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Crystallization from aqueous solution was studied in a range of dextran polysaccharides exhibiting low degrees of chain branching. The dextrans were characterised with respect to molecular weight by light scattering, viscosity, and end-group analytical procedures. Weight-average molecular weights lay within the range 3000 - 113,000.

Light scattering investigations performed on dextran solutions during crystallization, indicate the formation and growth of spherical particles. The diameter of these particles increases to approximately 200 nm before the onset of gelation. The time dependence of growth suggests that the crystallization kinetics are diffusion controlled.

The nucleation process may be predominantly homogeneous or predominantly heterogeneous, depending on the nucleation density of the dextran. In either case, the rate of formation of crystalline material agrees with that predicted from the Avrami analysis of crystallization kinetics.

Nucleation density in dextran can be increased, either by partial crystallization or by addition of nuclei from external sources; it can also be decreased, either by the action of heat or of specific chemicals. Kinetic studies of dextrans modified in this way confirmed that crystallization rate increased with increasing nucleation density, and vice versa.

Crystallization rate increases in response to decreases in the following parameters: (i) solvent concentration, (ii) solvent power, (iii) average molecular weight, and (iv) breadth of molecular weight distribution. A maximum occurred in the plot of rate against temperature, and the rate decreased as the temperature tended towards melting and glass points.
Crystalline dextran particles in aqueous suspension melt over a wide temperature range, from 30 °C up to the boiling point of the solvent. A small fraction resists melting at 100 °C. Light scattering studies of the melting process suggest that this fraction contains stable nuclei. The rate of development of stable crystalline material is much lower than the overall rate of crystallization, but both rates are controlled by the same factors. The fraction of the total crystalline material which resists melting is therefore very dependent on the crystallization history of the polymer.

As a result of these studies, it was possible to advance an explanation for turbidity formation during the fractionation and drying stages of the manufacturing process. Conditions for avoiding turbidity formation during preparation and storage of dextran solutions, have been defined.

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The effects of heterogeneous, enzymic, protein-peptide material (4 mg./l.) on mass transport rates were studied when 6-APA solutions were processed under reverse osmosis conditions (100 bar). The protein improves separation efficiency by increasing 6-APA retention without significantly decreasing water transport rate.

The effects of the protein-peptide mixture on mass transport rates were also studied when 6-APA solutions containing different levels of this material (1 - 100 mg./l.) were subjected to ultrafiltration. As the protein-peptide concentration increased, water transport rate, 6-APA transmission, and peptide transmission all decreased. Despite the increasing peptide concentration, the ratio of peptide to 6-APA in the permeate does not vary appreciably, since peptide transmission decreases much more rapidly than that of 6-APA.

As a result of these observations, a rapid method for determining the levels of proteinaceous material in 6-APA solutions was developed, based on measuring the transport rates of 6-APA and water across ultrafiltration membranes under closely controlled conditions.

The effects of protein on the transport rates of small molecules are interpreted in terms of membrane surface adsorption effects, which lead to the formation of an interfacial protein layer. This layer is believed to restrict the access of water and small solutes to the entrance of pores in the cellulose acetate membrane.
DYNAMIC ASPECTS OF CRYSTALLIZATION AND MASS TRANSPORT IN POLYMERS AND POLYMERIC MEMBRANES FOR PHARMACEUTICAL USE

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1975
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DECLARATION

The work described in this thesis was carried out in the laboratories of Inveresk Research International (IRI), Musselburgh, Midlothian, during the period 1969 - 1973.

I declare that this thesis has been composed by myself, and that the work described is my own. Any contributions to the work by other members of the IRI group are clearly indicated in the text.

Neil C. Beaton, B.Sc.

May 1975.
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Thanks are due to the directors and members of staff of Fisons Ltd., Pharmaceutical Division, and of Beecham Research Laboratories, for their kind co-operation, financial support, and permission to publish the results.

Finally, I am indebted to my wife for assistance in assembling the material for presentation, and to Mrs. D. Stagg for preparing the typescript.
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PART I

KINETIC STUDIES OF DEXTRAN CRYSTALLIZATION FROM AQUEOUS SOLUTION
INTRODUCTION

Dextran is the collective name for a large class of extracellular, bacterial polysaccharides, composed almost exclusively of the monomeric unit, \( \alpha-D - \) glucopyranose. Aqueous solutions of acid-hydrolysed, fractionated dextrans are used extensively as blood plasma substitutes. During preparation and storage of these solutions, the formation of insoluble, particulate matter has been frequently observed (1,2,3). This material causes turbidity, and renders the solutions unsuitable for intravenous injection. The initial objective of this work was therefore to investigate the phenomenon of turbidity formation in aqueous dextran solutions.

Various workers (4,5) have referred to the occurrence of crystallization in dextrans, a process by which the polymer may be rendered insoluble in cold water. While at the outset of this work, the formation of crystalline dextran was considered a strong possibility, the exact nature of the insoluble material responsible for turbidity had not been conclusively established. However, early in the investigations, it was demonstrated that the insoluble matter was indeed crystalline dextran. This finding was supported by the parallel studies of other workers (6,7), who were able to grow dextran spherulites of sufficient size to be resolvable in the optical microscope. Thereafter, the primary objective was to study the dextran crystallization process, with a view to uncovering preparative and storage conditions under which the formation of insoluble material might be controlled. In addition, it was intended to demonstrate that crystallization and melting processes in dextran were essentially similar to those in other polysaccharides, and in polymers generally.
1. BACKGROUND INFORMATION ON DEXTRANS.

(a) Historical Background.
For more than a century, dextrans were known principally through problems associated with their natural development in sucrose-containing solutions. The tendency of raw beet and cane sugar juices to thicken, gel, or form "scum" under certain circumstances, was documented in the early 1800's. The polysaccharide nature of the "scum" was also recognized, and in 1869, Scheibler coined the name "dextran" for this material because of its strong optical dextra-rotation (8). The microbial origin of dextran was demonstrated by Pasteur in 1861, and later, in 1878, the generic name "Leuconostoc" was proposed by van Tiegheim (10) to describe the dextran-producing micro-organisms.

In the following decades, it became recognized that the ability to synthesize polysaccharides was widespread among micro-organisms. The dextran-forming bacteria were identified and classified by Hucker and Pederson in 1930, and the most important species termed Leuconostoc Mesenteroides and Leuconostoc Dextricanum (6). These form a polysaccharide from sucrose, with release of fructose, and the structure and properties of the dextran formed depend primarily on the bacterial species and strain involved. The extracellular, bacterial enzyme, dextran sucrase, which effects polymerisation, was isolated by Hehre and Sugg (11) in 1962, and used for direct dextran synthesis. The specificity of the enzyme for sucrose substrate was also demonstrated.

In 1943, Gronwall and Ingelman (12) in Sweden, produced a partially hydrolysed, fractionated dextran with a weight-average molecular
weight in the region of 100,000. This material met the clinical requirements of a blood substitute, particularly since the \( \alpha-1 \rightarrow 6 \)-glucosidic bonds in the linear dextran are not split by the \( \alpha \)-amylases of the body. In the 1940's, dextran thus became the first microbial polysaccharide to be produced and used on an industrial scale. Developments were accelerated by anticipated needs for adequate supplies of therapeutically safe plasma extenders, for restoring blood volume in mass casualty situations. As a result, "clinical" dextrans soon assumed great importance, and they are now produced commercially in a number of countries (13).

In the 1940's and 1950's, production of native dextran was also initiated on an industrial scale. A large number of potential uses have been proposed in industries such as agriculture, petroleum and food (14). The major industrial use of acid-hydrolysed dextran is in the photographic industry, and production of low molecular weight dextran (m.wt. \( \approx 10,000 \)) for this application began in 1963 (15).

The most important laboratory use of acid-hydrolysed dextran is probably in the manufacture of cross-linked dextran structures for use in gel permeation chromatography (16). The application of such gels to molecular separation was first demonstrated in the late 1950's. In 1972, their use on a commercial scale for whey protein fractionation has also been reported (17).

(b) Utilization.

Large scale production of dextran occurs principally within the
pharmaceutical industry, where native dextran is converted into products suitable for use as plasma extenders. The molecular weight of native dextran is of the order of $5 \times 10^7$, which is too high for clinical use. To eliminate interference with normal blood coagulation mechanisms, and to avoid red blood cell aggregation, a molecular weight of less than 200,000 is required. Very low molecular weight dextran, with molecular weight less than about 30,000 is also unsatisfactory, since it is cleared too rapidly from the kidneys to be effective. The natural polymer must therefore be partially depolymerised by acid-hydrolysis, to give dextrans with weight-average molecular weights in the range 40,000 - 110,000. Fractionation is then necessary to eliminate high and low molecular weight ends of the molecular distribution. With dextrans prepared in this way, infusion is possible at concentrations such that the dextran osmotic pressure is equal to that of normal plasma; at these concentrations, solution viscosities do not prohibit intravenous supply. Dextran thus meets the recognized requirements for blood plasma substitutes, and is perhaps the most extensively studied and generally accepted substance available for blood replacement (8,13,16).

Dextrans for clinical usage must comply with molecular weight specifications drawn up by the various authorities legislating on pharmaceutical products. For use in the maintenance of blood volume and pressure, preventing shock in burns or haemorrhage, dextrans with molecular weight averages in the range 60,000 - 110,000 are required. In more recent years, lower molecular weight dextrans, with an average molecular weight of 40,000, have also
been employed for increasing the fluidity of blood and improving capillary flow (13).

The major industrial use of acid-hydrolysed, native dextran employs fractions of molecular weight in the region of 10,000. This product is used to increase the covering power, quality and developing speed of gelatino-silver halide emulsions for radiological and lithographic films (15).

On the laboratory scale, the availability of well-characterised dextran fractions has produced a polymer uniquely suited to medical, chemical and biochemical research. A number of research applications have been detailed by Jeanes (14). The use of cross-linked dextran structures in gel permeation chromatography is now well established (16). The degree of cross-linking is used to control the porosity of the gels, which are capable of fractionating water soluble substances on a size basis within the molecular weight range 2000 - 200,000.

Native dextrans also have a number of potential uses, such as drilling-mud additives, and protective coatings for seeds. They have applicability in foods, based on their ability to stabilize syrups against crystallization, and on their ability to stabilize texture in various products. Other uses of native water-soluble dextrans and their derivatives have been reviewed by Baker (19).

(c) Preparation.

The dextrans studied in this work consisted of a range of commercial products prepared by Fisons Ltd., Pharmaceutical Division, the
majority being produced for clinical use. The samples were received as free-flowing powders having a moisture content in the region of 10%. The preparation process employed was typical for this type of product, and included fermentation, hydrolysis, fractionation, purification and drying stages (20). A much wider range and quantity of samples than could be conveniently prepared in the laboratory was therefore available. During production, the degree of purity and molecular weight distribution of the dextrans were rigidly controlled, thereby eliminating the need for preliminary purification or fractionation. In accordance with usual practice, the dextrans were distinguished by their average molecular weight ($\bar{M_w}$), each molecular size present being averaged according to its weight fraction. The values of $\bar{M_w}$ for the dextrans studied lay in the range 3,000 - 113,000.

The native dextrans were prepared by fermentation using Leuconostoc
Mesenteroides, strain NRRL B512 (20). Native dextran has an extremely wide molecular weight distribution; the molecular weights present range from about 50 million down to less than 1000. The polymer is water soluble and gives rise to highly viscous solutions. Dextran was isolated from the fermentation liquors by precipitation with the non-solvent, ethanol. The properties of various native dextrans prepared in this way have been extensively studied by Jeanes and co-workers (21).

To prepare products for clinical usage, the chain length and molecular weight distribution—width of native dextrans were reduced by partial acid hydrolysis. Fractions of still narrower molecular weight distribution were then prepared from hydrolysed dextran by repeated partial precipitation with ethanol. After
removing the ethanol by distillation, the product was isolated from solution by evaporation and drying (20).

It was reported by Fisons Ltd. (20) that visible turbidity frequently developed in dextran solutions during fractional precipitation with ethanol. However, turbidity formed at the fractionation stage was generally destroyed during evaporation of solutions prior to drying. Despite this, significant turbidity was occasionally present in solutions reconstituted from the dried product, making them unsuitable for pharmaceutical use. This indicates either that particulate matter produced during fractionation had not been totally destroyed, or that additional material had been introduced at the drying stage.

For clinical purposes, the dextrans were reconstituted at concentrations of 10% w/v ($\bar{M} = 40,000$), or 6% w/v ($\bar{M} = 60,000$), in solutions of sodium chloride (0.9% w/v) or glucose (5% w/v). After sterilization by autoclaving, the solutions can remain stable for periods up to five years under suitable storage conditions (18). However, in certain instances, Fisons Ltd. (3) reported that during storage, particulate matter formed in dextran solutions which were initially of high clarity. This gave rise either to visible turbidity, or to the formation of "flakes". As a result, the solutions were rendered unsuitable for injection purposes.

(d) Chemical Structure.

Dextran is an anhydroglucose polymer which is synthesised from sucrose by growing cultures of certain bacteria, notably *Leuconostoc mesenteroides*. The structure of dextran is shown in
Most of the glucosidic linkages are $\alpha$-D-1→6, but to a lesser extent, 1→3 and 1→4 linkages also appear, i.e. a measure of chain branching occurs. The ratio of 1→6 to non-1→6 linkages varies within the range 3 - 50%, according to the source of the dextran (22). Much of the information relating to structure in dextrans has been derived from periodate oxidation studies (23), which permit a rapid determination of the proportion of various types of linkage present.

In dextrans prepared by fermentation using *Leuconostoc Mesenteroides* NRRL B512, the majority of the branch points are believed to occur in the 3-position (fig.1) (22,23,24). There is also considerable evidence to suggest that the side chains consist of single glucopyranose units (14,24)
The importance of branching in determining dextran solution viscosity has been demonstrated by Granath (24). For clinical use, the viscosity of solutions containing dextran of molecular weight 40,000 - 60,000, present at a concentration (6 - 10% w/v) giving the correct osmotic pressure, must be of the same order of magnitude as that of blood plasma. This is achieved by using dextrans containing ~5% non-1→6 linkages; such dextrans are prepared by fermentation using Leuconostoc Mesenteroides, strain NRRL B512 (25).

The degree of chain branching in dextrans is important in determining crystallization behaviour. If branch points are present, polymer chains may not be capable of assuming the local alignments required in the crystalline state. This effect has been considered in dextrans by Senti and co-workers (5), and is discussed below. Using periodate oxidation methods, the degree of branching in several of the dextrans used in this work, was measured by other workers within the IRI group (26). The results, which are described in the following section, indicated that the extent of branching lay within the range expected from the fermentation process conditions.

It has been established (22) that dextrans with high degrees of branching can be prepared using the appropriate bacterial strain; crystallization would be much less likely to occur in such dextrans (5). However, the incidence of allergic reactions is high when dextrans containing highly branched chains are used as plasma substitutes (13). It is not therefore possible to use this approach to eliminate problems associated with turbidity formation in clinical dextran solutions.
Methods for the characterisation of a range of clinical dextrans have been described by Beaton and Steinhoff (27). From the point of view of this work, it was logical to assume that aggregation or crystallization in dextran solutions would be affected by the molecular properties of the material. Before embarking on detailed studies of these processes, it was essential that the dextrans should be well characterised with respect to molecular branching, molecular weight and molecular weight distribution.

Dextrans are heterogeneous polymers and contain a mixture of molecules of different molecular weight, i.e., they are characterised by a molecular weight distribution. The physico-chemical parameters and techniques most commonly employed to characterise dextrans are as follows:

(i) **Number-Average Molecular Weight, $\bar{M}_n$.**

$\bar{M}_n$ is defined by,

$$\bar{M}_n = \frac{\sum n_i M_i}{\sum n_i} = \frac{\sum w_i}{\sum n_i}$$ \hspace{1cm} (1)$$

where $w_i$ and $n_i$ are respectively the weight and number of molecules of molecular weight, $M_i$. The number-average molecular weight represents the total weight of all molecules in a sample of polymer, divided by the total number of molecules present. This is equivalent to weighting the molecular weight of each species according to its mole fraction. $\bar{M}_n$ is thus very sensitive to changes in the content of low molecular weight material in the polymer, and relatively insensitive to similar changes in the amount of high molecular weight.
species. Values of $\bar{M}_n$ are determined by techniques such as osmometry or end-group analysis, which estimate the number of molecules present; the latter method was employed in this work.

(ii) Weight-Average Molecular Weight, $\bar{M}_w$.

$\bar{M}_w$ is defined by,

$$\bar{M}_w = \frac{\sum_i w_i M_i}{\sum_i w_i} = \frac{\sum_i n_i M_i^2}{\sum_i n_i M_i} \quad (2)$$

The weight-average molecular weight represents an average in which each of the molecular sizes present is weighted according to its weight fraction. Since $\bar{M}_w$ is particularly sensitive to the presence of high molecular weight species, whereas $\bar{M}_n$ is influenced more by molecules at the lower end of the molecular weight spectrum, the ratio $\bar{M}_w/\bar{M}_n$ is a measure of the polydispersity of the system. As the molecular weight distribution of a polymer becomes narrower, $\bar{M}_n$ will increase and $\bar{M}_w$ will decrease. In the limit, $\bar{M}_w = \bar{M}_n$ for a monodisperse system.

Values of $\bar{M}_w$ are determined by techniques such as ultracentrifugation or light scattering, which estimate the weight of molecules present. In light scattering methods, the intensity of light scattered from a single particle is proportional to the square of the amplitude of the wave. The amplitude of the scattered light is in turn proportional to the polarizability, and hence to the mass of the scattering particle. The intensity of light scattered by several particles is therefore proportional to the product of the number of particles and the square of the particle mass. It is this relationship which allows determination of weight-average molecular weight by light scattering techniques.
Light scattering methods, essentially as described by Beaton and Steinhoff (27), were employed for the determination of $\bar{M}_w$ in dextrans studied in this work.

(iii) Viscosity-Average Molecular Weight, $\bar{M}_v$.

$\bar{M}_v$ is defined by,

$$
\bar{M}_v = \left[ \frac{\sum_i w_i M_i^{1+\alpha}}{\sum_i w_i} \right]^{1/\alpha}
$$

where $\alpha$ is a constant in the Mark-Houwink equation (26),

$$
[\eta] = K M^\alpha
$$

The intrinsic viscosity, $[\eta]$, of a solution of polymer, concentration $c$, is given by (28),

$$
[\eta] = \lim_{c \to 0} \left( \frac{t - t_o}{t_o c} \right) = \lim_{c \to 0} \left( \frac{[\eta]_{sp}}{c} \right)
$$

$t$ and $t_o$ being the respective times required for a specific volume of solution and solvent to flow through a viscometer capillary tube.

For a monodisperse polymer, $[\eta]$ is related to molecular weight $M$ via eqn. 4; $K$ and $\alpha$ are constants obtained from a log-log plot of $[\eta]$ versus $M$ for a series of narrow polymer fractions approaching monodisperse systems. If viscosity measurements are performed on a polydisperse system, and a molecular weight is calculated from the Mark-Houwink equation, this will be the viscosity-average molecular weight. $\bar{M}_v$ lies between $\bar{M}_w$ and $\bar{M}_n$ and tends to $\bar{M}_w$ as $\alpha$ tends to 1.
The values of K and $\alpha$ have been determined for dextran by several workers (25,26,29,30).

The dextrans studied in this work were characterised by a narrow molecular weight distribution, comparable to that in the fractions used to establish these values of K and $\alpha$. The average molecular weight calculated from eqn.4 could therefore be taken as the weight-average, $\bar{M}_w$, rather than $\bar{M}_v$, without serious error. In other words, provided the molecular weight distribution remains very similar to that in the dextrans used to establish the values of K and $\alpha$, eqn.4 may be used to yield $\bar{M}_w$ directly. Experimental evidence in support of this contention is presented in part I.A.

Although viscosity measurement does not provide an absolute method of molecular weight determination, molecular weights can be estimated very quickly and simply, provided K and $\alpha$ have been determined. Accordingly, once the molecular weight-viscosity correlation had been established for the dextrans being studied, viscosity methods for molecular weight determination were employed extensively.

(iv) Molecular Weight Distribution.

The only absolute method for characterising a polymer in terms of its molecular weight distribution involves the process of fractionation. The polymer is divided into several fractions, each of which approximates to a monodisperse system. If the weight fraction, $w_i$, and molecular weight, $M_i$, are measured for each fraction i, then it is possible to construct an integral distribution curve for the sample. In Schulz's method, it is assumed that each half of the weight
of any fraction has a molecular weight above and below the average. The cumulative weight of the \( i \)'th fraction is calculated by adding half its weight, \( w_i \) to the weight fractions of all previous fractions (31), i.e.

\[
\text{Cumulative total} = \frac{1}{2} w_i + \sum_{i=1}^{i-1} w_i \quad (6)
\]

The integral distribution curve is obtained by passing a smooth curve through the points of a plot of cumulative total versus molecular weight. The differential distribution curve is obtained by graphical differentiation of the integral curve.

To fractionate dextran, two procedures are normally employed (26, 27), (a) fractional precipitation (b) preparative gel permeation chromatography. In the former method, use is made of the fact that polymer solubility decreases with increasing molecular weight; addition of a non-solvent, e.g. ethanol, to an aqueous dextran solution, precipitates the high molecular weight material first, and as more non-solvent is added, fractions of progressively lower molecular weight material are isolated. The chromatographic procedure is more rapid and convenient for routine purposes, and involves the separation of dextran molecules on a size basis. Dextran solution is applied to a column containing the fractionating medium (commonly Sephadex), which consists of swollen gel particles. Small molecules are able to diffuse rapidly into the gel, and their passage down the column is hindered. Larger species, on the other hand, can only penetrate the gel to varying degrees depending on molecular size. Molecules are therefore eluted in order of decreasing molecular weight.
Molecular weight determination in fractions isolated by precipitation or gel permeation chromatography enables the molecular weight distribution curve to be established. Values for $M_w$, $M_n$, and the ratio $M_w/M_n$ can also be computed from the data. Analytical gel permeation chromatography provides an alternative method of determining molecular weight distribution in dextrans and avoids the need for collection of fractions (32,33). In order to calibrate the column, it is however, first necessary to prepare a range of narrow dextran fractions by either one of the fractionation methods described.

The molecular weight distribution of a typical dextran (sample no. 12) used in the crystallization studies, was determined by other workers within the IRI group (26). Several sub-fractions were prepared by ethanol precipitation, and light scattering methods used to determine their molecular weight. The integral and differential distribution curves derived from the data are shown in fig.2. Computed values of $M_w$ and $M_n$ for the dextrans, were consistent with experimental values determined by the light scattering and end-group analytical methods described in part I.A.

