymatic synthesis of the same source of enzyme (NRRL B-512). This conclusion is based on the revelation of the six C 13 resonance peaks at the 99.3, 74.9, 72.9, 71.8, 71.3, and 67.5 ppm positions which are characteristic of linear dextran linked predominantly by

α 1→6 glycosidic linkage. This conclusion leads me to claim that there is no long range effect on an enzyme molecules' catalytic specificity associated with shear inactivation. In other words, this conclusion provides a clear proof that after the enzyme molecules are subjected to shear, eventually the molecular population will be divided into active molecules and inactive molecules (denatured by hydrodynamic shear) and there is no such state as sub-denaturation. The active molecules perform the catalysis as specific as non-sheared molecules, and the remaining activity of the enzyme solution represents the active enzyme concentration. The problems of reversible or irreversible inactivation and how fast or how slow the recovery of enzyme activity will occur after shear are circumvented in this study by observing the long range effect reflected by the molecular structures of the synthesized polymers.

10. The C 13 NMR spectra of the dextran precipitates reveal that the dextran precipitate synthesized by the dextransucrase of 1200 seconds shear history in 21 hours incubation contains a moderate quantity of levan (approximately 30%) indicated by its characteristic resonance peaks at the 105.4, 81.55, 78.40, 77.29, 64.73 and 62.28 ppm positions. Because the dextran precipitates from dextransucrase of 300 seconds shear history and non-sheared enzyme did not reveal any significant sign of levan resonance, I have

concluded that levansucrase presented in the purified dextransucrase preparation at a very low level (contributing less than 2% of the reducing power tested by contaminating enzyme assay) is much less susceptible to shear inactivation than the dextransucrase is.

11. The observation of the variation in molecular weight distribution and intrinsic viscosity of the dextran precipitates may serve as a impetus for future study, but the conclusion remains obscure at this stage.

VI. RECOMMENDATIONS

At the end of this study, I have obtained the experimental data that indicate that protective effect of substrate binding against the external shear field on dextransucrase, which strongly endorses the Ebert and Schenk's hypothesis on dextransucrase's insertion polymerization mechanism. I also found that one cannot modify an enzyme molecule's specificity by subjecting it to a moderate shear force, then by incubating it with substrate. One of the most direct, fruitful extensions of this work would be to perform a dextransucrase enzymatic reaction with sucrose in the deforming medium; that is, an enzymatic reaction takes place in the viscometer while the bob of the Couette system is rotating at a certain speed simultaneously. Repeated performances of the same experiment will be needed to generate enough dextran for its structural analysis. A C 13 NMR spectroscope technique will be one of the proper tools. The results of this type of work will reveal the information about possible branch mechanism of dextransucrase which remains unclear at the end of this study, but some reservations should be made on the intrinsic problems associated with this research: 1) the separation of the effect of conformation changes of enzyme due to shear, and 2) the effect of shear related scisson of dextran chains from the synthesized polymer to act

as acceptor molecules.

As a matter of fact, the crux of this problem provides me with the justification for my experimental design. I would shear the dextransucrase first, then let it react with sucrose so that if any structural variation is observed on synthesized dextran polymers, this could be exclusively attributed to the shear induced conformational changes on enzyme molecules, and could preclude the effect of the acceptor as the origin of branch formation. Apparently, any structural changes associated with shear on enzyme molecules cannot be maintained in the long range after the shear field is removed.

The presence of a small quantity of contaminating enzyme-levansucrase and its endurance under shear action gives the researcher another tacky problem. It is essential to resolve this before further study on the dextransucrase mechanism by shear without interfering with the experimental results. The best way out will be treating the membrane retentate with dextranase to strip the dextransucrase of any association with preformed dextran in the purification scheme and then by eluting through gel permeation chromatography with a "proper" column that will retard nascent dextransucrase but not levansucrase on the column so that the undesired levansucrase is not present in the preparation of dextransucrase, not even at a small level. A gel electrophoresis test procedure is recommended

after the purification work to guarantee the purity of the enzyme preparation.