Uniformity of molecular weight distribution in other dextrans (sample nos. 10,17,19) was confirmed by preparative gel permeation chromatographic studies; viscosity measurements were used to determine the sub-fraction molecular weights (26). The integral curves for these dextrans were essentially superimposable. The molecular weight distribution of dextran sample no.12 was also determined by the chromatographic procedure. The distribution was in excellent agreement with that of the other dextrans, and also with that obtained by the fractional precipitation method (27),
Fig. 2: Integral and differential molecular weight distribution curves for dextran (sample 12)
The relationship between $\bar{M}_w$ and $\bar{M}_n$ depends on the molecular weight distribution, the two averages being equal only for a homogeneous polymer, and otherwise $\bar{M}_w > \bar{M}_n$. The ratio, $\bar{M}_w/\bar{M}_n$, can be taken as a simple measure of the breadth of the distribution in situations where the general form of the distribution curve has been previously established. Measurements of $\bar{M}_w/\bar{M}_n$ were used to show that the width of the distribution remained similar over the range of dextrans studied in this work.

(v) Chain Branching.

The number of branch points in dextrans is known to vary according to the bacterial strain which is used during the fermentation process. *Leuconostoc Mesenteroides*, strain NRRL B512, which was used in this instance, normally gives rise to dextrans with low branch contents of the order of 5% (22). To verify that this was the case in the dextrans being studied, the degree of branching was determined in three samples (nos. 8, 9, 10). This work was conducted by other members of the IRL group (26), using periodate oxidation methods for structure determination (23).

The principle of the periodate oxidation method is that under carefully controlled oxidation conditions, linear 1→6 linked anhydroglucose units yield formic acid, whereas branched units do not. As shown in fig. 3 (a), the predominating 1→6 linked anhydroglucose unit takes up two moles of periodate and liberates one mole of formic acid. If the dextran unit constitutes a branching point, for example in the 3-position (fig. 3 (b), no reaction occurs; however, if the branch is in the 2- or 4- position (fig. 3 (c)), one mole of periodate is consumed but no formic acid released. Thus,
FIG. 3(a) PERIODATE OXIDATION OF DEXTRAN

FIG. 3(b) PERIODATE OXIDATION OF REDUCED DEXTRAN
the presence of 1→2, 1→3 or 1→4 linkages in a dextran molecule will cause it to liberate less formic acid than if it were entirely a linear, 1→6 linked polymer chain. The more branch points which are present, the greater will be the number of end-groups, but these do not make any more contributions to the yield of formic acid than if they were part of the chains. Reducing end-groups, of which there is only one per dextran molecule, do yield two molecules of formic acid (fig.3 (d)). However, this only amounts to an additional 0.4% of the total formic acid liberated from a dextran molecule of molecular weight 40,000.

The % branching (ratio of 1→6 to non-1→6 linkages) in the three dextrans studied, was estimated to be 2.2 ± 0.4%. No significant difference between the samples could be detected by this method. These % branching values are slightly lower, but of the same order of magnitude, as those quoted by other workers, viz. 4 - 5% (22,23,34). Senti and co-workers (29) have also shown that the % branching in dextrans is independent of molecular weight. It was therefore assumed that all the dextrans employed in this work with \( M_w \) in the range 3,000 - 113,000, had similar contents of non- 1→6 linkages.

These assumptions are reasonable on the basis that variations in dextran branch contents can only occur at the fermentation and hydrolysis stages of the preparation process. In the first instance, changes in branching could arise due to changes either in the bacterial strain, or in fermentation conditions. However, these were very closely monitored (20), and structural variations seem unlikely at this point. Secondly, during hydrolysis, 1→3 glucosidic linkages, for example, are known to be cleaved more rapidly than
l→6, types; it is possible that structural differences could arise at this stage (29). However, the content of non- 1→6 links in the polymer is low, and only about 0.2% of all the glucosidic bonds in native dextrans are cleaved during hydrolysis to a molecular weight of 100,000. For this degree of degradation, even if the difference between the rate of hydrolysis of 1→6 and non- 1→6 bonds were considerable, this would not cause a sufficient difference in the composition of the hydrolysis products to be readily detectable.

(f) Aggregation and Crystallization.
The development of particulate matter in dextran solutions during storage has been noted by workers at Fisons Ltd. (3). Similar experiences have been reported by Cadwallader et al (1); on average, these workers found that the amount of particulate matter which formed as "flakes" amounted to 0.003% of the total dextran present. The following observations by Cadwallader and co-workers (1) showed that the particulate material was in fact an aggregated form of dextran:–

(i) both soluble dextran and the particulate matter turned dark brown on heating to 270°C.
(ii) the particulate matter dissolved in hot water (60°C.), giving positive tests for carbohydrate.
(iii) the infra-red spectrum of powdered particulate material showed a strong resemblance to that of dextran.
(iv) examination of the hydrolysis products of both dextran, and of the particulate material, by paper chromatography, indicated glucose to be present.

The material could be redissolved either by heating a dispersion in water, or preferentially, by autoclaving the dispersion at
121 °C. for 30 minutes. Eirich (3) however, reported the existence of "flakes" which resisted dispersion on heating. This suggests that the heat stability of the particles may be dependent on the conditions under which they are formed.

Further studies on the nature of particulate material in clinical dextran solutions have been reported by Grasel (3). The indications were that the insoluble matter possessed the same molecular weight distribution and degree of branching as the soluble dextran from which it was derived. X-ray diffraction studies confirmed the crystalline nature of the large insoluble "flakes". The diffraction patterns observed bore a strong resemblance to those described by Jeanes (4), relating to dextran crystallized from aqueous solution by precipitation with ethanol. The birefringent nature of the "flakes" was also noted by Eirich (3); this is further evidence that their formation involves a process such as crystallization which leads to orientation of the polymer chains. It is thus considered very probable that the small insoluble particles, responsible for turbidity in dextran solutions, are precursors of these larger aggregates, and are also crystalline in structure.

The formation of particulate material in dextran solutions contained in vacuum-sealed bottles has been ascribed to storage conditions under which the top of the bottle is at a higher temperature than the bottom (18). In addition, the appearance of "flakes" can be accelerated by intermittent exposure of bottled solutions.
to elevated temperatures (3). Such conditions lead to the formation of surface skins. These arise due to evaporation of water, either from the surface of the bulk liquid, or from the surface of droplets of solution, which are present on the walls or stopper of the bottle in the space above the liquid. "Flakes" are believed to form in such skins, and drop into the bulk solution when the bottle is moved. No reasons as to why aggregation should occur preferentially in surface skins under such conditions have been put forward. It is well recognized that aggregation does not occur in the bulk solutions; under uniform temperature conditions, storage for extremely long periods (over five years) without the development of particulate matter, has been shown to be possible (35).

The formation of "flakes" occurred more rapidly and to a greater extent in fractions of low molecular weight dextran (23). Eirich (3) also reported an inverse relationship between turbidity and molecular weight ($M_w$) for dextrans in the molecular weight range 43,000 - 186,000; dextrans of $M_w$ above 100,000 exhibited very low turbidities. These observations suggested a dependence of crystallization rate on molecular weight, and the effect was investigated in the work reported in part I.C.

In the non-medical literature, early references to insoluble dextran are mainly concerned with correlating crystallinity, as measured by X-ray diffraction methods, with solubility properties (4). It is inferred that those dextrans producing X-ray diffraction powder patterns are partially crystalline in nature. Jeanes (4)
describes the preparation of a water-insoluble dextran by fermentation using *Leuconostoc Mesenteroides*, NRRL B523. This dextran was insoluble in boiling water, but dissolved in potassium hydroxide solution (0.75N). However, this dextran gave an essentially amorphous X-ray diffraction pattern. This indicates that, although molecular association must be extensive, a regular arrangement of chain segments in a periodic three-dimensional structure must not be present.

Jeanes et al. (4) also produced dextran by fermentation using *Leuconostoc Mesenteroides* NRRL B512; this was the strain employed to prepare the native dextran from which the acid-hydrolysed fractions used in this work were derived. While this dextran was normally water soluble, partial insolubility could be induced by three procedures, (a) by drying of aqueous pastes at high temperature, (b) by precipitation from aqueous solution with large amounts of ethanol, and (c) by humidification for extended periods at high relative humidity. In each case the transition from solubility to insolubility in water was accompanied by a change in X-ray diffraction pattern. As crystallization took place, the pattern reverted from one which was amorphous to one containing a distinct set of lines. The relative intensities of the lines and the background in patterns from the native dextrans indicated that the amount of crystalline material produced under these conditions was rather small.
It is significant that in this work, Jeanes (4) also briefly studied several products of acid hydrolysis of native dextran; the native polymer was produced by fermentation using *Leuconostoc Mesenteroides*, NRRL B512. Acid-hydrolysed fractions of low molecular weight were found to develop crystallinity during fractional precipitation with ethanol. Although higher molecular weight fractions remained amorphous during the fractionation procedure, crystallization could be induced either by humidification, or by extended contact with aqueous ethanol. These findings are in agreement with observations reported by Fisons Ltd. (3), that insoluble dextran can arise at this stage of the preparation process, and also that the rate of formation increases with decreasing molecular weight. In the work described in this thesis, (part I.D.), it was indeed verified that the development of turbidity accompanied fractional precipitation of the dextrans with ethanol.

Fisons Ltd. (3) also noted that insoluble dextran could be introduced into the product during drying stages. This observation is in accord with a similar observation of Jeanes (4), but relating to native dextrans, rather than to acid-hydrolysed products. However, it is almost certain that the crystallization behaviour of a native dextran will be reflected at least in part, in that of derived fractions of lower molecular weight.

The effect of structure on crystallinity in dextrans was investigated by Senti and co-workers (5,36). The general conclusion was that only those polymers with low degrees of branching, i.e. with high proportions of 1→6 linkages, were capable of crystallization.
For each native dextran, crystallization was induced by storage at high relative humidity. The dextran produced by fermentation using Leuconostoc Megenteroides, NRRL B512, contained 5% 1→4-linkages, and was amongst those dextrans in which strong X-ray diffraction patterns developed. It has been shown above that the dextrans to be studied in this present work, which were prepared using the same bacterial strain, had a similar content of non-1→6-linkages. The ability of these dextrans to crystallize is thus attributable with their low degree of molecular chain branching.

About the same time as this work was completed, it was reported by Pasika and West (1972) that small spherulites, with diameters in the range 8-25 μm, could be formed in fractions of acid-hydrolysed dextran (6). Films containing 50 - 60% crystallinity were prepared by replacement of water with another solvent in dextran gels. Following this report, more detailed investigations into the morphology of dextran spherulites was undertaken by Barham and co-workers (7). In this work, which has only been recently reported (1974), spherulitic dextran films were obtained by evaporation of dilute solutions (10% w/v) at relatively high temperatures. The dextrans were similar in branch content (5% non-1→6 links) to those used in this work, and three fractions with molecular weights (Mw) of 3000, 40,000 and >10^7 were studied. The solvents employed were dimethylsulphoxide (DMSO), formamide and water, at temperatures between 70 °C. and 180 °C. Using the evaporation procedure, dextran films with spherulitic diameters in the range 10 - 150 μm were obtained. The average size of the spherulites depended on the dextran molecular weight, on the evaporation temperature, and on the solvent used.
It is interesting to note that for any solvent, concentrations of <60% w/v, and high evaporation temperatures were necessary to produce structures which could be resolved in the optical microscope. Crystallization from aqueous solution below 100 °C gave rise only to very small spherulites (max. diameter 4 μm), in keeping with observations reported in part I.B. of this work.

Barham et al. (7) obtained a measure of growth rate by evaporating solutions at a fixed temperature for given lengths of time, and then measuring the mean diameter of the spherulites formed. However, since the early stages of crystallization could not be observed, no conclusions are possible regarding the mechanisms of nucleation and initial growth. In addition, the dextran would pass through a wide range of concentration conditions during crystallization, which would continuously alter the crystallization rate. Nevertheless, the results do indicate that growth is some 100 times faster with the dextran of molecular weight 40,000 than with the high molecular weight dextran ($M_w > 10^7$). Dextran of molecular weight 2400 had a growth rate lying between that of the two higher molecular weight samples.

The increase in rate with increasing molecular weight observed by Barham et al. (7) is consistent with established behaviour in synthetic polymers (37,38,39). The decrease in rate for the dextran of molecular weight 2400 relative to that for the sample of molecular weight 40,000 suggests a different mode of crystallization for the low molecular weight polymer. It is possible that the molecular weight is insufficiently high for typical polymeric behaviour to be exhibited (40). This is supported by the fact that the activation
energy for spherulitic growth measured for the low molecular weight sample, is lower than that for the two higher molecular weight dextrans. It is possible that the short chains of the low molecular weight dextran do not crystallize in the chain-folded configuration (41), which is typical of spherulites grown from higher molecular weight, synthetic polymers. Since no energy to fold the chains is required, a lower activation energy would be anticipated. The observed behaviour is very similar to that observed in polyurethanes (42), and in polyethylene (43), where a certain minimum molecular weight must be exceeded for chain-folding to be observed.

Barham et al. (7) observed two distinct types of spherulite, depending on the molecular weight of the dextran. Positive banded spherulites, i.e. with radial refractive index greater than tangential refractive index, were obtained from the high molecular weight dextran. In the case of the low molecular weight dextran, the spherulites were negative (tangential refractive index greater than radial refractive index), and unbanded. A review of this type of behaviour, which has also been noted in other polymers, is presented; a possible interpretation of the observations in terms of crystal morphology is also given (7).

When subjected to X-ray diffraction, all the spherulitic films prepared by Barham et al. (7) gave powder diagrams with the same interplanar spacings. These patterns were similar to the L-2 patterns obtained by Jeanes and co-workers (4), using dextran crystallized from aqueous solution by precipitation with ethanol. It is inferred that the crystalline structures produced from dextran solutions by
evaporating solvent, are basically similar to those formed by lowering the power of the solvent system.

In view of the previous investigations reviewed above, it was logical to assume at the outset that the insoluble material responsible for turbidity was crystalline dextran. This was justified on the basis that the particular dextran concerned was characterised by a low branched-chain content, and would therefore be liable to crystallize. However, the possibility, for example, of the occurrence of chemical cross-linking reactions, or of the presence of colloidal impurities, could not be totally excluded. A section of the work was therefore devoted to conclusively demonstrating that crystallization was responsible for turbidity in solutions of the dextrans provided by Fisons Ltd.

2. **CRYSTALLIZATION AND MELTING IN POLYMERS.**

Crystallization in polymers is normally studied by cooling from the melt, and much of the general information relating to polymer crystallization has been derived under such conditions (44,45). More recently however, studies of polymer crystallization from solution have become more frequent (46, 47). Single crystals, rather than polycrystalline growth units, can often be formed from dilute polymer solutions. The morphology and growth mechanisms of such single crystals are much simpler to evaluate than those of melt-crystallized polymers. Considerable interest in relating the structure of single crystals to that of the less well characterised structures evident in melt-crystallized samples has therefore arisen (48). In addition, with polymers such as dextrans, which decompose below their melting point, crystallization from solutions is the only convenient method by which the
crystallization behaviour of the polymer may be studied.

Irrespective of the manner in which polymer crystallization is induced, there are two important ways in which the crystallization process differs from that in low molecular weight species (45). Firstly, in the latter case, the process of crystallization is virtually complete. However, in polymer crystallization, a large fraction of the disordered structure characteristic of the amorphous state remains in the growth units. The amount of amorphous component present depends on the nature of the polymer and on the crystallization conditions; it generally constitutes between 20% and 80% of the total mass. The second difference is that melting in crystalline polymers takes place over a range of temperatures; the range may cover up to 100 °C. On the other hand, with low molecular weight crystals, a sharp melting point is observed. The melting range in polymers is considered to be due to variations both in the size and in the degree of perfection of the crystals (44,45).

(a) Morphology.
The morphology of crystalline polymers is complex. Regions of order known as crystallites, with dimensions in the range 10 - 100 nm, have been shown to exist in high polymers (44,45); these ordered regions are considered to be dispersed in an amorphous matrix. Polymer chains may pass through several crystalline and amorphous regions, with the chain segments in the crystalline regions being held together by primary valence bonds.

In many polymers, aggregates of crystallites are observed which are known as spherulites. These are spherically symmetrical, birefrin-
gent entities of sufficient size (>1 µm) that they can be seen to nucleate and grow in many polymers (45). In some instances, however, the spherulites may not be sufficiently large to be resolvable in the optical microscope. The presence of fibrous sub-units (fibrils) with cross-sections in the range 10 - 1000 nm is a characteristic feature of spherulitic structures. Fibrillar development during spherulitic growth is well established, with the fibrils radiating outwards from a central, single crystal nucleus (45). The crystallites are believed to be contained within these fibrils. It is interesting that in spherulites, the long-axes of the polymer chains are oriented across the radiating fibrils (49). Since the refractive index along a chain is normally different from the average value across it, because of this tangential orientation effect, light is extinguished in opposite quadrants when the spherulite is viewed in a microscope under crossed polariser. This gives rise to the Maltese cross pattern observed in all finely textured spherulites (44).

Polymeric single crystals with dimensions in the region of 10 - 1000 nm may be prepared by crystallization from dilute solutions (50,51). However, the relationship of these crystals to more complex morphological structures described above, which can be produced under melt crystallization conditions, is not completely clear. Much of the detailed work on single crystals has in fact been performed using polyethylene (52,53), although other polymers including the polysaccharide ester, cellulose triacetate, have also been studied (54). These single crystal structures take the form of a flat lozenge, about 10 nm thick, and with the chain molecules oriented across the thickness of the lozenge rather than in its
plane (50,52,55). It is evident that for polymer molecules up to 1 μm in length, chain folding must take place. The mechanism by which this occurs is however controversial (55).

One approach to determining the exact relation between single crystals and complex spherulitic structures, has been to study the progressive change in the morphology of species produced from solutions of increasing concentration (56). It is suggested that as the concentration increases, multi-layers of single crystals are produced which splay apart from a common axis; eventually, molecules will crystallize through several of these layers to give structures resembling incipient spherulites. Another approach (57) suggests that one face of the single crystal would develop fibrils preferentially, and this hypothesis is supported by the similarity in chain orientation observed in both single crystals and in the radiating fibrils of melt crystallized structures.

It is clear that a variety of structural features may exist simultaneously in polymers, at different levels of size. For example, in systems with a high nucleation density, the growth may be limited to a large number of partially developed pre-spherulitic structures e.g. truncated fibrils (45). At lower nucleation densities, a lesser number of fully developed structures may develop, with the fibrils being present as components of the larger structure. In kinetic studies, which are of interest in this work, it is important to know the form of the development; if several developing structures are present, the nature of the predominating growth species must be determined. If spherulites can be shown to develop, it is also necessary that crystallites should also form. However,
if the latter grow more rapidly than the former, and within the outlines of the larger structure, it is the nucleation and growth of the spherulite which is important, since this is the rate determining stage. In the work described below on dextran crystallization (part I.C.) the growth species formed were in fact spherulitic and their development from pre-spherulitic structures, also spherical in symmetry was demonstrated. Emphasis is therefore placed on the point that nucleation and growth of the spherulite or its precursor can be legitimately considered as the rate determining step in kinetic studies on dextran crystallization, even although smaller component units of structure may be present (45).

(b) Analysis of Crystallization Kinetics.

Structure characterisation, with the objective of establishing details of morphology in crystalline dextran particles, is not the purpose of this work (c.f. 6,7). The emphasis is on studying the kinetics of crystallization, and in order to do this, it is only necessary to be able to follow the development of structure.

Various methods are available for this purpose, and these are reviewed briefly below, as an introduction to section I.C. Light scattering methods were in fact selected as being most appropriate for the study of dextran crystallization, with a view to providing information both on nucleation and growth stages of the process.

The use of light scattering methods to follow rates of polymer crystallization and gelation in solution is well documented in the literature; procedures similar to those employed in this work have been used by Lanceley and Sharples (51) in studies on poly-(decamethylene terephthalate), and aggregation in amylose (58), a
polysaccharide bearing many resemblances to dextran, has also been investigated using light scattering techniques.

The general treatment of the kinetics of phase change were first developed by Avrami (59) in 1940, the principles being applied extensively to crystallizing polymer systems in the 1950's by Morgan and co-workers (60) in the U.K. and by Flory and Mandelkern in the U.S.A. (44). The analysis is based on various assumptions relating to the nature of the nucleation and growth processes involved.

If nucleation is random, or homogeneous, spontaneous aggregation of polymer chains occurs below the melting point of the polymer. The rate of appearance of regions where aggregation occurs involves a first order dependence on time. If \( n \) is the number of nuclei produced per unit volume at time, \( t \), then

\[
\frac{n}{Nt} = \frac{7}{7}
\]

where \( N \) is the nucleation constant.

Heterogeneous nucleation, on the other hand, arises from adventitious impurities or partially formed growth particles; with this type of nucleation, a limited number of growth centres becomes instantaneously effective once the conditions of crystallization are reached. The rate of appearance of nuclei thus has a zero-order dependence on time, and

\[
\frac{n}{N} = \frac{8}{8}
\]

After nucleation has occurred, growth may take place in one, two or three dimensions to give rods, discs or spheres respectively, until eventually the growth units impinge and growth is halted. During
growth, it is normally assumed that the rate of increase of the linear dimension, $r$, of the growing particles involves a first order dependence on time, i.e.,

$$r = G t$$ \hspace{1cm} (9)

where $G$ is a growth constant.

However, eqn. 9 only applies when the growth process is controlled by secondary nucleation (44). In this process, a molecule diffuses through the supercooled melt or solution, and takes up a position on the growing crystal face. Rapid migration to positions of lower free energy on the face will occur until all sites are filled and the surface layer is complete (45). After this, the rate determining step for growth is the formation of a new secondary nucleus on the crystal surface. However, it is evident that under certain conditions, for example, during crystallization from viscous polymer solutions with high rates of secondary nucleation, the overall growth process could become controlled by the diffusion of polymer to the growing face (44). In these circumstances, the growth rate is given by,

$$r = G' t^{\frac{1}{2}}$$ \hspace{1cm} (10)

where $G'$ is a growth constant containing the appropriate diffusion coefficient.

In the mathematical treatments of Morgan (60) and of Flory and Mandekern (44), a relationship is established between the amount of crystalline material formed, and the time of crystallization. The relationship depends on three factors: (i) the nature of the primary nucleation process, (ii) the geometry of the growth particles, and (iii) the process, either diffusion or secondary nucleation, which
controls growth rate. In the early stages of growth, the derivation of the appropriate equation is simple, no allowance being necessary for the effect of impingements of the growing bodies. Since it is shown in this work (part I.B.) that the extent of crystallization in dextran is low (<5%) over the period of observation concerned, the simpler treatment is considered satisfactory.

The relationship between the amount of crystalline phase, \( m_c \), formed from a given mass of liquid or amorphous polymer, and the time of crystallization, \( t \), takes the general form,

\[
m_c = kt^m \quad \text{(11)}
\]

\( k \) is a composite constant, which contains nucleation and growth constants, and the densities of liquid and crystalline phases. The most important parameter is the Avrami exponent, \( m \). This parameter is a combined function of (i) the number of dimensions in which growth takes place, (ii) the time dependence of the growth rate determining process, and (iii) the time dependence of the primary nucleation process. For three dimensional, spherulitic growth, the possible values which \( m \) may assume are detailed in Table 1.

<table>
<thead>
<tr>
<th>Primary Nucleation Process</th>
<th>Process Determining Spherulitic Growth Rate</th>
<th>Value of Avrami Exponent, ( m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogeneous</td>
<td>Secondary Nucleation</td>
<td>4</td>
</tr>
<tr>
<td>Homogeneous</td>
<td>Diffusion</td>
<td>2.5</td>
</tr>
<tr>
<td>Heterogeneous</td>
<td>Secondary Nucleation</td>
<td>3</td>
</tr>
<tr>
<td>Heterogeneous</td>
<td>Diffusion</td>
<td>1.5</td>
</tr>
</tbody>
</table>

TABLE 1: Dependence of Value of Avrami Exponent on Primary Nucleation and Spherulitic Growth Rate Determining Processes.
Data on crystallization kinetics may be fitted to an Avrami equation by plotting amount of crystalline material formed as a function of time, on a logarithmic basis. From the experimentally determined value of $m$, it is then possible to determine mechanism of crystallization, particularly if the geometrical form of the growth particles has been established by independent methods.

Some crystallization processes show deviations from the Avrami relationship. This may be due to the occurrence of simultaneous processes, such as the growth of similar units by different nucleation mechanisms. Another possibility is that consecutive processes may take place, in which, for example, spherulitic growth may initiate from rod or disc-like nuclei. All such cases can lead to the situation where the apparent value of $m$ is continually changing, and many of the experimentally observed deviations can probably be explained in this way (45).

In the case of crystallization from solution, as crystallization proceeds, solvent is excluded from the developing crystalline regions. This causes a corresponding decrease in polymer concentration in the surrounding liquid phase. Consequently, the density of the amorphous phase is changing, which means that some modification is necessary to the value of $k$ in eqn.11. The decreasing concentration of the liquid phase also means that the melting point of the polymer in this phase is decreasing. At any temperature, the degree of supercooling is reduced and rate of crystallization decreases. One consequence of this is that the diameter-time plots,
for growth of crystalline particle from solution, tend to be curved (44,45).

(c) **Nucleation and Growth Mechanisms.**

The number of nuclei initially present or appearing during the course of crystallization is important in that it determines the final size of the growth particles. This is due to the fact that the larger is the number of nuclei, the smaller is the fraction of the total crystalline material which can be distributed between the growth centres, and the smaller are the final dimensions of the particles.

In the case of homogeneous nucleation, nucleation rate is time dependent, and is considered to depend on temperature in much the same way as the overall crystallization rate (44,45). In the case of heterogeneous nucleation, the process is instantaneous, either with the nucleation density increasing with degree of supercooling, or constant, if a limit on the number of heterogeneous centres has been reached (61). The distinction between the two processes disappears if heterogeneous centres are brought into play successively; Sharples (45,62) in fact argues that apparently homogeneous nucleation processes may also involve heterogeneities. A nucleation process may be apparently heterogeneous or homogeneous, depending on the time at which the limiting value in the number of nuclei is reached, relative to the total crystallization time.

The nature of the heterogeneities responsible for instantaneous nucleation is not certain. It is argued that chemically different centres may not necessarily be present; regions of physical ordering
in the amorphous polymer, persisting above the melting point, may
be responsible for nucleation (63). Such regions would only dis-
perse very slowly on melting and the size and number would depend
strongly on the previous crystallization and melting history of the
polymer. For example, in poly(ethylene oxide) the existence of
stable heterogeneous nuclei above the melting point has been
demonstrated, the stability decreasing with increasing melt temp-
eratures (64,65).

A further suggestion by Price and Turnbull (66,67) is that stable
heterogeneous nuclei arise from particles containing crevices in
which crystallization can take place. On subsequent melting, the
stability of the particles is enhanced by elevation of its melting
point. This occurs due to adhesion of the polymer to the original
nucleating particle.

The phenomenon of self-seeding is important in controlling the
nucleation density in crystalline polymers (62,68,69). Because of
the existence of a melting range, small amounts of crystalline
material may persist when the bulk has melted, and such material is
capable of nucleating the crystallization process. The more effect-
ive the melting process is, the lower is the amount of crystalline
material capable of self-nucleating the crystallization process.
Particle growth into spherulitic structures commences from such
partial growth units, or seeds, in the same way as the seeds them-
selves develop from primary nuclei. With some polymers, it has also
been demonstrated empirically that nucleation density can be increased
by the addition of certain nucleating agents (70).
In polymer systems crystallizing in the absence of solvent, growth is generally controlled by secondary nucleation, rather than by diffusion, despite the viscous nature of the melt. However, as intimated by Mandelkern (44), it is probable that diffusion processes may control growth if solvent is present. The mechanism of growth can generally be established by observing the time dependence of one-dimensional growth in the crystallizing particles; an increase in linear dimensions with \( t^{1/3} \) indicates diffusion control of kinetics. During crystallization, various particle morphologies can develop depending on the particular conditions (solvent, temperature, etc.) involved, and for kinetic studies it is necessary to establish which is the dominant growth mechanism. The work of Keller (56) indicates that the platelets which frequently form in dilute solution, tend to assume a spherulitic shape as the polymer concentration increases. It is not therefore unreasonable to anticipate that spherulitic structures might also develop during dextran crystallization from concentrated solutions (67).

(a) Melting.
An understanding of the process of melting is often helpful in providing information as to the nature and stability of the crystalline particles undergoing the change of phase. The existence of a range of temperatures over which crystalline polymers melt (44,45), suggests that a corresponding range of crystallite stabilities exist. In all crystalline particles, a certain fraction of the surface will contain imperfect regions. However, with the smaller particles, the surface to volume ratio is high; the effect of the less perfect surface regions will therefore dominate, resulting in a lowering of the melting point relative to that of the hypothetical perfect crystal. It is thus generally observed that a variety of crystalline
particle stabilities exist, the melting range depending on the crystallization conditions employed (45,71). The effect of crystallization temperature on melting behaviour has been particularly noted (72,73); more stable crystalline entities are formed as the crystallization temperature increases, i.e. as the degree of supercooling, and hence crystallization rate, decreases.

The melting point, $T_m$, of a polymer is important, in that it is the prime factor determining whether crystallization can be induced at any given temperature. Crystallization rate becomes appreciable at some temperature, $T$, below $T_m$, increases until a maximum is reached, and then decreases to a value of zero, just above the glass point, $T_G$ (fig. 4). The dominant effect of supercooling, $(T_m - T)$, in determining rate is well established (45).

Crystallization and melting processes in polymers normally follow different paths (45,74). Crystallization involves nucleation and growth of birefringent units, whereas melting generally involves a decrease in birefringence which is uniform throughout the sample. However, where stable heterogeneous nuclei exist, the melting process may not be totally homogeneous in growth units derived from such nuclei (63,66,67). It is thus possible that the constituent crystalline units present in a large growth structure, may be induced to melt successively under different conditions. In this case, the melting process may follow, in reverse, the same stepwise path as that of the crystallization process by which the structure was built up.
(e) Factors Affecting Crystallization and Melting.

(i) Chemical Constitution.

The two factors which determine whether polymeric chains are capable of crystallization are, (i) chain mobility, and (ii) the free-energy change on crystallization. Chain mobility is necessary to allow motion of chain segments from their original positions to produce regions of local alignment. The more closely the chains can pack in this condition, the stronger will be the bonding forces acting between the segments, and the greater will be the free energy change for the process (44). Flexible linear molecules with no bulky side groups can readily take up the new configurations required by the crystalline state e.g. polyethylene, or cellulose. On the other hand, the presence of bulky side groups in a linear chain reduces rate of crystallization, and normally diminishes the stability of the final structure. In polystyrene, for example, and in related vinyl polymers, virtually no crystallinity is generally developed. However, in certain circumstances where stereo-regular sequences predominate in the chain, crystallinity may be induced (45). The presence of branch points or cross-links in polymer chains also reduces crystallization rate (44,45). The effect of chain branching in dextrans on crystallinity has in fact been considered by Senti et al. (5), and is discussed above.

In a number of polymers, including the polysaccharides, the hydrogen bond plays an important part in strongly binding together the polymer chains in the crystalline region (75,76). In these circumstances chemical agents, such as urea or sodium hydroxide, which tend to break hydrogen bonds (77), will be effective in dispersing crystalline material. The existence of hydrogen-bonded crystalline structures in
a water-insoluble polymer can therefore be demonstrated if the material dissolves under the action of these specific chemical agents.

(ii) **Temperature**

The effect of temperature on crystallization rate has already been considered in the section on melting. The relationship is well established for situations in which the kinetics are controlled by secondary nucleation, and takes the form shown in fig. 4. It is the temperature relative to the melting point which in fact controls rate, rather than the absolute temperature. To compare rates of crystallization under different conditions of solvent concentration etc., it is therefore only significant if comparisons are made at the same degree of supercooling. It is evident that the maximum in the rate-temperature plot will disappear if the growth rate is diffusion controlled at temperatures in the region of $T_m$.

![Temperature Dependence of Crystallization Rate](image)

**FIG. 4: TEMPERATURE DEPENDENCE OF CRYSTALLIZATION RATE**
(iii) Molecular Weight.

Melting point is depressed as molecular weight decreases, since the chain ends act as diluents and tend to be excluded from the crystallites (44). However, when account is taken of this effect, which is not generally large, lower molecular weight polymers invariably crystallize more rapidly (36, 38, 39, 45). Typical polymeric behaviour is not attained until a particular molecular weight is exceeded (39, 40, 43, 44). With very low molecular weight polymers, decrease in rate may be associated with changes to a new crystallization regime (7, 44). Also, for a given average molecular weight, rate of crystallization decreases as the distribution broadens (45).

As molecular weight decreases, diffusional transport to the growing crystal face increases. This is due to the fact that the diffusion coefficient increases, and the solution viscosity decreases, with decreasing molecular chain length. If the crystallization kinetics were diffusion controlled, a marked increase in rate with decreasing molecular weight would be anticipated.

(iv) Solvents.

Solvent, like molecular weight, affects melting point and hence crystallization rate. The free energy of the crystalline segments is not affected by solvent, since there is no solvent in the crystal structure. However, in the amorphous phase, the interaction of the polymer with the solvent affects the free energy of the segments considerably. The driving force for the melting process is the difference between the chemical potentials of the polymer in amorphous and crystalline states. This being the case, the melting point will depend on the amorphous polymer concentration, and decrease as the
amount of solvent increases. With poor solvent, due to the lower interaction between polymer and solvent, the melting point depression is less marked.

For a good solvent, the effect of polymer concentration on melting point is illustrated in fig.5 (a). However, with poor solvent, liquid-liquid phase separation may occur, as indicated in fig.5 (b), point X. Below the concentration defined by point X, the mixture may exist either as a single-phase liquid, or two-phase liquid; a third possibility is that crystallization of the polymer-rich phase can occur at lower temperatures. In crystallization studies from solution, it is therefore necessary to establish that a genuine liquid-crystal transition is involved. The opposite holds if the
The objective is to fractionate a polymer by non-solvent precipitation, in which case the transition required to ensure effective fractionation, is from single-phase liquid to two-phase liquid (31).

The effect of solvent on crystallization rate, when comparisons are made at the same degree of supercooling, has not been widely studied. With poly(ethylene oxide) solutions, Mandelkern (44) has shown that solvent has no dramatic effect on rate. However, it is pointed out that the diffusion of polymer segments to the crystallite-liquid interface must assume a more important role in the growth process as the system is diluted. Diffusion control of kinetics is therefore a distinct possibility over particular ranges of polymer concentration. In such a case, at the same degree of supercooling, the effect of solvent concentration on crystallization rate would be determined by the concentration dependence of the product, \( D \Delta C \). \( D \) is the diffusion coefficient of the polymer in the solution, and \( \Delta C \) is the concentration gradient between crystal surface and bulk solution.

(f) Crystallization in Polysaccharides.
Although crystallization in polysaccharides has been extensively studied from the point of view of the structures formed, kinetic studies of the process have received much less attention. Atkins and Mackie (78) have remarked on the lack of knowledge of the effects of temperature, solvent, etc. in promoting structural changes in natural polysaccharide systems. These workers studied crystallization in several unbranched biopolymers containing alginate and mucopolysaccharide groups. It was found that high temperature and high relative humidities favoured the rapid development of crystallinity. These conclusions are essentially similar
to the earlier findings of Jeanes, Senti, and co-workers (4,5,36), relating to crystallization in dextrans and in amylaceous polysaccharides. To explain these results, it is not unreasonable to assume that the crystallization processes may be treated in the same general terms as those in synthetic polymer systems. Although this approach has been widely adopted by workers in the area of crystallography, attempts to interpret polysaccharide crystallization kinetics in terms of the established behaviour of synthetic polymers have been much less frequent.

Crystallization in polysaccharides is also important from the point of view of gel formation. Again, in the studies reported, the emphasis has been placed on the investigation of structure rather than on the kinetics of gel formation. In the presence of aqueous solvents, polysaccharides typically form three-dimensional network structures known as gels. These gels have been extensively studied by Rees (79); they are believed to consist of amorphous regions, in which the chain segments exist in the random coil conformation, and crystalline regions in which the chain segments have an ordered structure. The differences between various polysaccharide gels appear to be determined largely by the strength and number of intermolecular linkages between chain segments. These, in turn, depend on the chemical structures of the polysaccharides and on the interactions between polymer and solvent, which enable the polymer chain segments to attain the necessary conformations in the crystalline zones. Studies relating to the structure of gels obtained from polysaccharides such as cellulose and its derivatives, chitin, and amylose have been reviewed by Rees (79). Dextran is also a gel-forming polysaccharide; in this work, the objective is to
study the initial crystallization process which takes place prior
to gel formation, rather than to examine changes in the nature of
the gel structures which are formed at a later stage.

Natural and regenerated celluloses are crystalline in nature. Al-
though spherulites have not been observed, the existence of micro-
fibrils is well established (75). Cellulose, in the regenerated
form, is crystallized during the spinning process by precipitation
from solution. At this stage, the number of nuclei present is
probably so large that the incipient spherulites develop only to
a limited extent, due to impingement of the growing centres.
Spherulites are generally considered to be rod-like in nature at
the early stages of development (44, 45), and it may be that only
the fibrillar stage is reached. In all probability, cellulose is
thus different from other crystallizable polymers only in that its
nucleation density is high, and the growth units are small. This
may be associated either with the basic nature of the cellulose
molecule, or more simply, with the way in which the polymer is
normally crystallized, i.e. from solution (80).

The similarity of cellulose to synthetic polymers is indicated by
the fact that spherulites can be formed in cellulose derivatives
(80). Manley (54) has also shown that cellulose triacetate can be
crystallized in the form of single-crystal lamellae, exhibiting the
characteristic chain folding of polymers such as polyethylene.

Amylose, the linear component of starch, is one polysaccharide in
which the crystallization process has been studied in some detail
(81, 82). Amylose crystallizes readily and completely from dilute
solution (<1%), to form lamellar single crystals; these exhibit the chain-folding structures characteristic of several synthetic polymers (83,84). More concentrated amylose solutions readily form gels (79).

Crystallization of amylose is responsible for the phenomenon of retrogradation in starch (81). In this process, starch in the soluble or hydrated state reverts to a water-insoluble form. The process also occurs in the solid state, and is believed to be responsible for the staling of bread (82). Starches precipitated by ethanol and left moist readily retrograde (82).

The factors affecting rate of crystallization in amylose, viz. molecular weight, temperature, concentration, etc. have been studied by Paschall and Foster (85), and by other workers (81,86, 87); the following conclusions were evident: -

(i) rate increases with increasing temperature up to about 60-70°C.; above this temperature, amylose solutions remain stable (81).
(ii) rate increases with increasing amylose concentration, and an initial induction period is present (87).
(iii) rate goes through a maximum in the 50,000 molecular weight region (86).
(iv) rate is retarded by the presence of urea, and by salts of monovalent ions (87).

Jeanes et al. (4) and Taylor et al. (36) report that amylose responds to humidification and to contact with aqueous-ethanol in the same way as dextran. Further similarities between amylose and
dextran exist, in that with both polymers, rate of crystallization has been found to depend on the conditions of precipitation and drying (85). Additional comparisons between the behaviour of the two polymers will be made throughout the course of this work.

No explanations for these empirical observations, relating to crystallization in amylose and dextran, have been proposed. It is therefore an objective of this work to rationalize these findings in terms of the established behaviour of polymeric systems. It is also envisaged that a study of the crystallization process in dextrans may lead to a greater understanding of crystallization in other polysaccharide systems.

3. OBJECTIVES OF WORK.

The main objective of this work was to determine the factors responsible for the formation of particulate matter in dextran solutions. This material causes turbidity, and renders the solutions unsuitable for clinical use as blood plasma substitutes. In the first instance, it was necessary,

(i) to identify the nature of the particulate material.
(ii) to identify the processes responsible for its formation.
(iii) to define conditions under which the formation process might be studied in the laboratory.
(iv) to identify processes by which the particulate material might be destroyed, or its formation minimised or prevented.

Early in the study, it was shown that crystallization was responsible for the formation of particulate material in dextran solutions. Furthermore, it was found that the crystallization process could be conveniently studied in concentrated aqueous solutions. A range of
dextrans was therefore characterised so that a systematic study of the crystallization process could be made.

The overall objectives of detailed studies on dextran crystallization were as follows:

(i) to investigate crystallization and melting processes in dextrans, and determine whether they are similar to those in other polysaccharides, and in polymers generally.

(ii) to identify critical stages in the preparation process at which formation or destruction of crystalline material might occur.

(iii) to indicate how conditions at critical preparation stages might be adjusted to minimise the formation of crystalline dextran during preparation.

(iv) to indicate how preparation and storage conditions might be controlled to minimise the formation of crystalline material during storage of clinical dextran solutions.

In order to achieve these objectives, it was necessary to conduct experimental studies with the following specific aims:

(i) to establish light scattering methods of measuring crystallization rate.

(ii) to identify the nucleation and growth mechanisms involved.

(iii) to study the effects of nucleation density on crystallization rate.

(iv) to evaluate effects of parameters such as temperature, dextran concentration and molecular weight of dextran,
on crystallization rate.

(v) to compare rate of crystallization in aqueous solution with that in aqueous-ethanol solution; the latter solution arises when the polymer is fractionated by partial precipitation from aqueous solution with ethanol.

(vi) to identify methods for destroying crystalline material.

(vii) to identify crystallization conditions conducive to the formation of stable particles resistant to melting.
RESULTS AND DISCUSSION
I.A. CHARACTERISATION OF DEXTRANS.

Following an assessment of the methods available, the weight-average molecular weight (\( M_w \)) of several dextrans was determined by light scattering procedures. Viscosity measurements were also performed on these dextrans, and the viscosity - \( M_w \) correlation shown to be in good agreement with results quoted in the literature (25,29, 30). Using the established correlation, \( M_w \) was then determined for several additional dextrans by means of viscosity measurements alone.

An end-group analytical procedure was selected for determination of number-average molecular weight (\( M_n \)), and \( M_n \) was measured for selected dextrans within the range under study. The characterisation data are summarised in table 17 at the end of part I.A.

1. DETERMINATION OF WEIGHT-AVERAGE MOLECULAR WEIGHT.

(a) Assessment of Available Methods.

Two absolute methods exist for determining the weight-average molecular weight of a polymer (88,89), viz. light scattering and ultracentrifugation. In the latter procedure, if sedimentation velocity measurements are performed, whereby the sedimentation coefficient, \( S_o \) is evaluated, a simultaneous determination of the diffusion coefficient, \( D_o \), is also required. However, the average molecular weight derived by combining weight-average values for \( S_o \) and \( D_o \) is not generally \( M_w \), but some other average which depends on the shape of the polymer molecule. Sedimentation pseudo-equilibrium methods are somewhat simpler to apply since diffusion measurements are not required. Nevertheless, the experimental procedure is still lengthy.

The light scattering method was therefore considered to be better suited for the rapid determination of \( M_w \) in dextrans.
Viscosity measurement is frequently employed as a very rapid and convenient procedure for determining $\bar{M}_w$ in polymers (28,89). However, the method is not absolute, in the sense that empirical correlation constants, by which $\bar{M}_w$ and viscosity can be related, must first be determined (eqn. 4). Such correlation constants were established for dextran by performing both viscosity and light scattering measurements on selected samples. It was then possible to determine $\bar{M}_w$ in several further dextrans, using viscosity measurements alone.

Sedimentation coefficients may also be correlated empirically with molecular weight. The correlation established by Senti et al. (29) for fractionated, acid-hydrolysed dextrans, prepared by fermentation using *Leuconostoc mesenteroides*, NRRL B512, is given by,

$$S^o = 0.0245 \bar{M}_w^{0.44} \quad (12)$$

$S^o$ is the sedimentation coefficient at zero concentration. It is obtained by measuring the sedimentation coefficient, $S$, at a range of concentrations, $c$, and extrapolating to zero concentration by means of a plot of $1/S$ vs. $c$.

Procedures for determining dextran sedimentation coefficients were established by other IRI workers (26) using a Beckman Spinco Division ultracentrifuge (90). Solutions of several dextrans (sample nos. 12,17,19) were examined at a single low concentration ($0.0025$ g./ml.), and in each case a constant value of $2.50 \times 10^{-13}$ c.g.s.u. was obtained for $S$. Using the data of Senti et al. (29) to give the gradient of the $1/S$ vs. $c.$ plot in the region of $\bar{M}_w$ close to 40,000, the value of $S^o$ was calculated as $2.63 \times 10^{-13}$ c.g.s.u.
From eqn. 12, the corresponding value of $\bar{M}_w$ was determined as 41,000. This figure is in excellent agreement with the value of 40,000 for the mean $\bar{M}_w$ of the three dextrans, as determined by light scattering procedures in this work (table 14). Single peaks were also observed in the Schlieren patterns obtained in ultracentrifugation, indicating no abnormalities in the molecular weight distribution of the dextrans.