Another future work needed is the understanding of the native state or three-dimensional structure of the dextransucrase molecule by more sophisticated methods such as circular dichrosim or optical rotatory dispersion. Without such knowledge, the conformtional changes accompanying shear inactivation and on substrate binding cannot be quantified and interpreted from any physical measurements performed on the system. This is ture that the UV spectra of dextransucrase solution, of the native enzyme, and with different shear histories shown in Figure 24 bear little value except to indicate the researcher's intention and technique. UV spectra for a totally denatured dextransucrase such as a boiled enzyme should be taken in order to serve as the negative control.

In order to be more informative on the enzymatic synthesis of dextran, the observation on molecular weight distribution and viscosity should be done by experimental designs that would take into account other possible factors such as reaction time, and the uniformity of the molecular weight of the polymer.

VII. SUMMARY

The extracellular enzyme dextransucrase (α 1,6-glucan: D-fructose 2-glucosyltransferase EC 2.4.1.5) was concentrated and purified from the fermentor culture of Leuconostoc mesenteroides (ATTC 10830) by a three-step procedure: centrifugation, membrane ultrafiltration and dialysis, and gel permeation chromatography. This enzyme preparation had a protein content of 0.0125 mg/ml and a dextransucrase activity of 0.95 U/ml where dextransucrase U is defined as the amount of enzyme that liberates one micromole of reducing sugar taken as fructose per minute at 25°C. The contaminating enzyme levansucrase content was determined to be less than 2%. In another preparation procedure, a hollow fiber membrane device was used after gel permeation chromatography. The enzyme solution was concentrated down to 3/5 of its original volume which contained 0.02 mg/ml protein and 1.59 U/ml dextransucrase activity.

Shear inactivation study was performed on a batch reactor after subjecting the enzyme solution to a shear rate of 1046 $second^{-1}$ in the Couette viscometer for several different periods of shear exposure time. The kinetic data showed dextransucrase lost part of its catalytic power due to shear inactivation.

A correlation curve of the remaining activity versus a dimensionless group--shear strain was generated in the absence of substrate. There was not much inactivation until shear strain reached 10^5.

Two sets of the correlation curves were generated on the same system but with the presence of substrate at two different levels of enzyme loading. Both results indicated less shear inactivation effect in the presence of substrate and this protective effect on substrate binding strongly supports Ebert and Schenk's hypothesis that there are substrate induced conformation changes of the enzyme molecule.

In order to find any long range effect on the catalytic specificity of the enzyme molecule associated with shear inactivation, the dextransucrase solution with different shear histories was incubated with sucrose. The synthesized dextrans were then precipitated out by an 83% ethanol-water mixture. When the molecular structure of these dextran precipitates was analyzed on C 13 NMR spectroscopy at 90°C, it was found that there was no change in the polymer structure. But one of the spectra (which corresponded to the dextransucrase preparation with the longest shear history) contained resonance peaks of levan. All these findings lead me to the conclusion that shear induced conformational changes of the enzyme molecules can not alter their catalytic specificity, and that the sensitivity toward shear inactivation is much greater by

dextransucrase than by a small quantity of levansucrase.

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APPENDIX 1

A sample calculation for dextransucrase enzyme activity from the absorbance of Somogyi-Nelson reducing sugar assay.

The results of the Somogyi-Nelson reducing sugar assay yielded values of adsorbance at 500 nanometer:

$$\text{Abs } 500 = Y$$

Step 1

Use the standard curve, Figure 19, to find out what is the equivalent fructose amount in 1 ml portion of the reaction solution assayed, which had been brought into the range of the fructose standard curve by a proper dilution factor.