(b) $\bar{M}_w$ Determination by Light Scattering.

The theory of light scattering has been well covered in the literature (89,91,92), and only a summary is presented here. The discussion is centred around those considerations relevant in the application of this technique to $\bar{M}_w$ determination in dextrans. In particular, calibration procedures and experimental techniques aimed at producing maximum accuracy are described. The suitability of the light scattering method for purposes of quality control during the manufacture of clinical dextrans has already been described by the author (27, appendix 1).

(i) Light Scattering Theory.

When a light beam hits a molecule of dimensions smaller than the wavelength of the light, energy received is re-emitted as light in all directions; this phenomenon is known as light scattering. Most of the incident light is scattered without change in wavelength. Such scattering is referred to as Rayleigh scattering, and is the only mode of scattering of concern in this discussion. It can be shown that light-scattering measurements, when performed on polymer solutions, enable the weight-average molecular weight, $\bar{M}_w$, and the
second virial coefficient, $B$, to be determined (89,91).

Particles or molecules small in relation to the wavelength of the incident light can be considered as single-point scattering sources. Scattered light emitted from all points on the particle will be in phase. However, when particle size approaches the wavelength, $\lambda$, of the incident light, an out-of-phase condition will appear between scattered light beams emitted from various points on the scattering source, and interference will be observed between them. It has been found experimentally that if the molecule has dimensions in excess of $\lambda/20$, this must be taken into account when relating scattered light intensity to molecular weight (91). With particles of such dimensions, a study of the interference phenomenon can often be used to derive information regarding the shape and size of the scattering entity.

(ii) Turbidity and Rayleigh Ratio.
Light passing through an optically inhomogeneous medium is scattered in all directions; the intensity of the transmitted beam decreases exponentially with distance traversed. The turbidity of the medium is then defined by (91),

$$I = I_0 e^{-Tl} \quad (13)$$

where $I_0$ and $I$ are the intensities of the beam before and after passing through a length, $l$, of the medium.

Integration of eqn. 13 over all directions in space gives the total scattered intensity, $i_9$, per unit scattering volume, viewed at an angle $\theta$ to an unpolarised incident beam, and at a distance, $r$, from
the scattering centre. Hence,

\[ \Upsilon = \frac{16\pi}{3} \cdot \frac{r^2}{I_0 (1 + \cos^2 \Theta)} \]  \hspace{2cm} (14)

The quantity, \( \frac{i_9 r^2}{I_0 (1 + \cos^2 \Theta)} \), is known as the Rayleigh ratio, \( R_\theta \), and

\[ \Upsilon = \frac{16\pi}{3} \cdot R_\theta \]  \hspace{2cm} (15)

In simple systems exhibiting Rayleigh scattering, measurements of scattered intensity are usually made at an angle normal to the incident beam \((91)\). In this case,

\[ R_{90} = \frac{i_{90} r^2}{I_0} \]  \hspace{2cm} (16)

and,

\[ \Upsilon = \frac{16\pi}{3} \cdot R_{90} = \frac{16\pi}{3} \cdot \frac{i_{90} r^2}{I_0} \]  \hspace{2cm} (17)

Values of \( i_{90} \) are obtained from the light scattering instrument in arbitrary units; they may be related (without knowledge of \( I_0 \) or \( r^2 \)) to absolute values of \( \Upsilon \) or \( R_{90} \) by a calibration procedure using substances of known scattering power (see below). In other words, it is possible to evolve calibration relationships of the form,

\[ \Upsilon = K_i i_{90} \]  \hspace{2cm} (18)

The \( i_{90} \) values obtained from the instrument may be used to determine \( \Upsilon \) directly, provided two conditions are satisfied. Firstly, the polymer molecules must be optically isotropic (see below). Secondly, the molecular dimensions must be less than \( \frac{\lambda}{20} \), where \( \lambda \) is the incident light wavelength; this ensures that the observed value of \( i_{90} \) will not be reduced as a result of destructive interference effects \((91)\).
Molecular Weight Determination.

The basic equation for determining weight-average molecular weight from turbidity measurements on polymer solutions takes the form (89, 91),

$$\frac{H_0}{\Delta \gamma} = \frac{1}{\bar{M}_w} + 2B_0$$  \hspace{1cm} (19)

where,

- $c$ = solute concentration ($g/ml$),
- $\Delta \gamma$ = excess turbidity of solution over solvent ($cm^{-1}$),
- $\bar{M}_w$ = weight-average molecular weight,
- $B_0$ = second virial coefficient ($ml/g$),

and,

$$H = \frac{32\pi^3 n^2}{3 \lambda^4 No} (dn/dc)^2 \hspace{1cm} (mole \ cm^2/g.-2)$$

$H$ is constant for any polymer - solvent combination, and contains the parameters,

- $n$ = refractive index of solvent,
- $\lambda$ = wavelength of light (cm.),
- $No$ = Avogadro's number = $6.02 \times 10^{23}$,

and,

$$(dn/dc) = \text{specific refractive index increment (ml.g.-1)}$$

the rate of change of refractive index with polymer concentration.

Following eqn. 18, for any solution of concentration, $c$, the turbidity excess, $\Delta \gamma$, will be given by,

$$\Delta \gamma = K \left[ i_{90} \hspace{1cm} \text{(solution)} - \ i_{90} \hspace{1cm} \text{(solvent)} \right] = K I$$  \hspace{1cm} (20)

$i_{90}$ (solution) and $i_{90}$ (solvent) are determined experimentally for unpolarised incident light, and $K$ is a calibration constant. To determine the molecular weight of a polymer, the turbidity of several
solutions must be measured at a series of concentrations. If \( \frac{c}{\Delta T} \) is plotted against \( c \), extrapolation to zero will give the reciprocal of the molecular weight of the solute. To determine \( M_w \), the only other experimental measurement required is the variation of the refractive index of the solution with concentration (\( dn/dc \)). The second virial coefficient, \( B \), can also be determined simultaneously from the gradient of \( [c/\Delta T] - c \) plots.

(iv) Anisotropy and Depolarisation.

Unpolarised light can be resolved into two polarised beams at right angles to each other. If an isotropic molecule is subjected to the action of an electromagnetic wave of polarised light, a dipole will be induced which will oscillate in the same plane as that of the incident radiation. Light scattered normal to the incident unpolarised beam and observed in the horizontal plane will then be vertically polarised, with intensity equal in all directions. However, if the scattering molecule is anisotropic, the polarisibility (dipole amplitude) will vary with direction, and the induced dipole will no longer be parallel to the plane of polarisation of the incident light. As a result, light scattered normal to the incident beam will no longer be completely polarised in the vertical plane, but will be reinforced by an additional horizontal component. The measured scattering of anisotropic molecules is therefore greater than that of isotropic molecules of the same size (91). When determining the molecular weight of anisotropic molecules by light scattering, it is possible to correct for this effect by means of a factor involving the depolarisation ratio, \( \rho_{90} \).

\[
\rho_{90} = \frac{I_{90}}{I_{90}}
\]

\[
\rho_{90} = \frac{I_{90}}{I_{90}} \quad (21)
\]
$i_{90}^H$ and $i_{90}^V$ are the scattered light intensities at $90^\circ$ for incident light polarised in the horizontal and vertical planes, respectively. For isotropic molecules, the scattered light at $90^\circ$ is totally polarised in the vertical plane, and $\rho_{90} = 0$. The depolarisation factor, $\rho_{90}$, is thus a measure of the optical symmetry of the molecule (91,92).

(v) **Internal Interference with Large Particles.**

Large particles with dimensions greater than $\lambda/20$ cannot be considered as single scattering centres. This is due to the fact that light waves emitted from different parts of the particle will no longer be in phase, and interference will be evident between these scattered beams. As the angle of scatter increases, the path difference will increase between scattered light received from the different parts of the particle, and the intensity of scatter is reduced due to destructive interference. Scatter is no longer symmetrical about the $90^\circ$ position and $R_g$ now depends on the angle at which it is measured.

At $0^\circ$, the interference phenomenon is zero, which means that eqn. 19 is applicable at this angle. To obtain the molecular weight of a large particle, measurements can be made of the scatter at several concentrations and at several angles; the molecular weight may be calculated from the data by extrapolation to zero angle and to zero concentration using the procedure devised by Zimm (89,91,92). No assumptions as to the shape of the particle need be made in this approach.
The dissymmetry coefficient, $Z$, is defined as,

$$Z_G = \frac{i(\Theta)}{i(\Pi - \Theta)} \quad (22)$$

$Z_G$ is the ratio of the intensities of scatter at angles, $\Theta$ and $(\Pi - \Theta)$, which are symmetrical about the $90^\circ$ position. For any value of $\Theta$, $Z_G$ increases as the size of the particle increases. If it is assumed that the particle has the shape of a rod, sphere, coil, etc., then its size can be directly related to the value of $Z_G(91)$. The ratio $i_{450}/i_{1350}$ is called the dissymmetry, and if its value differs appreciably from unity, the measured value of $i_{90}$ will require correction before being used in eqn. 20.

Dissymmetry of scatter should not be observed in low molecular weight polymer solutions, provided the molecules present have dimensions less than $\lambda/20$. On the other hand, contaminants in the solution, such as dust and other large extraneous particles, will exhibit high dissymmetries of scatter. These particles must be separated from the polymer molecules, since their presence will lead to errors in the values of $i_{90}$ which are measured during $M_w$ determination by light scattering. With low molecular weight polymers, measurements of dissymmetry are therefore frequently employed to demonstrate that contaminating particulate materials have been effectively removed from solution.
(vi) **Light Scattering Photometer.**

A description of the SOFIGA Photo-Gonio Diffusometer is given in the experimental section and only a brief account of the principal features is required at this point. Light from a mercury vapour lamp passes through one of two optical filters, enabling either blue ($\lambda = 436 \text{ nm}$) or green ($\lambda = 546 \text{ nm}$) lines of the spectrum to be selected. A polariser is also incorporated in the optical path; the incident light may therefore be vertically or horizontally polarised as required. Before the light reaches the scattering medium, which is contained in a cylindrical glass cell, the incident beam intensity can be adjusted by means of a variable slit. The scattering cell is immersed in a vat of benzene in order to minimise reflection which would otherwise occur at the air-glass interface. The intensity of light scattered at angles of $0^\circ - 180^\circ$ to the direction of the transmitted beam can be measured using a rotatable photomultiplier tube.

**Optical Alignment:** To ensure that the incident light beam was correctly adjusted so as to pass horizontally through the centre of the cell, the following beam alignment check was performed (92,93). A dilute solution of sodium fluorescein was introduced into the scattering cell, and a green filter (max. transmission, $\lambda = 546 \text{ nm}$) was placed in front of the detector. Fluorescence occurs with blue incident light ($\lambda = 436 \text{ nm}$), and the angular dependence of emitted light intensity was measured over the range $30^\circ - 150^\circ$. The blue incident light is absorbed by the fluorescein molecule, and emitted with equal intensity in all directions, but at a longer wavelength. This being the case, provided the beam alignment is correct, the emitted light intensity, when corrected for changes in observed
scattering volume, should be independent of angle of observation. By placing the green filter in the detected beam, effects of scatter associated with the blue light are totally eliminated.

Table 2: Check on Optical Alignment of Light Scattering Photometer by Measurement of the Angular Dependence of Light Emission from Fluorescein Solution.

<table>
<thead>
<tr>
<th>Angle of Measurement, θ (degrees)</th>
<th>Observed Emitted Light Intensity, ( i_0 ) (arbitrary units)</th>
<th>( \sin θ )</th>
<th>( i_0 \cdot \sin θ )</th>
</tr>
</thead>
<tbody>
<tr>
<td>30.0</td>
<td>3.70</td>
<td>0.500</td>
<td>1.85</td>
</tr>
<tr>
<td>37.5</td>
<td>3.00</td>
<td>0.609</td>
<td>1.83</td>
</tr>
<tr>
<td>45.0</td>
<td>2.56</td>
<td>0.707</td>
<td>1.81</td>
</tr>
<tr>
<td>60.0</td>
<td>2.09</td>
<td>0.866</td>
<td>1.81</td>
</tr>
<tr>
<td>75.0</td>
<td>1.87</td>
<td>0.966</td>
<td>1.81</td>
</tr>
<tr>
<td>90.0</td>
<td>1.80</td>
<td>1.000</td>
<td>1.80</td>
</tr>
<tr>
<td>105.0</td>
<td>1.86</td>
<td>0.966</td>
<td>1.80</td>
</tr>
<tr>
<td>120.0</td>
<td>2.08</td>
<td>0.866</td>
<td>1.80</td>
</tr>
<tr>
<td>135.0</td>
<td>2.56</td>
<td>0.707</td>
<td>1.81</td>
</tr>
<tr>
<td>142.5</td>
<td>2.98</td>
<td>0.609</td>
<td>1.82</td>
</tr>
<tr>
<td>150.0</td>
<td>3.68</td>
<td>0.500</td>
<td>1.84</td>
</tr>
</tbody>
</table>

The scattering volume observed at 90° depends on the width of the incident beam and on the dimensions of the photomultiplier slit. At other angles, the observed scattering volume will be greater. If all measurements are to apply to the same scattering volume, the true scattered intensity should be related to the observed intensity, through the equation (94),

\[
i_0 \text{(correct)} = i_0 \text{(observed)} \times \sin θ \quad \text{(23)}
\]
Eqn. 23 will only apply if the incident beam passes horizontally through the centre of the scattering cell. Under the experimental conditions described, this means that the observed intensity of light emitted at any angle, $\theta$, when multiplied by $\sin \theta$ to correct for changes in the observed volume of fluorescein solution, should be constant. The results of the test are given in table 2. The constant value of $i_0 \sin \theta$ observed over the range $45^\circ - 135^\circ$ confirmed that the optical alignment of the instrument was correct. A slight error was evident at high and low angles, but this will not affect the results in this work, where all measurements were performed within the $45^\circ - 135^\circ$ range.

(vii) Correction Factors.

The values of $i_0$ from which Rayleigh ratio's are calculated may sometimes require correction for optical effects associated with the instrument before they can be regarded as absolute. One such correction, already referred to above, is necessary due to the fact that the photomultiplier sees a scattering liquid volume greater than that defined by the incident beam; eqn. 23 provides the necessary correction to $i_0$ in this instance.

A second correction arises due to the fact that the intensity of light seen by the photomultiplier may be increased by refraction effects. These occur when light passes from the liquid (refractive index, $n$) present in the cell, to the external liquid (refractive index, $n_e > n$) surrounding the cell (94). In this instance, the cell is surrounded by benzene. The correction for refraction effects is given by,

$$i_0^{(correct)} = i_0^{(observed)} \times \frac{n^2}{n_e^2} \quad \text{(24)}$$
In molecular weight determinations by light scattering, the ratio of two scattered light intensity measurements requires to be measured. One measurement is performed on the sample liquid, and the other on a calibration standard, under the same conditions. Since a ratio is involved, either if both sample and standard are organic liquids, or if both are aqueous, the need for the refraction correction disappears; scattered beams from both sample and standard will undergo equal intensity reductions due to refraction as the light passes from the cell into the benzene. However, if the calibration standard is organic, and the sample liquid is aqueous, or vice versa, eqn.24 must be applied to account for errors in the observed scattered light intensities which result from refraction effects.

A third correction stems from the fact that a small fraction of light which is incident normally on an interface between two transparent media, is reflected (91,92). The SOFICA instrument is remarkably free from reflection effects, largely due to the existence of the benzene vat in which the sample cell is immersed. The entire interior surface of the vat, except for the entrance window for incident light, is blackened with material stable in benzene. A special light absorbing glass, placed on the inner surface of the vat opposite the entrance window, serves as a trap for the transmitted primary beam without reflection. Scattered light which is incident on the surface of the vat should be mostly absorbed by the black coating, and reflection of scattered light from the benzene-black coating interface is probably negligible (95). The only reflection effect of any consequence for this instrument will be a small effect occurring at the solvent-glass interface when light
scattering is conducted in aqueous solvents. It has in fact been shown (95) in light scattering measurements on aqueous dextran solutions, that the error which arises if this reflection effect is neglected, amounts only to about 1% in the molecular weight.

Finally, if solutions contain molecules which absorb the incident light, or which fluoresce at the incident beam wavelength, further corrections have to be considered if accurate light scattering measurements are to be performed (91,92).

(viii) Calibration of Instrument.
The objective of light scattering measurements on polymer solutions is to determine the solution turbidity, a quantity from which the weight-average molecular weight of the polymer can be derived. The readings, $i_0$, which are obtained from the galvanometer on the light scattering photometer, and which signify the intensity of scattered light at an angle $\theta$, are in arbitrary units. They must therefore be related to those readings observed under the same conditions when some substance of known turbidity is present in the scattering cell.

Two methods of calibrating the light scattering photometer were investigated.
Benzene Calibration.

The turbidity of pure benzene has been frequently measured (91, 92, 96). Widely accepted values for the Rayleigh Ratio of benzene at 90°, \( R_{90} \), are shown in table 3.

Table 3: Rayleigh Ratios for Benzene at 25°C. (94).

<table>
<thead>
<tr>
<th>Wavelength of Incident Light (nm)</th>
<th>Rayleigh Ratio, ( R_{90} \times 10^6 ) (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>436 (blue)</td>
<td>48.5</td>
</tr>
<tr>
<td>546 (green)</td>
<td>16.3</td>
</tr>
</tbody>
</table>

To calibrate the photometer, benzene was placed in the scattering cell. With unpolarised incident light (e.g. \( \lambda = 436 \) nm), the incident intensity was adjusted until the magnitude of the scattered intensity at 90° was signified by an arbitrary galvanometer reading of 0.50. If an unknown solution is placed in the cell, all other conditions remaining constant, a different galvanometer reading will result, which will be proportional to the turbidity of the solution. This unknown turbidity or Rayleigh ratio can then be found from,

\[
\frac{R_{90} \text{ (unknown solution)}}{R_{90} \text{ (benzene)}} = \frac{48.5 \times 10^{-6}}{0.50} \quad (25)
\]
To avoid the necessity of maintaining a benzene standard in suitable condition, it is convenient to employ a secondary glass standard whose scattering power relative to that of benzene, has been determined. Thus, the incident light intensity (both blue and green light) was adjusted to produce a scattered light intensity reading of 0.50, with benzene in the cell. With the glass standard in place of the benzene the readings observed under the same conditions were as shown in table 4.

Table 4: Turbidity of Glass Standard Relative to Benzene and Water.

<table>
<thead>
<tr>
<th>Wavelength of Incident Light (nm)</th>
<th>Scattered Light Intensity at 90°, 190° (Galvanometer Readings)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Benzene</td>
</tr>
<tr>
<td>436</td>
<td>0.50</td>
</tr>
<tr>
<td>546</td>
<td>0.50</td>
</tr>
</tbody>
</table>

The experimental conditions under which eqn.25 can be employed to determine the absolute turbidities of polymer solutions may now be reproduced without using the primary benzene standard. With the glass standard in position, the galvanometer readings are simply adjusted to that value, listed in table 4, which corresponds to the wavelength of the incident light being used.

Calibration with Colloidal Silica.

This method of calibration is absolute, in the sense that a suspension is employed, the turbidity of which can be directly measured in a spectrophotometer. When the turbidity has been determined in this way, the suspension is placed in the scattering cell, and the scatter at 90° is determined.
Following eqn.13, the spectrophotometric turbidity is given by,

\[
\gamma = \frac{2.303}{1} \log \frac{I_0}{I} = \frac{2.303}{1} D \quad \text{------------(26)}
\]

where \(D\) is the optical density of the solution, and \(l\) is the path length in the spectrophotometer cell.

One aspect of the suitability of colloidal suspensions as calibration media may be confirmed by measuring turbidity as a function of wavelength (91,92). Eqn.19 shows that if \(c\) and \(\bar{M}_w\) are constant, \(\gamma\) is proportional to \(H\). \(H\), in turn, is proportional to \((1/\lambda)^4\), provided the term \(n^2 (dn/d\alpha)^2\), does not change with wavelength. In the spectrophotometer, \(\gamma\) is proportional to \(D\) (eqn.26); a plot of optical density against \((1/\lambda)^4\) should therefore yield a straight line.

Colloidal silica suspensions were prepared in sodium chloride solution at silica concentrations in the range 0.5 - 3.0%; the sodium chloride serves to stabilise the suspension. The suspensions were filtered through membranes of mean pore diameter, 0.22 \(\mu\)m, and their optical densities measured at several wavelengths in the range 400 - 600 nm. The plots of optical density against the fourth power of the reciprocal wavelength are shown in fig.6 for five silica suspensions. It is evident from the straight line plots that the fourth power law is obeyed. Colloidal silica is therefore a potentially suitable calibration medium for light scattering studies, provided no dissymmetry of scatter or depolarisation effects are observed (91,92).

The scattering of light by optically dense particles such as
Figure 6: Dependence of turbidity on incident light wavelength, $\lambda$, for colloidal silica dispersions.
colloidal silica, is so great that at any appreciable concentration, the observed scatter is considerably less than the true value. This is attributable to the loss in intensity of the primary beam due to scattering before it reaches the centre of the cell. In addition, there will also be a loss in the scattered light intensity, due to further scattering before it reaches the photomultiplier tube. A correction for this effect, which is observed in the use of colloidal suspensions for calibration, has been pointed out by Maron and Lou (97); if the path lengths of the incident and scattered beams are $l_1$ and $l_2$ respectively, the intensity $I_0'$ reaching the scattered volume is related to the incident intensity, $I_0$, by:

$$I_0' = I_0 e^{-l_1} \quad \text{(27)}$$

The intensity of scattered light observed, $i'_{90}$, is related to the true initial scattered intensity, $i_{90}$, by:

$$i_{90}' = i_{90} e^{-l_2} \quad \text{(28)}$$

Hence, utilizing eqn.17,

$$\log \frac{\Upsilon}{i_{90}'} = \log \frac{16 \pi}{3} \cdot \frac{r_2}{I_0} + \frac{l_1 + l_2}{2.303} \quad \text{(29)}$$

Eqn.29 may be written as,

$$\log \frac{\Upsilon}{i_{90}'} = k_1 + k_2 \Upsilon \quad \text{(30)}$$

For any given wavelength, $\lambda$, $\Upsilon/i_{90}$, is only a constant when $k_2 \Upsilon$ is negligibly small. The calibration constant, $k_1$, may be obtained graphically from a plot of $\log \Upsilon/i_{90}$, against $\Upsilon$; $k_1$ is the value of $\log \Upsilon/i_{90}$, at $\Upsilon = 0$ (97)

The five silica suspensions, the turbidity of which had been previously determined as a function of wavelength, were each filtered
into the scattering cell. Using both blue and green incident light, the intensity of scatter at 90°, i90, was measured in each case; all galvanometer readings were related to those for the secondary glass standard given in table 4. The absolute turbidities, T, of these solutions at wavelengths 436 nm and 546 nm were obtained from fig.6. For each wavelength, plotting log 10 T/i90 against T resulted in a linear plot. Both lines which are shown in fig. 7, were extrapolated to T = 0 in accordance with the procedure outlined above. The values of the observed calibration constants, k1 derived from fig.7 are given in table 5.

Table 5: Calibration of Light Scattering Photometer with Colloidal Silica Suspensions.

<table>
<thead>
<tr>
<th>Wavelength of Incident light (nm)</th>
<th>436</th>
<th>546</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed Calibration Constants, k1 x 10^3 (cm^-1)</td>
<td>1.29 ± 0.02</td>
<td>4.32 ± 0.04</td>
</tr>
<tr>
<td>Refraction Correction (Correct/Observed)</td>
<td>0.776</td>
<td>0.786</td>
</tr>
<tr>
<td>Absolute Calibration Constants, k1A x 10^4 (cm^-1)</td>
<td>1.66 ± 0.02</td>
<td>5.50 ± 0.04</td>
</tr>
</tbody>
</table>

The high scattering power of the silica suspensions, which is a requirement in a suitable calibration medium, is demonstrated by the high values of i90' observed in the photometer.

Silica particles are approximately 20 nm in diameter. Their size is thus less than 1/20 of the wavelength of the incident light, which means that they should behave as ideal scattering particles and show little dissymmetry of scatter. This was confirmed by the
FIG. 7: CALIBRATION OF SOFICA LIGHT SCATTERING PHOTOMETER USING COLLOIDAL SILICA DISPERSIONS
The fact that the measured dissymmetries of the silica suspensions did not exceed 1.10. The depolarisation of the silica suspensions was also measured and shown to be essentially zero. This fact is in accordance with the optical isotropy associated with the known symmetrical (spherical) shape of the silica particles. These observations confirm the suitability of colloidal silica suspensions for calibrating the light scattering photometer. In this calibration procedure, values of $i_{90}'$ were measured when aqueous solution was in the scattering cell, and benzene was the external liquid. The calibration constants, $k_1$, will thus only apply when scattering measurements are performed on aqueous solutions. This remains true throughout the work reported here, and no refraction correction need be applied.

Table 6: Rayleigh Ratios for Water and Benzene Calculated from Colloidal Silica Calibration.

<table>
<thead>
<tr>
<th>Wavelength of Incident Light (nm)</th>
<th>Rayleigh Ratio at $25^\circ C.$, $R_{90} \times 10^6$ (cm$^{-1}$)</th>
<th>Water</th>
<th>Benzene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calculated</td>
<td>Literature</td>
<td>Calculated</td>
</tr>
<tr>
<td></td>
<td>Kraut (93)</td>
<td>Bischoff (30)</td>
<td>Kraut (93)</td>
</tr>
<tr>
<td>436</td>
<td>2.70 ± 0.15</td>
<td>2.89</td>
<td>2.68</td>
</tr>
<tr>
<td>546</td>
<td>1.03 ± 0.03</td>
<td>1.05</td>
<td>1.01</td>
</tr>
</tbody>
</table>

Water was filtered into the scattering cell; the galvanometer readings which were recorded for the intensity of scatter at $90^\circ$, relative to those for the glass standard, are shown in table 4. Values for $\gamma$ were computed using the above constants, $k_1$ (table 5), and the Rayleigh ratios for water were calculated using eqn.15.
These are shown in table 6, where a comparison with literature values is made. The excellent agreement between measured and literature values supports the accuracy of the silica calibration procedure for molecular weight determinations in aqueous solutions.

For both blue and green incident light, the value of ϕ90 for benzene is known to be 0.50 when the glass standard scatter takes the values given in table 4. However, in order to calculate the turbidity or Rayleigh ratio for benzene using the constants derived from silica calibration, these constants, k1, determined above (table 5) must be made absolute. This is achieved by correcting all values of ϕ90 which were observed with aqueous solution in the cell, for refraction effects associated with the benzene-glass-water interface.

Literature values for the refractive indices of benzene and water are given in table 7.

Table 7: Refractive Indices of Benzene and Water (98).

<table>
<thead>
<tr>
<th>Wavelength of Incident Light (nm)</th>
<th>Refractive Indices at 25 °C.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Benzene</td>
</tr>
<tr>
<td>436</td>
<td>1.524</td>
</tr>
<tr>
<td>546</td>
<td>1.506</td>
</tr>
</tbody>
</table>

Substituting these values into eqn.24, the refractive index correction factors were determined; these are given in table 5. The absolute values for the calibration constant, k_A, which were derived from the observed constants, k1, are also shown in this table.
The Rayleigh ratios calculated for benzene are shown in table 6, together with literature values given in table 3. The good agreement between experimental and literature ratios is indicative of the validity of the refractive index correction, which is applicable when the refractive indices of sample and standard are appreciably different.

Throughout the light scattering studies on aqueous dextran solutions which are described below, the calibration constants employed to relate \( \tau \) to \( i_{90} \) were those observed constants, \( k_1 \), determined by calibration with aqueous colloidal silica suspensions. Their use without correction for refraction effects is valid. With benzene as the external liquid, the enhancement in scattered intensity, \( i_{90} \), will be the same with aqueous samples as with calibration standards. Since the same correction for refraction effects applies in each case, values of turbidity derived by using \( k_1 \) will be absolute. This will remain true provided only aqueous, rather than organic dextran solutions are employed.

(ix) Measurement of Specific Refractive Index Increment.

The constant, \( H \), in the light scattering equation contains the term \((dn/dc)^2\), where \((dn/dc)\) is the rate of change of refractive index with concentration. Being a squared term, it must be measured accurately if serious error is to be avoided in the determination of molecular weight. The refractive index increment of dextran in water was measured using a differential refractometer of the type supplied by Polymer Consultants Ltd. In this instrument, light from a mercury vapour lamp is passed through a filter to select either blue (\( \lambda = 436 \) nm) or green (\( \lambda = 546 \) nm) lines of the mercury
spectrum. After passing through a divided cell and an aperture, the beam is finally directed into a telescope, the eyepiece of which has a calibrated horizontal scale at right angles to the light beam. A vertical image of the slit is observed in the eyepiece. When a solution is placed in one side of the cell, and solvent on the other, the shift of the slit image, $\Delta d$, relative to its position with solvent on both sides of the cell, can be determined. The difference in refractive index between solution and solvent, $d_n$, is proportional to the shift, $\Delta d$. That is,

$$d_n = K \cdot \Delta d \hspace{1cm} (31)$$

The calibration constant, $k$, for the refractometer was determined using sucrose solutions, $d_n$ being calculated from the known sucrose concentrations and the literature values for the refractive index increment given in table 8.

**Table 8: Calibration of Differential Refractometer with Sucrose Solutions.**

The mean values of $K$ determined in this way are also shown in table 8.

<table>
<thead>
<tr>
<th>Wavelength of Incident Light (nm)</th>
<th>Sucrose Refractive Index Increment (99)(ml./g.)</th>
<th>Calibration Constant, $K \times 10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>436</td>
<td>0.1449</td>
<td>9.06</td>
</tr>
<tr>
<td>546</td>
<td>0.1430</td>
<td>9.09</td>
</tr>
</tbody>
</table>
Table 9: Refractive Index Increment for Dextran in Water.

<table>
<thead>
<tr>
<th>Wavelength of Light (nm)</th>
<th>Measured Value</th>
<th>Refractive Index Increment at 25 °C. (dn/dc),(ml.g.⁻¹)</th>
<th>Literature Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Senti et al. (29)</td>
<td>Granath (25)</td>
</tr>
<tr>
<td>Blue, 436</td>
<td>0.151</td>
<td>0.151</td>
<td>0.151</td>
</tr>
<tr>
<td>Green, 546</td>
<td>0.149</td>
<td>0.154</td>
<td>0.151</td>
</tr>
</tbody>
</table>

Values of Δd were similarly measured for several dextran solutions, and dn was determined from eqn.31, using the calibration constants given in table 8. The value of (dn/dc) was calculated from the known dextran concentrations; full results are given in the experimental section. The measured values of (dn/dc) for dextran are given in table 9, together with several literature values. The value for blue light (λ = 436 nm) is in very close agreement with the findings of several other workers. When green light (λ = 546 nm) is used, discrepancies appear between literature values, although it is generally the case that (dn/dc) is marginally higher at 546 nm than at 436 nm. The change of (dn/dc) with wavelength is in accordance with results obtained on other polymers e.g. amylopectin (102), dn/dc = 0.156 (λ = 436 nm) and dn/dc = 0.154 (λ = 546 nm). In view of these findings the observed values would appear justifiable, and were employed for all molecular weight determinations in this work.

(x) Preparation of Solutions.

In light scattering studies on polymer solutions, if dust, large colloidal particles, or molecular aggregates are present, these
will contribute to the 90° scattered intensity associated with the polymer molecules. Careful preparation of solutions is therefore essential to ensure that the measured turbidities and derived molecular weights are not in error (91,92). Where molecular size is sufficiently small that dissymmetry associated with the molecular components can be ruled out, the clarity of solutions for light scattering studies is frequently assessed by measurement of the dissymmetry value. Visual inspection of clarified solutions is also a remarkably sensitive test for the presence of dust particles. Both approaches were employed in this work; samples were required to satisfy a visual examination before dissymmetry measurements were recorded.

Before introducing solutions and solvents into the glass light scattering cells, the cells were carefully cleaned to eliminate dust particles. Final rinsing is an important step. This was carried out using acetone which had been filtered through a membrane of mean pore diameter, 0.2 μm. All solutions and solvents employed in the light scattering studies were clarified by filtration through Millipore membranes; the liquids were filtered directly into the scattering cells.

Clarification of dextran solutions was studied using several Millipore membranes with mean pore diameters in the range 0.10 - 0.45 μm. Portions of a dextran solution (sample no.22, 0.01g./ml.) were filtered through these membranes, and the dissymmetry and 90° scattered intensity measured in each case. The effect of removing molecular aggregates by filtration to various particle size levels is illustrated in fig.8, where C/I is plotted against
FIG. 8: EFFECT OF AUTOCLAVING AND FILTRATION ON TURBIDITY AND DISSYMMETRY OF DEXTRAN SOLUTIONS
dissymmetry, $Z$. Only with the $0.10 \mu m$ and $0.22 \mu m$ pore diameter membranes does the dissymmetry approach an acceptably low level close to unity. It is inferred that particles which are capable of permeating the $0.45 \mu m$ pores, and which contribute to dissymmetry, can be removed by the membranes of smaller pore size. As the larger sized particles are removed by increasingly finer filtration, the dissymmetry tends to unity; the value of $I$ also decreases, and $C/I$ approaches the constant value associated with the molecular scattering of the solution.

The possibility of obtaining further reductions in dissymmetry and in $90^\circ$ scattered intensity, over those levels attainable by filtration, was examined. The process of autoclaving was investigated. In the section which follows, autoclaving is firmly established as a method for reducing the visible turbidity associated with crystalline dextran particles; a crystal melting process is shown to be involved. Accordingly, the dextran solution used in the filtration studies was autoclaved (120 °C., 30 mins.), and samples were filtered through the three membranes as before. Dissymmetry and $90^\circ$ scatter measurements were again performed on the autoclaved and filtered solutions; the results are displayed in fig.8. Comparing the results with those for non-autoclaved solutions, it is evident that prior autoclaving does not reduce dissymmetry to any extent over and above that attained by filtration to the $0.10 \mu m$ particle size level. Autoclaving, however, does marginally reduce dissymmetry in solutions filtered through the $0.22 \mu m$ membrane, while with the $0.45 \mu m$ membrane, the effect is even more marked. The heat treatment procedure is evidently dispersing particles in the $0.2 - 0.5 \mu m$ size range. The absence of any residual dissymmetry after extensive solution clarification confirmed that the dextran molecules them-
selves exhibited no dissymmetry of scatter.

The work of Bischoff (30) with visibly turbid dextran solutions exhibiting dissymmetries as high as 1.7 prior to autoclaving, but after filtration through a 0.2 μm pore diameter membrane, clearly demonstrates the necessity of this heat treatment approach with certain dextran samples. In Bischoff's study, solutions were autoclaved until dissymmetries approach unity, and the 90° scattered light intensity reached a constant value. One of the samples of dextran employed in his work was of low molecular weight (Mw = 14,000); a high visible turbidity had been introduced into the product as a result of prolonged contact with ethanol during preparation. Later investigations in this thesis (section D) show that such turbidity would be associated with small crystalline particles. These would not be filterable using a 0.2 μm pore diameter membrane, and would be difficult to disperse by heat treatment. It is thus possible to explain the difficulties experienced by Bischoff (30) in clarifying such solutions prior to conducting light scattering measurements. In the dextrans studied in this work, the level of turbidity associated with crystalline particles was much lower, which made clarification a simpler and less troublesome operation.

As a result of these investigations, autoclaving of solutions, followed by filtration through a 0.10μm pore diameter filter, was adopted as the standard sample preparation procedure. It is understood from the above study that the autoclaving step may be superfluous in many instances; nevertheless, autoclaving was included as a precautionary measure for the elimination of non-filterable
aggregates which are occasionally present in dextran solutions.

In all samples examined for molecular weight determination by light scattering, solution dissymmetries in no case exceeded 1.05. This was true even for dilute solutions where the molecular scatter is small, and extraneous particle scatter, if present, can amount to a considerable proportion of the total. For this reason, with comparable degrees of clarification, dissymmetries tend to increase with decrease in polymer concentration, being greatest for pure water. Water dissymmetries of less than 1.10 were in fact attainable by the filtration procedures described. Non-aqueous liquids such as benzene, are much simpler to clarify and it was possible to obtain dissymmetries of less than 1.01.

(xi) Molecular Weight Determination.

For low molecular weight dextrans such as are being investigated in this work, the polymer molecule dimensions are small compared with the wavelength of visible light. No dissymmetry of scatter was therefore anticipated, and this was confirmed by the low values (<1.05) measured on the dextran solutions. In determining molecular weight, it is not therefore necessary to measure the angular dependence of scatter, and observations of scattered intensity at 90° to the incident beam will suffice. The work of Senti et al. (29) shows that dextran molecular weights (Mw) must exceed 2,700,000 before measurable dissymmetry of scatter is evident.

Dextran molecules are optically isotropic, so that the depolarisation factor, ρ90°, is effectively zero (95); this was confirmed by
actual measurements of $I_{90}$, in which no scatter at 90° was observed with horizontally polarised incident light. No correction factor to the 90° scattered intensity resulting from depolarisation effects is therefore required.

At the wavelengths employed, the dextran solutions did not exhibit any significant absorption of light. The possibility of fluorescence was also considered by examining dextran solutions in a fluorescence spectrophotometer; scanning over the complete range of exciter wavelengths, within the bandwidths of the two filters used in the light scattering instrument, did not uncover any fluorescent effects. No corrections to the 90° scattered intensity associated with absorption or fluorescence phenomena were therefore required.

To determine the molecular weight of a dextran sample, a range of aqueous solutions at several concentrations was prepared. The intensity of scatter was measured for each solution at an angle of 90° to the incident beam. The solutions were clarified by autoclaving and filtration, as described above, to give asymmetries of less than 1.05. The scatter due to these dextran solutions was related to the turbidity associated with a glass scattering standard. The latter was in turn related to an absolute turbidity, by calibration using colloidal silica suspensions. Dextran solutions were studied at concentrations within the range 0.002 - 0.015 g./ml.; for accurate extrapolation to zero concentration, a minimum of six solutions were prepared for each dextran. Dextran concentrations were determined by polarimetry, which in view of the high specific rotation of dextran,
To calculate the molecular weight of a dextran, it is necessary to evaluate the constant, \( H \), in eqn.19. \( H \) was calculated using the values of \( n \) and \( (dn/dc) \) given in tables 7 and 9; these values, together with those of the constant, \( H \), are presented in table 10.

### Table 10: Values of Constants Required for Light Scattering Determination of \( M_w \) in Dextrans.

<table>
<thead>
<tr>
<th>Wavelength of Incident Light (nm)</th>
<th>Refractive Index of Water, ( n ), at 25 °C.</th>
<th>Refractive Index Increment for Dextran in Water ( (dn/dc) ) at 25 °C. ( (\text{cm}^3 \text{g}^{-1}) )</th>
<th>( H \times 10^7 ) (mole ( \text{cm}^3 \text{g}^{-2} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>436</td>
<td>1.342</td>
<td>0.151</td>
<td>6.23</td>
</tr>
<tr>
<td>546</td>
<td>1.334</td>
<td>0.149</td>
<td>2.44</td>
</tr>
</tbody>
</table>

Introducing eqn.20, the calibration constant, \( K \), can now be incorporated into eqn.19, which becomes,

\[
\frac{H_0}{T} = \frac{H_0}{K} = \frac{1}{\bar{M}_w} + 2B_c \quad \text{--------- (32)}
\]
Table 11: Typical Light Scattering Data on Dextran Solutions. Dextran Sample No.17. (25 °C).

<table>
<thead>
<tr>
<th>Solution Concentration ( c \times 10^2 ) (g.mL(^{-1}))</th>
<th>( 90° ) Solution (galvanometer reading)</th>
<th>( 190° ) Solution (galvanometer reading)</th>
<th>( c/I \times 10^3 ) (g.mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 0.413 )</td>
<td>0.75</td>
<td>0.71</td>
<td>5.86</td>
</tr>
<tr>
<td>( 0.471 )</td>
<td>0.80</td>
<td>0.76</td>
<td>6.20</td>
</tr>
<tr>
<td>( 0.769 )</td>
<td>1.17</td>
<td>1.13</td>
<td>6.80</td>
</tr>
<tr>
<td>( 0.854 )</td>
<td>1.25</td>
<td>1.21</td>
<td>7.05</td>
</tr>
<tr>
<td>( 1.067 )</td>
<td>1.46</td>
<td>1.42</td>
<td>7.51</td>
</tr>
<tr>
<td>( 1.277 )</td>
<td>1.64</td>
<td>1.60</td>
<td>7.98</td>
</tr>
<tr>
<td>( 1.481 )</td>
<td>1.77</td>
<td>1.73</td>
<td>8.56</td>
</tr>
</tbody>
</table>

Incident Light \( \lambda = 436 \) nm (blue)

Incident Light \( \lambda = 546 \) nm (green)
Utilising the experimentally determined values for \( K (k_1, \text{table } 5) \) then,

\[
\lambda = 436 \text{ nm (blue); } \frac{c}{I} \times 4.79 \times 10^{-3} = \frac{1}{\bar{M}_w} + 2Bc \quad -----(33)
\]

\[
\lambda = 546 \text{ nm (green); } \frac{c}{I} \times 5.65 \times 10^{-3} = \frac{1}{\bar{M}_w} + 2Bc \quad -----(34)
\]

To determine molecular weight, the value of \( c/I \) is plotted against \( c \) and the results extrapolated to \( c = 0 \).

A typical set of experimental data measured during a light scattering determination on dextran (sample no.17) are given in table 11; the \( c/I \) versus \( c \) plot is shown in fig.9, for both blue and green incident light. The values of \( (c/I)_{c=0}, \bar{M}_w \), gradient \( m \), and second virial coefficient, \( B \), which were determined from the plot are given in table 12.

Table 12: Values of \( \bar{M}_w \) and \( B \) Derived from a Typical Plot of \( c/I \) against \( c \).

<table>
<thead>
<tr>
<th>Wavelength of Incident Light (nm)</th>
<th>((c/I)_{c=0} \times 10^3) (g.ml.(^{-1}))</th>
<th>(\bar{M}_w)</th>
<th>Gradient ( m )</th>
<th>Second Virial Coefficient,( B \times 10^4 ) (mole cm(^3).g.(^{-2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>436</td>
<td>4.96</td>
<td>42,000</td>
<td>0.240</td>
<td>5.75</td>
</tr>
<tr>
<td>546</td>
<td>4.30</td>
<td>41,100</td>
<td>0.222</td>
<td>6.28</td>
</tr>
</tbody>
</table>

Plots of \( (c/I) \) against \( c \) for several other dextrans are shown in fig.9. Molecular weights and second virial coefficients which were determined from these plots are given in table 13.

The values of molecular weight shown in table 13 appear to depend on the wavelength of the light used in the determination; the difference between determinations based on blue and green light amounts to approximately 5%. Few examples of molecular weight determinations at two or
FIG. 9: EXTRAPOLATION OF DEXTRAN SOLUTION TURBIDITIES TO ZERO CONCENTRATION
more wavelengths could be found in the literature. While Senti et al. (29) performed light scattering measurements on dextran solutions with incident light at wavelengths of both 436 nm and 546 nm, only average values are quoted without mention of the agreement between them. Bischoff's determinations (30) were conducted only at 436 nm, because of absorption effects at 546 nm, which were associated with dextran solutions which had been autoclaved for long periods of time. Parfitt and Wood (103) have observed a difference of 14% between dextran -40 molecular weights determined from measurement at 436 nm and 546 nm, the difference being in the same direction as measured in this work.

Table 13: Molecular Weights and Second Virial Coefficients of Dextrans.