The fructose content is the quantity X in the linear curve:

$$Y = mX+b$$

Doublet is required, so an average value is taken

$$(X1 + X2)/2 = F1$$

Step 2

Find out what are the values of equivalent fructose contents for boiled enzyme solution which serves as a negative control:

$$(X3 + X4)/2 = F0$$

Step 3

Calculate the amount of fructose liberated from dextransucrase activity in 1 ml portion assayed volume:

$$F1 - F0 = F_{liberated}$$

Step 4

Final calculation of dextransucrase activity U, which is defined as the amount of enzyme that will liberate 1 micro mole of reducing sugar taken as fructose per minute at 25°C.

$$\frac{\text{F micrograms Fructose}}{\text{1 ml solution}} \Big| \frac{\text{1 micro mole Fructose}}{\text{180 micrograms Fructose}} \Big| \frac{}{\text{30 minutes incubation}}$$

$$\frac{}{\text{0.1 ml enzyme solution delivered to the reaction mixture}} \Big| \frac{\text{10.1 ml total reaction solution volume}}{} \Big| \frac{}{\text{dilution factor of 1/10}}$$

= dextransucrase U/ml enzyme solution

e.g. for retentate of one of the enzyme preparation batches:

$$\frac{17.16 \times 10^{-6}\text{g Fructose liberated}}{180 \times 10^{-6}\text{g-ml}} \quad \frac{10.1\text{ ml}}{30\text{ minutes}} \quad \frac{}{0.1\text{ ml}} \quad \frac{}{1/10}$$

= 3.178 U/ml enzyme solution.

APPENDIX 2

A sample calculation for remaining percentage of enzyme after different periods of time of shear.

I followed the same procedure as in Appendix 1 to calculate the dextransucrase activity of the enzyme solutions.

For non-sheared enzyme solution, I delivered 0.1 ml portion enzyme solution to 8 ml potion of sucrose solution and incubated at 25°C for 30 minutes, then I made a dilution (1/2) and assayed the fructose content of this final solution in 1 ml volume.

53.8×10^{-6} fructose liberated				8.1 ml	
1 ml	180×10^{-6} g	30 minutes	0.1ml		1/2

= 1.59 U/ml enzyme solution.

For sheared enzyme solution at a shear rate $\dot{\gamma}$ = 1046 $second^{-1}$ for 300 seconds shear time, $\dot{\gamma} = 3.14 \times 10^5$, dextransucrase activity is 1.00 U/ml, so the percentage of the remaining activity = 1.00/1.59 = 63.7%.

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THE STUDY OF SHEAR INACTIVATION OF DEXTRANSUCRASE PREPARATION FROM LEUCONOSTOC MESENTEROIDES

by

Simon S. Shih

(ABSTRACT)

The extracellular enzyme dextransucrase (α 1,6-glucan: D-fructose 2-glucosyltransferase EC 2.4.1.5) was concentrated and purified from the fermentor culture of Leuconostoc mesenteroides (ATCC 10830) by a three-step procedure: centrifugation, membrane ultrafiltration and dialysis, and gel permeation chromotography. Two enzyme preparations which contained dextransucrase activity of 0.95 U/ml and 1.59 U/ml were used for this study. The contaminating enzyme levansucrase was determined to be less than 2%.

Shear inactivation study was performed on a batch reactor after subjecting the enzyme solution to a shear rate of 1046 second^{-1} in the couette viscometer for several different periods of shear exposure time. The kinetic data showed dextransucrase lost part of its catalytic power due to shear inactivation.

A correlation curve of the remaining dextransucrase activity versus a dimensionless group-shear strain was generated in the absence of substrate. The data revealed there was not inactivation until shear strain reached 10^5. Two sets of the correlation curves were generated on the same system but with the presence of substrate at two different levels of

enzyme loading. Both results indicated less shear inactivation effect in the presence of substrate and this protective effect on substrate binding strongly endorses Ebert and Schenk's hypothesis that there are substrate induced conformation changes of the enzyme molecule.

In order to find any long range effect on the catalytic specificity of the enzyme molecule associated with shear inactivation, the dextransucrase solution with different shear histories was incubated with sucrose. The synthesized dextrans were then precipitated out by a 83% ethanol-water mixture. When the molecular structure of the dextran precipitates was analyzed on ^{13}C NMR spectroscopy at 90°C, it was found that there was no change in the polymer structure. But one of the spectra which corresponded to the dextransucrase preparation with the longest shear history contained resonance peaks of levan. All these findings lead to the conclusion that shear induced conformational changes of the enzyme molecule can not alter their catalytic specificity, and that the sensitivity toward shear inactivation is much greater by dextransucrase than by the small quantity of levansucrase present.