<table>
<thead>
<tr>
<th>Dextran Sample No.</th>
<th>Molecular Weight, Mw</th>
<th>Mean* Molecular Weight, Mw</th>
<th>Second Virial Coefficient, B x 10^4 (mole cm^3.g^-2)</th>
<th>Mean* Second Virial Coefficient, B x 10^4 (mole cm^3.g^-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λ=436 nm (blue)</td>
<td>λ=546 nm (green)</td>
<td>λ=436 nm (blue)</td>
<td>λ=546 nm (green)</td>
</tr>
<tr>
<td>4</td>
<td>17,700</td>
<td>16,200</td>
<td>17,000</td>
<td>6.57</td>
</tr>
<tr>
<td>5</td>
<td>20,000</td>
<td>18,600</td>
<td>19,300</td>
<td>7.07</td>
</tr>
<tr>
<td>7</td>
<td>28,200</td>
<td>27,200</td>
<td>27,700</td>
<td>4.45</td>
</tr>
<tr>
<td>12</td>
<td>36,800</td>
<td>35,400</td>
<td>36,100</td>
<td>5.06</td>
</tr>
<tr>
<td>17</td>
<td>42,000</td>
<td>41,100</td>
<td>41,600</td>
<td>5.76</td>
</tr>
<tr>
<td>20</td>
<td>56,400</td>
<td>53,500</td>
<td>55,000</td>
<td>5.04</td>
</tr>
<tr>
<td>22</td>
<td>69,500</td>
<td>65,400</td>
<td>67,500</td>
<td>4.32</td>
</tr>
</tbody>
</table>

* Mean of determinations with blue and green incident light.
Various possible causes for the observed discrepancy were considered. One possible source of error is in the accuracy of the values quoted for the incident light wavelengths. These are determined by the emission spectrum of the mercury vapour lamp, and by the transmission characteristics of the optical filters employed. The transmission characteristics of both blue and green filters were therefore examined, and it was found that all lines in the mercury spectrum close to 436 nm and 546 nm would be effectively excluded by the filters.

A second possible source of error arises from the profound effects which measured values of \((dn/dc)\) have on the value of the constant, \(H\) (eqn.19). If the value of \((dn/dc)\) is accurate to \(\pm 0.002\), or \(\pm 1.4\%\), then at each wavelength \(\bar{M}_w\) will carry an associated uncertainty of \(\pm 2.8\%\). The observed discrepancy in determinations of \(\bar{M}_w\) at two wavelengths, which amounts to an average difference of \(5\%\) \([\bar{M}_w (\lambda = 436 \text{ nm}) > \bar{M}_w (\lambda = 546 \text{ nm})]\) can therefore be explained on the basis of systematic errors in the determination of \((dn/dc)\). That is, \((dn/dc)\) is low when \(\lambda = 436 \text{ nm}\), and high when \(\lambda = 546 \text{ nm}\). The uncertainty in values of \((dn/dc)\) for dextran, particularly at \(\lambda = 546 \text{ nm}\), has been noted by Zebec (102); at this wavelength, literature values range from 0.140 to 0.154. In view of these findings, an uncertainty in the value of \(\bar{M}_w\) of \(\pm 2.8\%\), associated with measurements of \((dn/dc)\), is not an unduly large source of error.
### Table 14: Fractionation of Dextran Sample No.12 (26).

<table>
<thead>
<tr>
<th>$w_1$ (g.)</th>
<th>$M_1 \times 10^{-3}$</th>
<th>$w_1 M_1 \times 10^{-4}$ (g.)</th>
<th>$(w_1/M_1) \times 10^5$ (g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.53</td>
<td>77.0</td>
<td>11.78</td>
<td>1.99</td>
</tr>
<tr>
<td>1.37</td>
<td>66.0</td>
<td>9.04</td>
<td>2.08</td>
</tr>
<tr>
<td>0.82</td>
<td>57.0</td>
<td>4.67</td>
<td>1.44</td>
</tr>
<tr>
<td>1.85</td>
<td>51.0</td>
<td>9.44</td>
<td>3.63</td>
</tr>
<tr>
<td>1.94</td>
<td>45.0</td>
<td>8.73</td>
<td>4.13</td>
</tr>
<tr>
<td>1.97</td>
<td>37.0</td>
<td>7.29</td>
<td>5.32</td>
</tr>
<tr>
<td>1.32</td>
<td>32.0</td>
<td>4.22</td>
<td>4.31</td>
</tr>
<tr>
<td>0.82</td>
<td>29.0</td>
<td>2.38</td>
<td>2.83</td>
</tr>
<tr>
<td>1.31</td>
<td>27.0</td>
<td>3.54</td>
<td>4.85</td>
</tr>
<tr>
<td>2.09</td>
<td>24.0</td>
<td>5.02</td>
<td>6.71</td>
</tr>
<tr>
<td>1.47</td>
<td>20.0</td>
<td>2.94</td>
<td>7.35</td>
</tr>
<tr>
<td>1.22</td>
<td>15.0</td>
<td>1.63</td>
<td>8.13</td>
</tr>
<tr>
<td>1.21</td>
<td>12.5</td>
<td>1.51</td>
<td>9.68</td>
</tr>
</tbody>
</table>

$$\sum_i w_1 = 18.92; \sum_i (w_i M_i) = 72.39; \sum_i (w_i / M_i) = 64.45$$

$$\bar{M}_W = \frac{\sum_i (w_i M_i)}{\sum_i w_i} = 38,300$$  $$\bar{M}_n = \frac{\sum_i w_i}{\sum_i (w_i / M_i)} = 29,400$$

The overall accuracy of the light scattering determination is governed by those errors arising from three sources (2) measurement of $(dn/dc)$, $(\pm 2.0\%)$, (b) determination of the calibration constant, $k_1$ $(\pm 1 - 2\%)$, and (c) extrapolation to zero concentration $(\pm 1\%)$. The uncertainty in the value of $\bar{M}_W$ at any wavelength is therefore of the order of $\pm 6\%$. 

90
In order to determine the molecular weight distribution, dextran sample no. 12 was fractionated by precipitation with ethanol; this work was carried out by other members of the IRI group (26). Sub-fraction molecular weights were determined by light scattering methods, and the fractionation data are given in table 14. The weight-average molecular weight calculated from the fractionation data was $38,300$. This value is in good agreement with that obtained by light scattering methods, viz. $36,100$, which confirms the accuracy of the latter procedure.

The second virial coefficients of the dextran samples were determined from the gradients of the $c/I$ vs. $c$ plots (fig. 9) and are shown in table 13. The values are in accord with those of Senti et al. (29), being of the order of $5 \times 10^{-4}$ mole cm.$^3$ g.$^{-2}$. Although there is some scatter in the results, a decrease in $B$ with increasing molecular weight is evident, which is in agreement with the results of various workers (29, 30). This is interpreted as being due to a slow decrease in excluded volume, which results from an increasing preference for polymer-polymer contacts in the random coil.

2. VISCOSITY-MOLECULAR WEIGHT CORRELATION.

The objective of this work was to establish a correlation between molecular weight and viscosity, in order that molecular weight might be determined from viscosity measurements alone. Viscosity determinations were therefore conducted on those dextrans which had been characterised with respect to $M_w$ by light scattering methods.
(a) *Viscosity Measurement.*

Solutions of each of the dextrans listed in table 13 were prepared at several concentrations within the range 0.002 - 0.02 g.ml⁻¹. Flow times for solution and solvent (water) were measured in a suspended level viscometer. The concentration dependence of viscosity was expressed in terms of the Huggins equation (28),

\[
\frac{\eta}{c} = [\eta] + k_1 [\eta]^2 c \tag{35}
\]

The intrinsic viscosity, \([\eta]\), was determined by extrapolating linear plots of \(\eta_{sp}/c\) against \(c\) to zero concentration. In several instances, the viscosity data was also extrapolated to zero concentration by means of the equation defining inherent viscosity,

\[
[\eta]_{inh} = (1/c) \ln (t/t_0) \tag{36}
\]

Typical results are indicated in fig.10, from which it is evident that the value of \(\eta_{inh}\) measured when \(c < 0.005\) g.ml⁻¹ is in excellent agreement with the intrinsic viscosity obtained by extrapolation. This finding remained valid throughout the range of dextrans studied. Since \(\eta_{inh}\) and hence \([\eta]\), may be determined from flow measurements on a single solution, the experimental work required to measure \([\eta]\) is considerably reduced. All dextrans subsequently studied in this work were therefore characterised by viscosity determinations at a single concentration in the region of 0.005 g.ml⁻¹.

Many polymer solutions show non-Newtonian behaviour, in that the viscosity varies with the rate of shear in the viscometer (28). The dependence of viscosity on shear rate was examined in several dextrans by comparing the viscosities of dilute solutions (0.005 g.ml⁻¹), measured in two viscometers with different capillary diameters. A factor of four in the shear rate had negligible effect on
FIG. 10: EXTRAPOLATION OF DEXTRAN SOLUTION VISCOSITY TO ZERO CONCENTRATION
the viscosity number. This finding was confirmed by determining
the shear rate dependence of viscosity in a rotational coaxial
cylinder viscometer, at shear rates between 50 and 500 sec.\(^{-1}\).
The viscosity of a solution of dextran (sample 12, 0.3 g/ml.\(^{-1}\))
showed very little shear rate dependence when examined in this
way. The work of Oene and Cragg (104) also shows that dextran
solutions have a negligible shear rate dependence even at mole-
cular weight weights as high as 10\(^{7}\).

(b) Determination of Correlation.

In order to utilise viscosity measurements for determining mole-
cular weight in dextrans, it is first necessary to determine the
viscosity-molecular weight correlation obtained when both quantit-
ies are independently measured. The results of viscosity measure-
ments on those dextrans which had been previously characterised
with respect to \(M_w\) by light scattering methods, are given in table
15. In accordance with eqn.4, log \(\eta_p\) was plotted against log \(M_w\),
and the results are shown in fig.11. Viscosity-molecular weight
data obtained by other IRI workers (26) on five sub-fractions of
one of the dextrans (sample 12), are also given in table 15, and
presented graphically in fig.11. The correlation between viscosity
and molecular weight derived by Senti et al. (29) for similar
dextran fractions is also shown.

It is evident from fig.11 that the correlation for the dextrans
being characterised in this work agrees very closely with that of
Senti et al. (29); the best correlation is given by,
FIG. 11. VISCOSITY–MOLECULAR WEIGHT CORRELATIONS IN DEXTRANS
\[
\eta = (9.68 \times 10^{-4}) \bar{M}_w^{0.50} \quad (37)
\]

This is to be compared with the correlation of Senti et al. (29), which is,

\[
\eta = (9.78 \times 10^{-4}) \bar{M}_w^{0.50} \quad (38)
\]

It is also clear that the viscosity-molecular weight data for the dextran sub-fractions fits the derived correlation (eqn.37) to a good degree of accuracy.

If the viscosity measurements have been performed on polydisperse polymers and the correlation has been established using essentially monodisperse fractions, eqn.37 would normally give the viscosity-average molecular weight, \( \bar{M}_v \). However, in this case, dextran fractions and derived sub-fractions have been shown to fit the correlation equally well. The inference is that the molecular weight distributions of the fractions and sub-fractions are insufficiently different to influence the correlation. This would appear to be a reasonable assumption in view of the fact that the dextrans possess low (\( \bar{M}_w/\bar{M}_n \)) ratios in the range 1.3 - 1.5 (table 17) and are thus approaching monodispersity. The excellent agreement with the results of Senti et al. (29) suggests that fractions of a similar molecular weight distribution were employed to establish both correlations. (\( \bar{M}_w/\bar{M}_n \)) values reported by Senti et al. (29) (1.1 - 1.5) are in fact comparable with those measured in this work (table 17).

The intrinsic viscosities of the remaining dextrans were determined by the procedures outlined above, and the corresponding molecular weights (\( \bar{M}_w \)) calculated using eqn.37. Results are given in table 17 which follows at the end of section I.A.
Table 15: Dextran Viscosity and Molecular Weight Determinations.

<table>
<thead>
<tr>
<th>Dextran Sample No.</th>
<th>([\eta] \text{ (dl.g.}^{-1})</th>
<th>(M_w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.126</td>
<td>17,000</td>
</tr>
<tr>
<td>5</td>
<td>0.135</td>
<td>19,300</td>
</tr>
<tr>
<td>7</td>
<td>0.160</td>
<td>27,700</td>
</tr>
<tr>
<td>12</td>
<td>0.184</td>
<td>36,100</td>
</tr>
<tr>
<td>17</td>
<td>0.197</td>
<td>41,600</td>
</tr>
<tr>
<td>20</td>
<td>0.227</td>
<td>55,000</td>
</tr>
<tr>
<td>22</td>
<td>0.250</td>
<td>67,500</td>
</tr>
<tr>
<td>S1</td>
<td>0.107</td>
<td>12,500</td>
</tr>
<tr>
<td>S2</td>
<td>0.136</td>
<td>20,000</td>
</tr>
<tr>
<td>S3</td>
<td>0.168</td>
<td>30,000</td>
</tr>
<tr>
<td>S4</td>
<td>0.222</td>
<td>48,700</td>
</tr>
<tr>
<td>S5</td>
<td>0.242</td>
<td>62,200</td>
</tr>
</tbody>
</table>
3. **DETERMINATION OF NUMBER-AVERAGE MOLECULAR WEIGHT.**

(a) **Assessment of Available Methods.**

Several techniques, notably membrane osmometry, vapour phase osmometry and end-group analysis, are applicable to the determination of number-average molecular weights in dextrans (89). To assess the relative accuracy and convenience of such methods, a dextran of well-defined number-average molecular weight was selected for study. This dextran (sample 12) had been subjected to fractionation by ethanol precipitation; a value of 29,400 for $M_n$ was calculated from the fractionation data (table 14). The technique giving a value close to this figure and with good reproducibility of results was selected as most appropriate for this work.

(i) **Osmometry.**

In principle, membrane osmometry is a simple technique to apply (88, 89, 105). Solution in an osmometer is separated from pure solvent by a membrane impermeable to solute molecules. Solvent then flows to the solution compartment until equilibrium is established, and a hydrostatic pressure develops corresponding to the osmotic pressure of the solution. To measure the osmotic pressure, a rising or falling level of solution in the measuring capillary is monitored until equilibrium is attained. This work is concerned with dextrans characterised by values of $M_n$ in the range 10,000 - 100,000; the osmotic pressure of solutions of concentration 0.01 g.ml.$^{-1}$ ($10^{-3}$ - $10^{-4}$ M) will be in the range 5 - 50 cm. water. Such pressures are readily measurable in a conventional osmometer.

In theory, when pure solvent is present on both sides of the membrane, no asymmetry in pressure should develop. However, in many cases, a difference is in fact observed in the heights of liquids in
the measuring capillaries. A correction factor, or membrane constant, must therefore be applied to account for this difference. Membrane constants have been observed in a large number of membrane-solvent systems, and are generally considered to be due to effects associated with adsorption of solutes at the membrane surface (105).

Attempts were made to use osmometry for determining \( \bar{M}_n \) in dextrans. In the Pinner-Stabin osmometer employed (106), which was fitted with gel cellophane membranes, irreproducible membrane constants were observed. Problems also arose due to the high surface tension of water, which leads to sticking of menisci, both in the measuring capillary and in the reference capillary used to correct for capillary rise. It was therefore difficult to obtain a good degree of accuracy in the application of osmometry to aqueous dextran solutions. Additionally, the time requirement for reaching equilibrium, together with the necessity to account for solution non-ideality by performing measurements at various solute concentrations, and extrapolating to infinite dilution, made the osmometry method rather lengthy. Nevertheless, after extensive studies on membrane conditioning, the number-average molecular weights of several dextrans \( (\bar{M}_n \approx 50,000) \) were determined successfully by Fisons Ltd. (20). The values of \( \bar{M}_n \) obtained were in good agreement with those determined below by end-group analytical procedures (table 16).

Under certain conditions, \( \bar{M}_n \) may also be determined by vapour phase osmometry (107). This technique involves measuring the temperature difference between two thermistors, one in contact with solution, the other in contact with solvent, and both in an atmosphere.
saturated with solvent vapour at constant temperature. The difference in temperature between the two thermistors, as recorded in arbitrary units of resistance, is proportional to the lowering of the vapour pressure, which in turn depends on the molar concentration of dissolved solute. To relate resistance readings to solution molarities, calibration curves are prepared using solutions of known concentration, containing a solute such as sucrose. When a polymer solution of known concentration (w/v) is contacted with the solution thermistor, the molar concentration corresponding to the observed resistance reading can be obtained from the calibration graph, and $\bar{M}_n$ can be determined. Again, to account for non-ideality in polymer solutions, it is necessary to measure osmotic pressure as a function of concentration, and extrapolate to infinite dilution.

Since water has a relatively low vapour pressure, vapour phase osmometry is not particularly sensitive when applied to aqueous solutions, particularly when $\bar{M}_n$ is greater than 20,000 and the vapour pressure lowering is very small. Attempts were made to determine $\bar{M}_n$ in dextrans using an Hitachi-Perkin-Elmer vapour phase osmometer. Low values of $\bar{M}_n$ were observed, which suggested that the dextrans might be contaminated by traces of low molecular weight materials; these small molecules would have a significant effect on the value of $\bar{M}_n$ determined by this method. Traces of glucose, ethanol, sodium chloride etc. are known to be present (20), and on removal of these components by dialysis, more realistic $\bar{M}_n$ values were obtained. However, in view of the low sensitivity of the method and the extensive experimental work involved in extrapolating results to infinite dilution, further refinement of the
technique was not attempted.

(ii) End-Group Analysis.

Examination of the structure of dextran (fig.1) shows that one reducing end-group is present on each dextran molecule. By reacting this group with an oxidising agent, and measuring the quantity of reaction product formed, it is possible to determine the molar concentration of a dextran solution. If the weight concentration (w/v) of the solution is also known, the number-average molecular weight of the polymer can be calculated.

Reducing end-groups in polysaccharides are normally estimated by oxidation with hypoiriode or with alkaline ferricyanide solutions (89,108). In the latter procedure, ferrocyanide ion, the reduction product, requires to be measured accurately at low concentration. This is commonly effected by formation of the Prussian blue complex. However, the method suffers from several disadvantages associated with the colloidal nature of the coloured complex. An alternative procedure for the determination of ferrocyanide is based on a method which involves the formation of ferrous ions, and their subsequent reaction with a reagent for iron, viz. 2,4,6, -tri-(2'-pyridyl )-1,3,5-triazine (109). The reactions involved are as follows:-

\[
\begin{align*}
H_2O + ROH + 2 Fe^{3+} & = RCOOH + Fe^{2+} + 2H^+ \\
Fe^{2+} + 2TPTZ & = Fe(TPTZ)_2^{+2}
\end{align*}
\]

TPTZ acts as a tridentate ligand towards ferrous ion, and gives a violet colour which is measurable at 595 nm. This reagent is highly sensitive and has been used to measure glucose quantitatively down to a concentration of \(2 \times 10^{-9}\) M (109).
The ferricyanide oxidation method, using TPTZ for iron determination, was evaluated by other members of the IRI group (26). Absorbance-concentration curves which were linear up to a concentration of $10^{-7}\, \text{M}$, were obtained on ferricyanide oxidation of glucose and isomaltotriose. However, an experimental determination of $M_n$ for a dextran of known number-average molecular weight (table 14) gave results which were lower than anticipated. Although this method may be highly sensitive, and hence even applicable to higher molecular weight dextran fractions, the number of glucose groups is over estimated by about 20%. This method was therefore abandoned in favour of an alternative end-group analytical procedure which proved to be more specific than the simple oxidation methods referred to above. The method involved dextran end-group reduction with borohydride, followed by oxidation with periodate, and colorimetric determination of liberated formaldehyde with chromotropic acid (110). On application of the method to the dextran of known $M_n$ (table 14), consistent and reproducible results were obtained. This procedure was therefore considered as being suitable for $M_n$ determination in dextrans.

(b) $M_n$ Determination in Dextrans by End-Group Analysis.

A procedure for reducing end-group assay, involving reduction with borohydride, oxidation with periodate, and colorimetric determination of the liberated formaldehyde with chromotropic acid, has been described by Unrau and Smith (110), and later elaborated in greater detail by Smith and co-workers (111). The method is suspect for native undegraded polysaccharides of very high molecular weight, because of the uncertain nature of the "reducing" end-group. However, it is well suited to degraded polysaccharides such as the
Dextrans being studied in this work; such polysaccharides have been depolymerised by acid hydrolysis, and can thus be expected to have a true reducing terminal aldose residue. Unlike the ferricyanide oxidation procedure, which is susceptible to interference from traces of reducing components present in solution, the borohydride reduction method is believed to be much more specific for Mn determination in dextrans.

Dextran will be oxidised by sodium periodate according to the scheme shown in fig.12 (a). It is evident that slightly more than 2 moles of periodate per anhydroglucose (C₆H₁₀O₅) residue are required for complete oxidation, and no formaldehyde is released. On reduction with borohydride, however, the reducing glucose end-group of dextrans is converted to a 6-substituted D-glucitol residue, which should liberate 1 mole of formaldehyde on complete oxidation. The reaction is illustrated in fig.12 (b).

High concentrations of the oxidised dextran have been shown to give interfering colour in the formaldehyde-chromotropic reaction, and must be removed by dialysis as described by Smith and co-workers (111). It is, however, not necessary to remove the periodate and iodate ions by precipitation (111), although they must be converted to iodide by addition of excess sodium metabisulphite (112) :-

\[ 2 \text{Na}_2 \text{S}_2\text{O}_5 + 2\text{H}_2\text{O} + \text{NaIO}_4 = 4 \text{Na} \text{HSO}_4 + \text{NaI} \]

Formaldehyde concentrations were related to absorbance through linear plots obtained by oxidising mannitol solutions of known concentration under conditions identical to those used in the
FIG. 12: REACTIONS OF DEXTRAN UNITS WITH PERIODATE
Dextran assay procedure:

\[
C_6H_{14}O_6 + 5NaIO_4 = 2C H_2O + 4HCOOH + H_2O + 5NaIO_3
\]

It is evident that 1 mole of mannitol yields 2 moles formaldehyde on complete oxidation.

The number-average molecular weight of several dextrins was determined by the end-group assay method described above; results are indicated in table 16. The accuracy of the method was confirmed by the $\bar{M}_n$ value of 28,000 obtained for sample 12; this agrees with the value, 29,400, calculated from the fractionation data (table 14). The $\bar{M}_n$ value of 47,000 for sample 20 also compares well with that obtained from osmometry measurements (20). The main potential source of error lies in incomplete reduction of the polysaccharide; an attempt to allow for slow reaction was made by selecting a relatively long reduction period of 72 hours. The small blank readings which were observed are most probably associated with oxidised dextran products which may partially permeate the dialysis membrane. Studies on the reproducibility of the method indicate an uncertainty in $\bar{M}_n$ of the order of $\pm 5\%$ when $\bar{M}_n$ is in the region of 40,000.
Table 16: Number-Average Molecular Weights of Dextrans.

<table>
<thead>
<tr>
<th>Dextran Sample No.</th>
<th>Number-Average Molecular Weight, Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Determination by End-Group Analysis</td>
</tr>
<tr>
<td>2</td>
<td>6,200</td>
</tr>
<tr>
<td>7</td>
<td>19,000</td>
</tr>
<tr>
<td>12</td>
<td>28,200</td>
</tr>
<tr>
<td>20</td>
<td>46,000</td>
</tr>
<tr>
<td>23</td>
<td>80,000</td>
</tr>
</tbody>
</table>

4. SUMMARY OF DEXTRAN CHARACTERISATION RESULTS.

The $M_w$, $M_n$, $[\eta]$ and $(M_w/M_n)$ data characterising the twenty three dextrans which were employed in the investigations of crystallization, are indicated in table 17. Details of three dextran subfractions which were studied in this work are also given. Several dextrans with a range of average molecular weight were selected for $M_n$ determination. The objective of the $M_n$ determinations was simply to demonstrate that the width of the molecular weight distribution (as characterised by $(M_w/M_n)$) did not alter significantly throughout the range of dextrans being studied.
Table 17: Summary of Dextran Characterisation Data.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>$\eta$ (dl.g$^{-1}$)</th>
<th>$M_w$</th>
<th>$M_n$</th>
<th>$\frac{M_w}{M_n}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.051</td>
<td>2,800</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.095</td>
<td>9,600</td>
<td>6,200</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>0.095</td>
<td>9,600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4*</td>
<td>0.126</td>
<td>17,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5*</td>
<td>0.135</td>
<td>19,300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.160</td>
<td>26,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7*</td>
<td>0.160</td>
<td>27,700</td>
<td></td>
<td>19,000 1.5</td>
</tr>
<tr>
<td>8</td>
<td>0.161</td>
<td>28,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.162</td>
<td>28,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.180</td>
<td>34,600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.184</td>
<td>36,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12*</td>
<td>0.184</td>
<td>36,100</td>
<td></td>
<td>28,000 1.3</td>
</tr>
<tr>
<td>13</td>
<td>0.187</td>
<td>37,200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0.189</td>
<td>38,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.193</td>
<td>39,600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>0.197</td>
<td>41,200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17*</td>
<td>0.197</td>
<td>41,600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>0.197</td>
<td>41,600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>0.199</td>
<td>42,000</td>
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<td></td>
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<tr>
<td>20*</td>
<td>0.227</td>
<td>55,000</td>
<td></td>
<td>46,000 1.2</td>
</tr>
<tr>
<td>21</td>
<td>0.240</td>
<td>61,400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22*</td>
<td>0.250</td>
<td>67,500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>0.325</td>
<td>113,000</td>
<td></td>
<td>80,000 1.4</td>
</tr>
<tr>
<td>s1**</td>
<td>0.107</td>
<td>12,500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>s2**</td>
<td>0.136</td>
<td>20,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>s3**</td>
<td>0.168</td>
<td>30,000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Samples in which both $\eta$ and $M_w$ were experimentally determined.

** Sub-fractions of sample no. 12.
1. INTRODUCTION.

The dextrans studied in this work were prepared by Fisons Ltd. for pharmaceutical use; details of the method of preparation are given in section I.F. Although rigorous conditions of quality control were enforced at each stage of the manufacturing process, when aqueous dextran solutions were prepared, it was visually evident that some were much more turbid than others.

The turbidity of several aqueous dextran solutions (10% w/v) was measured by the nephelometric method described below. Some dextrans gave rise to particularly turbid solutions \((N_{100} = 100 - 200)\), whereas others contained only small amounts of insoluble material \((N_{100} = 20 - 40)\). This turbidity must be due to the presence of dispersed, insoluble particles, which are almost certain to be composed chemically of molecular aggregates of dextrans.

There was no tendency for the insoluble particles to settle out on standing, or even on prolonged centrifugation. No significant reduction in turbidity could be achieved by filtering the solutions through a membrane of mean pore diameter 10 \(\mu\)m. It was therefore assumed that particles of colloidal dimensions were present. Investigations described in this section were aimed at studying the nature of the particulate material responsible for turbidity. Further objectives were to uncover procedures for the assessment, development, and destruction of the insoluble material, and to identify the process responsible for dextran insolubilisation.
2. LIGHT SCATTERING METHODS.

(a) Simple Assessment of Turbidity.

Following the observation that wide variations existed in the turbidity of aqueous dextran solutions, the first stage was to develop a simple, rapid method of measuring turbidity. Spectrophotometric measurement of absorbance, which is proportional to turbidity, is one possible technique; it is however rather sensitive to the presence of colour, of which there were traces in some of the lower molecular weight dextrans. A nephelometric method, using natural incident light, overcomes this to some extent, and is more rapid and convenient. This procedure was used throughout the turbidity studies described below.

In the nephelometric method, a beam of light is shone vertically up a test-tube which contains the test solution. Light which is scattered at a range of small angles to the incident beam impinges upon a photoelectric cell, the output being recorded on a galvanometer scale. The method requires a standard reference turbidity, and a scratched perspex rod inside a test-tube was used for this purpose. For low turbidity measurements, the galvanometer sensitivity control was adjusted such that a full-scale deflection of 100 was obtained with this standard in place. For measurements of turbidities exceeding 100, the galvanometer full-scale deflection reading was re-set to some lower value (50, 25 or 10), and readings scaled up by the corresponding factor. Thus, all the nephelometric readings \(N_{100}\) extending up to 2,000, which were measured in this work, are related to a galvanometer reading of 100 observed with the perspex rod standard.
Several suspensions of silica particles in sodium chloride solution (0.05 M) were prepared, with turbidities both smaller and greater than 100, as measured by the above procedure; the absorbance of each of these suspensions was measured in a spectrophotometer at 400 nm. A plot of nephelometer turbidity vs. absorbance is shown in fig.13. The linearity of the plot up to an absorbance of 0.5 shows that the procedure of using the scaling factor for determining turbidities greater than 100, is justifiable. Above an absorbance of about 0.5, the nephelometer readings are somewhat lower than anticipated. This is most probably due to the onset of inter-particle interference (loss of scattered light intensity) as the concentration of silica particles increases. This phenomenon has already been reported (section I.A.1 (b) viii), and the necessary corrections were applied when silica suspensions were used for calibrating the SOFICA light scattering photometer (eqn.30).

(b) Detailed Light Scattering Measurements.

In studies where the objective was to follow changes in the detailed light scattering behaviour of visibly turbid dextran solution, the SOFICA light scattering photometer, described in part I.A., was employed.

The complications which arise in the application of light scattering methods to molecular weight determination when large particles are present, have already been considered briefly in section I.A.2. In the case of particles having dimensions comparable to the incident light wavelength, scattering becomes dependent on the shape of the particle. The scattered intensity is no longer symmetrical about the 90° position, and $R_g$ becomes dependent on the angle of measurement.
FIG. 13: CORRELATION OF ABSORBANCE OF SILICA DISPERSIONS WITH NEPHELOMETER TURBIDITIES AS MEASURED BY SCALE FACTOR METHOD
However, the relationship derived from small particles (eqn.19) can still be applied if the observed turbidities are corrected for interference effects (91,92). If the particle scattering factor $P(\theta)$ is defined as,

$$ P(\theta) = \frac{R_\theta}{R_0} \quad \text{(39)} $$

Then eqn.19 becomes,

$$ \frac{P(\theta) \times c}{\gamma} = \frac{1}{\tilde{F}_w} + 2B_c \quad \text{(40)} $$

$P(\theta)$ is the intensity of scatter at any angle, $\theta$, relative to that at zero angle; the latter is independent of the size and shape of the particle. In order to measure true turbidities under conditions where $P(\theta) \neq 1$, $P(\theta)$ must either be known, or the results evaluated in such a way that $P(\theta)$ approaches unity. The latter approach is the one originally proposed by Zimm (89,91), in which the intensity of scatter is measured at several angles and the results are extrapolated to zero angle.

$P(\theta)$ depends on the shape of the scattering particle; if this is known, $P(\theta)$ can be related to particle size. For example, in the case of spherical particles, $P(\theta)$ is given by (91),

$$ P(\theta) = \left[\frac{3}{x^3} (\sin x - x \cos x)\right]^2 \quad \text{(41)} $$

where $x = \frac{2\pi \text{noD}}{\lambda} \sin(\theta/2)$

and $D = \text{sphere diameter}$.

Using this expression for $P(\theta)$, it is possible to calculate the ratio $P(\theta)/P(\Pi - \theta)$ for a series of values of $(\text{noD}/\lambda)$ at any angle $\theta$. Thus, if it is assumed that the particle is spherical, the size can be directly related to the dissymmetry coefficient, $Z$. 

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The factor \( \frac{1}{P(90)} \), by which observed turbidities \( i_{90} \) are corrected for interference effects, can also be calculated as a function of \( Z \). The appropriate data relating to values of \( P(\Theta) \) and \( Z \) as a function of \( (\text{mol} / \lambda) \) for spheres, random coils, and rigid rods are given by Stacey (91).

Since the dissymmetry coefficient is concentration dependent, it is necessary to extrapolate experimental data to zero concentration. This can often be achieved conveniently by means of a plot of \( \frac{1}{(Z-1)} \) against \( c \); such plots are frequently linear. The value \( Z(c = 0) \) can be used to determine the characteristic dimension of the particle if the particle shape is known.

When the size of the scattering particles exceeds about 200 nm, the methods for particle size determination described above can no longer be applied with accuracy (113). For monodisperse systems, the scattering parameters are no longer monotonic in particle diameter and scattering intensity becomes strongly periodic in both scattering angle and particle size (91,114,115). In heterodisperse systems, the periodicities observed in the monodisperse envelope are broadened and diminished by an amount which depends on the particle size distribution. Sufficient heterodispersity may yield a non-periodic scattering envelope, and the scattering parameters may become only slightly dependent on the average particle diameter (115,116).

One scattering parameter which retains a strong dependence on particle size for large heterodisperse particles is the \( 90^\circ \) depolarisation ratio, \( \sigma_{90} \). Values of \( \sigma_{90} \) have been computed for monodisperse spherical systems as a function of particle size and refractive
index (11,7). These may be used to obtain particle size information. Graessley and Zufall (115) have additionally shown that if the oscillations in the value of $\text{d}_0$ are removed by heterodispersity, $\text{d}_0$ should remain proportional to the average particle diameter of the system. Measurements of $\text{d}_0$ can therefore be used to investigate changes occurring in the average dimensions of spherical particles present in systems with a wide particle size distribution.

3. STUDIES OF INHERENT TURBIDITY.

For the purpose of this work, inherent turbidity is that which arises from insoluble material originally present in dextrans. This is to be distinguished from developed turbidity, which is associated with insoluble particles which can be deliberately produced in dextran solutions under certain conditions.

(a) Microscopy and Light Scattering Studies.

A turbid solution of dextran (sample 7, 10% w/v) was examined under high magnification (x 200) in a polarising microscope. No discrete particles were identifiable, and under crossed polarisers, no birefringence was exhibited. The inference is that the particles responsible for turbidity are below the limits of resolution, i.e. with dimensions less than approximately 1 $\mu$m.

Solutions of two dextrans, one (sample 7) exhibiting high turbidity, the other (sample 17) of low turbidity, were studied by light scattering methods using the SOFICA photometer. In each case, it proved necessary to remove large extraneous particles by filtration through
a membrane of mean pore diameter, 0.8 μm. Using blue incident light (λ = 436 nm), dissymmetry ratios were measured as a function of dextran concentration. Assuming that the insoluble material is homogeneously dispersed, the dextran concentration will be directly proportional to the concentration of insoluble particles. The dissymmetry data was extrapolated to zero particle concentration using an empirical \(1/(Z - 1)\) vs. c plot (91,113); results are shown in fig.14.

It is evident from fig.14 that in each case, dissymmetries approach constant values below those levels of insoluble particles which correspond to dextran concentrations in the region of 10% w/v. The rapid rise in dissymmetry at higher concentrations is most probably due to inter-particle interactions, the increase being more pronounced in the case of the more turbid sample.

The depolarisation ratio, \(\phi_{90}\), was also measured for each solution at a dextran concentration of 10% w/v. The low values observed for \(\phi_{90}\) viz. 0.02 - 0.03, point to the presence of small symmetrical scattering centres. For the purposes of calculating particle size from dissymmetry measurements, a spherical particle shape was therefore assumed.

A graphical relationship between spherical particle diameter, \(D\), and dissymmetry, \(Z\), was constructed from tables of \((n_o \cdot D / \lambda)\) as a function of \(Z\) (91); values of solvent refractive index, \(n = 1.33\), and wavelength, \(\lambda = 436\) nm, were used to calculate \(D\) from \((n_o \cdot D / \lambda)\). The plot is shown in fig.15. For each dextran, the values of \(Z(c = 0)\) and \(D\) which were determined from figs.14 and 15 are given in table 18.
FIG. 14: EXTRAPOLATION OF CRYSTALLINE PARTICLE DISSYMMETRY, Z, TO ZERO CONCENTRATION
Fig. 15: Dependence of sphere dissymmetry on particle diameter.

\[ \lambda = 436 \text{ nm} \]
\[ n_o = 1.33 \]
The insoluble material present in dextrans, which is responsible for turbidity, therefore probably consists of sub-microscopic, spherical particles, with diameters of the order of 100 nm. The turbidity of dextran solutions will depend both on the number and size of the insoluble particles. It is also evident that the number of particles present can vary considerably from one dextran to another.

(b) Filtration Studies.
To gain further insight into the size of particles responsible for turbidity, a membrane filtration approach, using membranes with graded pore diameters in the range 10 - 450 nm, was adopted. Because of the considerable viscosity of dextran solutions, it was necessary to use moderately low dextran concentrations (2% w/v) to ensure practical filtration rates. Turbid solutions were recirculated over the membrane surface under pressure in an ultrafiltration cell system. This procedure facilitates the dispersal of membrane surface layers associated with retained particles; it offers the best possibility of estimating particle size from retention measurements, with minimum complications from membrane surface effects. Wherever possible, asymmetric membranes with
a pseudo-conical shaped pore arrangement, were selected (118). This was to avoid the possibility of anomalous retention effects which can sometimes occur due to internal blockage in isotropic filters (depth-type filtration) (119).

Four membrane filters were selected; the turbidity and dextran content of two solutions, one of high turbidity, the other of low turbidity, were measured before and after passage through each of the membranes. In each case, a constant volume of permeate was withdrawn through the membrane; the volume of retained solution was sufficiently large that its turbidity did not alter significantly during filtration. Results are shown in table 19.

With sample 7 of higher turbidity, the insoluble particles are not removed by membranes with pore diameter above 10 nm. With the 10 nm membrane, the permeate turbidity \( N_{100} = 9 \) is comparable with that of water. The existence of particles in the 10 - 500 nm diameter range is indicated, with at least a proportion being in the range 100 - 500 nm. The presence of particle sizes above 500 nm is considered unlikely in view of the fact that no clarification of the solution was possible in a laboratory centrifuge. With sample 17 of lower turbidity, clarification was effected with membranes of pore diameter, 100 nm or less, but not with the 450 nm membrane; the presence of particles within the range 100 - 500 nm is indicated.
Table 19: Retention of Soluble and Insoluble Dextran during Filtration of Solutions through Graded Pore Diameter Membranes.

<table>
<thead>
<tr>
<th>Membrane Type and Mean Pore Diameter (nm)</th>
<th>Dextran Sample 7 Retentate Solution 2% w/v</th>
<th>Dextran Sample 17 Retentate Solution 2% w/v.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Turbidity of Membrane Permeate (N100)</td>
<td>Dextran Retention (%)</td>
</tr>
<tr>
<td>Asymmetric 10</td>
<td>9</td>
<td>93</td>
</tr>
<tr>
<td>Asymmetric 50</td>
<td>19</td>
<td>78</td>
</tr>
<tr>
<td>Asymmetric 100</td>
<td>19</td>
<td>51</td>
</tr>
<tr>
<td>Isotropic 450</td>
<td>28</td>
<td>5</td>
</tr>
</tbody>
</table>

* Retention (%) = \left( 1 - \frac{\text{Permeate Concentration}}{\text{Retentate Concentration}} \right) \times 100

Turbidity of Water: N100 = 8
It is not possible to place an upper limit on particle size based on the pore dimensions of membranes in which 100% transmission of turbidity is observed. This is because of secondary membrane formation, which occurs due to the accumulation of retained particles at the membrane surface; as a result, a barrier with selectivity properties of its own is formed. This secondary membrane may be retentive of particles to which the primary membrane is freely permeable. In the dextran filtration studies, evidence for secondary membrane formation is provided by the high retentions observed for molecular dextran (table 19). All the membrane filters utilised in this study were non-retentive of dextran molecules in the region of 40,000 molecular weight (120). In the same way, the passage of small particles of insoluble dextran could well be impeded by traces of larger retained particles. Total transmission of turbidity is therefore unlikely to be observed. The decreasing dextran retention with increasing membrane pore diameter is also consistent with secondary membrane formation; this shows that the pore size of the primary membrane, as well as the particle size distribution, is important in determining the overall transmission characteristics of the membrane system.

In principle, filtration using a membrane of pore diameter 10 nm could be used to quantitatively measure the insoluble material content of dextran solutions. However, difficulties in washing the retentate free of dissolved dextran and in subsequently removing the insoluble material from the membrane surface in a quantitative manner, made this an impractical approach.
In summary, these filtration studies demonstrated that the size of particles responsible for turbidity in dextrans may vary from one source to another, and that there is possibly a distribution of particle dimensions in the range 10 - 500 nm. These findings support those obtained above from light scattering studies, and are not inconsistent with the presence of crystalline entities at various stages of development, i.e. small, partially developed spherulites.

(a) Dispersal of Inherent Turbidity.
If the insoluble material present in dextrans is indeed crystalline in nature, it should be possible to clarify turbid solutions in two ways, (i) by the action of chemicals which would promote the solution process, and, (ii) by the action of heat, which would cause melting of the crystalline material. Both approaches were studied in an attempt to eliminate or reduce turbidity in a dextran (sample 8) which contained a high level of insoluble material.

(i) Action of Chemical Agents.
The effect on turbidity of adding various chemicals to the dextran solution was studied in two ways, (a) the chemicals were added during preparation of the solutions, and (b) the chemicals were added to freshly prepared dextran solutions. In each case, the effects were quantified by measuring the turbidities of solutions of known dextran concentration (10% w/v) after a 30 minute period. The results were compared with the turbidity of a similar dextran solution into which no additional substances had been introduced.

The addition of several species to dextran solutions was considered, each additive representing a general class of chemical substance;
the effect of these additions on turbidity is indicated in table 20. It is evident that sodium hydroxide is particularly effective in reducing turbidity, irrespective of whether it is included during preparation of the dextran solution, or is added after solution has taken place. The addition of urea also produces a similar, but less pronounced effect. Sodium chloride, glycine and glucose addition each proved less effective in reducing turbidity, whereas hydrochloric acid addition was totally ineffective in this respect.

Table 20: Effect of Added Chemical Species on the Turbidity of Dextran Solutions.

<table>
<thead>
<tr>
<th>Added Chemical Species</th>
<th>Additive Concentration (M)</th>
<th>Time of Adding Chemical:</th>
<th>Turbidity of Dextran Solution (10% w/v) after 30 min. (N₁₀₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>---</td>
<td>-</td>
<td>93</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>1.0</td>
<td>A</td>
<td>18</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>1.0</td>
<td>D</td>
<td>17</td>
</tr>
<tr>
<td>Urea</td>
<td>8.0</td>
<td>A</td>
<td>33</td>
</tr>
<tr>
<td>Urea</td>
<td>8.0</td>
<td>D</td>
<td>34</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>4.8</td>
<td>A</td>
<td>46</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.1</td>
<td>A</td>
<td>70</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0</td>
<td>A</td>
<td>69</td>
</tr>
<tr>
<td>Hydrochloric Acid</td>
<td>1.0</td>
<td>A</td>
<td>94</td>
</tr>
</tbody>
</table>
The action of sodium hydroxide and urea in reducing the turbidity of dextran solutions is particularly relevant in that these substances are generally recognised as being capable of breaking hydrogen bonds in polymer systems (77). The results suggest that aggregates of dextran in which inter-chain hydrogen bonding plays an important role, are being brought into solution. The presence of urea or sodium hydroxide causes hydrogen bonds between hydroxyl groups on adjacent polymer chains to be destroyed. In all possibility, the aggregation of the polymer chains would in the first instance, have resulted from the process of dextran crystallization.

The smaller effects on turbidity associated with the addition of other substances may be due, at least in part, to a reduction in scatter associated with the lower refractive index difference between the insoluble particles and the suspending medium.

There is a close similarity between the observed behaviour of insoluble dextran and that of 'retrograded', crystalline amylose derived from starch. Insoluble amylose cannot be dissolved in 8 M urea solution, but it will dissolve in 1 M sodium hydroxide solution (81). With both dextran and amylose, alkaline conditions lead to oxidative degradation (121), a process which gives rise to the formation of small amounts of yellow-brown colour. However, it is not considered that the extent of degradation which occurs within a 30 minute period would be sufficient to aid solubilisation to a degree that would explain the turbidity reductions observed.
(ii) **Action of Heat.**

The effect on turbidity of heating a solution of dextran (sample 8, 10% w/v) for 15 mins. at various temperatures, is shown in fig.16. A reduction in turbidity was observed only at temperatures above 60 °C; this reduction was not reversible on cooling the solutions. These findings suggest that a melting process is responsible for bringing the insoluble particles into solution. The fact that the insoluble dextran can be redissolved by heat suggests that the insolubility is due to crystallinity in the dextran, rather than, for example, a chemical cross-linking process. The presence of a range of particles with different melting points, thereby giving a melting range rather than a sharp melting point, is evident from fig.16.

This behaviour is typical of the melting process in polymer solutions (44,45), and is further evidence for identifying the insoluble material responsible for turbidity as crystalline dextran. More detailed studies on the melting process are reported in a later section (I.E.).

4. **Turbidity Development Studies.**

In order to study the formation of turbidity in dextran solutions, it was necessary to identify conditions under which the insolubilisation process would occur at measurable rates. It was strongly suspected that a crystallization process was responsible. Accordingly, since crystallization processes are concentration dependent, studies were conducted to determine whether turbidity development could be induced by varying the dextran concentration.

When the turbidity of moderately dilute solutions (10% w/v) of several dextrans was monitored at ambient temperature over a period
Aliquots of stock dextran solution (10% w/v) heated at each temperature for 15 min.

**FIG. 16: MELTING OF CRISTALLINE DEXTRAN OVER A RANGE OF TEMPERATURES**
of twelve months, no significant changes were evident. However, preliminary experiments showed that dextran solutions at higher concentrations (40 - 70% w/v) became highly turbid, and eventually gelled. The rate of development of turbidity was reproducible and characteristic of particular samples, and the process could be conveniently followed in the laboratory over periods ranging from 1 - 100 hours.

This turbidity, developed at high dextran concentration, was assumed to be of the same nature as that inherently present in the dextrans. During final drying of the dextrans, conditions favourable towards the development of turbidity must clearly have existed, albeit for a short period of time. In this way, small amounts of insoluble material would have been introduced into the products. This material can thus be considered as crystalline dextran at an early stage of growth. Extensive crystallization is improbable since the process would have been terminated when the product was isolated from solution in the amorphous state.

(a) Dispersal of Developed Turbidity.

The assumption has been made that both developed and inherent turbidity in dextrans originate by one and the same mechanism, which is most likely to be that of crystallization. If this is so, then both forms of insoluble material should respond similarly to methods of dispersal. The methods identified above, by which low levels of inherent turbidities could be reduced, were therefore examined when applied to the higher levels of induced turbidity. Such high turbidities were developed in concentrated dextran solutions which had been allowed to stand for several hours at ambient temperature.
(1) Action of Chemicals.

Three solutions of dextran (sample 8, 60% w/w) were prepared, and turbidity allowed to develop over a period of one hour at 20 °C. The solutions were then diluted to a lower concentration (10% w/v), at which turbidity development does not occur. In one sample, urea was incorporated during dilution, to give a final urea concentration of 8 M, while in a second, sodium hydroxide was introduced to give a final concentration of 1 M. No chemical was added to the third sample, which was used as a control. The turbidities of each dextran solution (10% w/v) were measured in the nephelometer after a 30 minute period and the results are shown in table 21. It is evident that both urea and sodium hydroxide are partially effective in dispersing induced turbidity. As was found in the case of inherent turbidity dispersal (table 20), sodium hydroxide is the more effective agent. The fact that turbidity is not completely dispersed again suggests that insoluble species having a range of stabilities may be present.

A dextran gel was prepared by allowing a solution (sample 8, 60% w/w) to stand at 20 °C, for 24 hours. After breaking the gel into small pieces, it could be redissolved within one hour by shaking with sodium hydroxide solution (1 M) at 20 °C. Urea solution (8 M) would not dissolve the gel under similar conditions. As mentioned above, this behaviour has been observed with crystalline amylose(81,82) a fact which suggests that the insoluble dextran may also be crystalline in nature.
(ii) **Action of Heat.**

Turbidity was developed in a dextran solution (sample 20, 60% w/w) over a period of 5 hours at 20 °C. The solution was then diluted to a lower concentration (10% w/v), and the turbidity measured in the nephelometer. After the solution had been boiled for 5 mins., the turbidity was remeasured. Under the same turbidity development conditions, another dextran (sample 8) formed a gel, which was redissolved after being broken up, suspended in water, and the suspension boiled for 30 minutes. The results of turbidity measurements on the diluted solutions, before and after heating, are given in table 22. It is evident that developed turbidity, and even gels, can be redispersed by the action of heat in the same manner as inherent turbidity.

Thus, both the insoluble material inherently present in dextrans, and also the insoluble matter developed in dextran solutions at high concentration, behave very similarly under the action of specific chemicals, viz., urea and sodium hydroxide, and under the action of heat. This is good evidence in support of the view that the insoluble particles originating from the two sources are of a similar nature. The manner in which the insoluble material responds to dispersal also suggests that it is crystalline in composition.
Table 21: Effect of Adding Urea and Sodium Hydroxide on Developed Turbidity.

<table>
<thead>
<tr>
<th>Chemical Added after Turbidity Development (Concentration)</th>
<th>Developed Turbidity (Dextran Sample 8, 10% w/v Solutions) ($N_{100}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea (8 M)</td>
<td>300</td>
</tr>
<tr>
<td>Sodium Hydroxide (1 M)</td>
<td>150</td>
</tr>
<tr>
<td>None</td>
<td>950</td>
</tr>
</tbody>
</table>

Table 22: Action of Heat on Turbidity Developed in Concentrated Dextran Solutions.

<table>
<thead>
<tr>
<th>Dextran Sample No.</th>
<th>Turbidity of 10% w/v Solution ($N_{100}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Heating</td>
</tr>
<tr>
<td>20</td>
<td>490</td>
</tr>
<tr>
<td>8</td>
<td>Gel</td>
</tr>
</tbody>
</table>

(b) Identification of Insoluble Material.

To conclusively establish the nature of the insoluble material formed in dextran solutions at high concentrations, two samples of the insoluble product were isolated for study. Firstly, insoluble material was produced in a solution of dextran (sample 7, 60% w/w) which had been allowed to stand at 25 °C for one hour. After diluting the solution to a lower concentration (10% w/v) to prevent complete gelation, the insoluble fraction was isolated by centrifugation, and
washed free of soluble dextran. A second sample of insoluble material was prepared, by allowing insolubilisation and gelation to take place over a longer, two hour period. The gel was re-dispersed as small particles in water, and the insoluble material isolated as before.

In each case, the insoluble solids were dried to approximately 90% dry matter; the moisture content was determined by extended drying of a small sample. The insoluble products isolated before and after gelation, amounted respectively to 4% and 6% of the total dextran present. These could be redissolved by boiling dilute aqueous dispersions, and in this way, aqueous solutions of known concentration (0.5% w/v) were prepared. Optical rotation and viscosity measurements were conducted on each solution, and the results are given in table 23. The specific rotations and viscosities of the isolated materials, which were insoluble in cold water but soluble in hot water, are virtually identical with those of the dextran from which they were derived. This demonstrates that the insoluble substance produced in dextran solutions at high concentration is indeed an insoluble form of dextran. The insolubility in cold water is almost certainly due to the presence of a crystalline structure.

Although fractionation during crystallization is sometimes observed (122), it is generally considered that crystallization processes do not result in fractionation on a molecular weight basis (31,123). For example, in adding a non-solvent to a polymer solution for the purpose of fractional precipitation, it is essential to ensure
that when separation occurs, it is due to liquid-liquid separation, and not to liquid-solid separation, or crystallization. It is evident from the viscosity measurements in table 23, that there is no fractionation on a molecular weight basis during insolubilisation of dextran. This finding is consistent with the premise that crystallization is responsible for the aggregation and insolubilisation of dextran. These observations are also in accordance with those of Grassl (2) who reported that no molecular fractionation occurred during the formation of flakes in clinical dextran solutions.

(c) Microscopy Studies.
The insoluble materials which were isolated from a concentrated dextran solution by the methods described in the previous section, were examined in a polarising microscope. Under crossed polarisers with a magnification factor of 200, both samples exhibited birefringence effects. The background was highly granular, but no discrete structures were resolvable. More significantly, on examining the insoluble material which was isolated after the dextran solution had gelled, several very small Maltese cross patterns were observed, estimated as being about 5 μm in diameter. This is indicative of the presence of finely-textured spherulites, the exclusion of light in opposite segments being caused by the orientation of the polymer chains across the radiating fibrils. These findings provide strong evidence in support of the assumption that the insoluble dextran is indeed crystalline in nature. Barham et al. (7) have since reported that dextran spherulites can be grown to even larger dimensions by crystallization at high temperatures from non-aqueous solvents.
Table 23: Identification of Insoluble Material Isolated from Dextran Solutions.

<table>
<thead>
<tr>
<th>Contents of Solution</th>
<th>Specific Rotation $\left[ \alpha \right]_{D}^{20^\circ C}$</th>
<th>Intrinsic Viscosity (dl.g.$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insoluble material isolated before gelation of solution and redissolved</td>
<td>$+196^\circ$</td>
<td>0.158</td>
</tr>
<tr>
<td>Insoluble material isolated from gelled solution, and redissolved</td>
<td>$+197^\circ$</td>
<td>0.160</td>
</tr>
<tr>
<td>Dextran (sample 7), from which insoluble material was produced</td>
<td>$+198^\circ$</td>
<td>0.160</td>
</tr>
</tbody>
</table>

* Estimated as inherent viscosity at 0.005 g.ml$^{-1}$. 
At the point just after gelation had occurred in the dextran solution, examination of the isolated insoluble material indicated that only a very small proportion of the spherulites were resolvable in the optical microscope. No spherulitic structures were detectable either in the particles responsible for inherent turbidity, or in the insoluble material which had been isolated from the solution before gelation. These observations suggest that during the development of turbidity, the diameter of the spherulites is increasing with time. This is what would be expected in a crystallization process. It is only after a certain period of growth that the spherulites develop to a sufficient size (>1 μm) to be resolvable in the optical microscope. In later studies on crystallization kinetics, the actual growth of the spherulite precursors is followed by light scattering measurements, and by filtration methods, using membranes of known pore dimensions.
5. **CONCLUSIONS.**

The turbidity observed in dilute solutions of clinical dextrans is attributable to the presence of insoluble particles; these are spherical in shape, and approximately 100 nm in diameter. The particles can be redissolved either by the action of chemicals, such as urea and sodium hydroxide, which are known to break hydrogen bonds, or by the action of heat. In the latter case, the particles dissolve over a wide range of temperatures; this suggests that a crystalline polymer melting process may be involved.

High turbidities are rapidly developed in concentrated aqueous dextran solutions (60% w/w) at 20 °C. The action of heat and of hydrogen-bond-breaking agents on insoluble material produced in this way, and on insoluble material originally present in the dextrans, is very similar. The particles responsible for both the original and the developed turbidity are therefore assumed to be of a similar nature, and are assumed to have been formed by identical processes. These assumptions can be further justified on the basis that in the drying stage of the preparation process, the dextrans must have been subjected to high concentration conditions which would have been conducive to the development of insoluble material.

The insoluble material which develops in concentrated dextran solutions is in fact an insoluble form of dextran. In view of the observed similarities between the properties of this material and those of the insoluble material originally present, the latter can also be assumed to be insoluble dextran.
Insoluble material formed in concentrated aqueous dextran solutions has a molecular constitution identical to that of the soluble dextran. In other words, no fractionation on a molecular weight basis takes place during the insolubilisation process.

Spherulitic structures are present in the insoluble particles formed in concentrated dextran solutions. Such structures can only be detected after the insolubilisation process has reached the stage of gel formation. In view of the fact that no resolvable structures could be detected in the insoluble material originally present in the dextrans, it is evident that particle growth must have occurred during the process of insolubilisation in concentrated solution.

All of these findings suggest that insolubilisation in dextrans is caused by a crystallization process. The crystalline material can be dispersed either by the reverse process, i.e. by melting, or by the action of chemicals which disrupt the hydrogen-bonded crystal structure.

Conditions have been uncovered under which the crystallization process in dextrans can be followed at measurable rates, and under which the melting process can be studied. The relevant factors affecting these processes can now be investigated, and the results interpreted in terms of the established facts relating to crystallization and melting in polymer systems.
1. INTRODUCTION.

In this section, the overall objectives were as follows:

(i) to identify procedures for measuring rates of development of crystalline structure in dextrans.

(ii) to investigate factors affecting the crystallization process.

(iii) to compare the crystallization behaviour of dextran with that of polymer systems generally.

An investigation of structure in the crystalline particles was not attempted in this work, except in so far as it was possible to deduce information on particle size and shape from light scattering studies.

Although crystallization processes involve distinguishable stages of nucleation and growth, most methods available for measuring crystallization rates enable only the overall kinetics to be determined. An assessment of the methods available for rate determination was therefore carried out. The aim was to determine those procedures which would yield most information relating to nucleation and growth processes taking place during dextran crystallization from solution.

As indicated in the introduction, the nucleation process normally has a strong influence on the overall crystallization kinetics. An investigation into methods of altering nucleation density in dextrans was therefore undertaken. Molecular parameters, such as molecular weight and molecular weight distribution, and external
factors, such as solvent concentration and temperature, also affect polymer crystallization rates. This arises due to their influence both on the degree of supercooling to which the amorphous polymer is subjected, and on the rate of mass transport by diffusion. Specific objectives of the work described in this section were therefore to investigate the effects of the following parameters on rate of dextran crystallization:

(i) nucleation density
(ii) molecular weight
(iii) molecular weight distribution
(iv) solvent concentration
(v) temperature

2. MEASUREMENT OF CRYSTALLIZATION RATES.

(a) Assessment of Available Methods.

The technique of light microscopy has been frequently employed to study growth rates in crystallizing polymer systems. Developing spherulites are highly birefringent; growth may therefore be observed in the polarising microscope, provided the dimensions of the spherulites exceed the limit of resolution, i.e. 1 μm. It has been shown above (section I.B.4) that crystalline dextran particles do not grow to dimensions of this order, at least during the period over which crystallization rates can be determined, i.e. up to the point at which the concentrated dextran solutions revert to gels. The method is not therefore applicable to the study of dextran crystallization under the conditions employed in this work.
Electron microscopy and X-ray diffraction methods have been employed to study crystallization in polymers, but are more frequently used to characterise crystal morphology rather than to follow crystallisation rates. Kinetic studies would involve quenching the crystallisation process at various stages of growth, followed by an examination of the products. Because of the difficulties of preparing samples in suitable form, such techniques were not pursued. In any event, X-ray diffraction studies on crystalline dextran have already been reported (4,7), and the existence of highly ordered regions in the polymer conclusively identified. It was not considered that further studies in this direction would contribute further to an understanding of the factors responsible for crystallization in dextrans. It is this, rather than a detailed study of the morphology of the crystalline species, which is the primary objective of this work.

Dilatometry is perhaps the most common technique applied to study crystallization kinetics; in this method, the overall crystallization kinetics can be investigated by following changes in volume of the crystallizing mass. The fact that the density of the crystalline regions can be up to 10% greater than that of the amorphous regions means that as the crystallization process advances, a progressive reduction in volume will be observed. However, in studying crystallization of dextran from concentrated aqueous solutions, where only small changes (<4%) towards complete crystallization take place before the onset of gelation, the volume changes which require to be detected will be very small. Additionally, the practical problems associated with adequately out-gassing concentrated dextran solutions mitigate against this approach.
The application of differential thermal analysis to the problem of studying crystallization in concentrated dextran solutions was briefly evaluated. When a sample of concentrated solution (60% w/w) was held at constant temperature in the cell of a du Pont Differential Thermal Analyzer, no exothermic peaks were detected. It is assumed that because of the low extent of crystallization, coupled with the presence of solvent, the amount of heat evolved per unit mass of solution is too low to be detectable.

Light scattering methods are particularly applicable to the study of crystallization kinetics in solution, and are suitable for investigating the behaviour of rapidly changing systems. Turbidity, dissymmetry and depolarisation ratio can be measured continuously as a function of time, thereby providing information as to the number, size and shape of the developing crystalline species. However, interpretation of the data in an unambiguous manner is not always a simple exercise. Nevertheless, light scattering methods are especially suitable for the measurement of relative rates. They were therefore employed extensively in this work to determine the effects of various parameters on the kinetics of dextran crystallization.

The kinetic data obtained from light scattering measurements can also be evaluated in the light of various possible crystallization mechanisms. In this way, those mechanisms predominating under specific conditions can be identified. In addition, once a particular crystallization mechanism has been proposed, the observed kinetic data can be compared with that which would be predicted by the Avrami analysis relating to that particular mechanism. This
serves as a method of checking that the proposed pathway is correct.

Values for particle size determined by light-scattering methods can be checked by measuring particle retentions when suspensions are filtered through a series of membranes of graded pore dimensions. This method can also be used to provide information on particle size in suspensions which are too turbid for quantitative light-scattering measurements to be carried out.

(b) Detailed Light-Scattering Studies.
Two dextrans were selected for study, one (sample 17) containing a small number of insoluble particles, the other (sample 7) containing a much larger number. Initially, concentrated dextran solutions were investigated, in which the development of turbidity, dissymmetry and depolarisation ratio was observed directly and continuously in a light scattering cell. The dextran concentrations were 60% w/w and 40% w/w for samples 17 and 7 respectively. Because of the large difference in the crystallization rates of the two dextrans, it proved difficult to study them both at the same concentration.

In order to determine the rate of crystallization under identical concentration conditions, a second procedure was followed; the crystallization process was frozen at various points in time by diluting the concentrated solutions with water. Light-scattering measurements were then performed on the diluted solutions, providing additional information as to changes in the number, dimensions and nature of the growing crystalline entities. There is a possibility that in the latter procedure, the crystalline aggregates
may undergo changes on dilution e.g. dissociation. However, no evidence to support this was found in comparative studies on dilute and concentrated solutions.

Difficulties have already been experienced in using light-scattering methods on concentrated solutions to give quantitative dimensions from dissymmetry measurements. (Section I.B.3) There were also practical problems in rapidly preparing highly viscous solutions for light-scattering work. The dilution approach eliminates these problems, and can also be used when turbidity is measured both in the SOFICA light-scattering photometer, and in the simple nephelometer.

(i) Dissymmetry Studies.
To follow changes in the dimensions of insoluble particles present in the dextran solutions, the increase in dissymmetry was determined as a function of time. The values of $Z$ observed in concentrated solutions, and also in solutions diluted with water, are shown in fig.17 and fig.18 for dextran samples 17 and 7 respectively.

The increase in dissymmetry can be best interpreted in terms of the growth of spherical particles. The assumption that spherical particles are present is based on the fact that the measured values of dissymmetry are higher than those which would arise due to the presence of rod or disc-shaped particles. This conclusion is supported by the low depolarisation ratios observed in the early part of growth (see below), which indicates the presence of optically isotropic (symmetrical) particles.
FIG. 17: DISSYMMETRY INCREASE ASSOCIATED WITH PARTICLE GROWTH DURING DEXTRAN CRYSTALLIZATION FROM SOLUTION
FIG. 18: DISSYMMETRY INCREASE ASSOCIATED WITH PARTICLE GROWTH DURING DEXTRAN CRYSTALLIZATION FROM SOLUTION

Dextran sample 7

Crystallization - 40% w/w
Measurement of Z - 40% w/w

Crystallization - 60% w/w
Measurement of Z - 10% w/v

Onset of multiple scatter effects at 30 mins.

Dissymmetry, Z

Crystralization Time

mins. 60% w/w
hours 40% w/w
The results obtained with the concentrated dextran solutions directly demonstrate the occurrence of particle growth. However, for quantitative determination of particle dimensions from dissymmetry measurements, it is necessary, for the reasons given in section I.B.3, to use dissymmetry values obtained with more dilute solutions. Accordingly, the dissymmetry values measured using the dilute dextran solutions (10% w/v) were related to spherical particle diameter, \( D \), using fig.15. Plots of \( D \) vs. \( t \) and of \( D \) vs. \( t^2 \) are given in fig.19 and fig.20 respectively, for both dextrans.

Considering sample 17, over an initial crystallization period of approximately 16 hours, the dissymmetry which is associated with insoluble particles originally present in the dextran, complicates the interpretation of the data. While some growth is apparent during this period, presumably associated with those particles present at zero time, the growth of particles with dissymmetries less than those originally present cannot be followed. However, turbidity measurements (see below) provide some information relating to this period of crystallization. After 16 hours, a rapid increase in dissymmetry, associated with particles attaining dimensions of the order of 200 nm, is observed. It is not possible to study the crystallization process beyond the 26 hour period due to the onset of multiple scatter effects. Thus, because of the relatively small dimension range over which growth can be quantitatively observed, the time dependence of growth is difficult to determine accurately. The relationship between particle diameter, \( D \), and time, \( t \), would not appear to be linear (fig.19). However, over at least part of the growth period (16 - 20 hours), the \( D \) vs.
FIG. 19: RATE OF SPHERICAL PARTICLE GROWTH DURING DEXTRAN CRYSTALLIZATION FROM SOLUTION
FIG. 20: RATE OF SPHERICAL PARTICLE GROWTH DURING DEXTRAN CRYSTALLIZATION FROM SOLUTION
The deviation from linearity observed in the $D$ vs. $t^{1/2}$ plot for sample 17 over the 20 - 26 hour period, may simply be a feature of the process of polymer crystallization from solution. During crystallization, there will be a progressive reduction in the concentration of polymer in the liquid phase in which the crystals are growing. This will result in a lowering of the melting point, causing a reduction in the degree of supercooling, and hence a decrease in crystallization rate. In the case of the dextran solutions (60% w/w), a 4% change towards complete crystallization will decrease the concentration of liquid-phase polymer to 57.6% w/w. Since results quoted below (Section I.C.4) show that crystallization rate is highly concentration dependent within this region, a curved growth plot is therefore to be expected.

In principle, it should be possible to derive information relating to the original size of the growth particles by extrapolating the
D vs. \( t^{\frac{1}{2}} \) plot to zero time or to zero dimensions. However, unambiguous conclusions cannot be reached if, as in this case, the nature of the equation on which the extrapolation is based, has not been defined. Nevertheless, in this case, the conclusions based upon extrapolation appear to be meaningful, and are supported by later findings relating to turbidity measurements.

Extrapolation of the D vs. \( t^{\frac{1}{2}} \) plot for dextran sample 17 (fig.20), to zero D, indicates that there is an apparent induction period of some four hours before spherulitic growth commences. It is therefore probable that homogeneous nucleation is a necessary prerequisite for growth in this dextran. Pre-spherulitic growth in the form of rods or discs can also be observed in crystallizing polymer systems (51). However, the absence of strongly depolarizing species suggests that only optically isotropic entities are present in this instance.

The dissymmetry-time plot observed for sample 7 can be interpreted in a manner similar to that for sample 17, viz. in terms of spherical particle growth. However, the results obtained with both concentrated and dilute solutions indicate that up to the point where multiple scatter effects make quantitative deductions impossible, the particle dimensions are not as great as with sample 17. A dependence of particle diameter, D, on \( t^{\frac{1}{2}} \) is again observed over a particular crystallization period, viz. 16 - 27 minutes (fig.20). After this time, the D vs. \( t^{\frac{1}{2}} \) plot becomes curved, presumably due to the effect of changing solution composition on the growth kinetics. The data suggests that the growth rate is 2.6 times faster than for sample 17, it being assumed that the starting time for growth is the end of the
induction period. This finding is in accordance with the lower molecular weight of sample 7 ($M_w = 28,000$) compared with that of sample 17 ($M_w = 42,000$); this matter is further discussed below (Section I.C.4).

In the case of sample 7, extrapolation from the region in which growth rate can be measured leads to the conclusion that growth may initiate from particles of the order of 25 nm in diameter. This is not unreasonable, since earlier filtration studies on the particles initially present in dextrans revealed the existence of species of this magnitude (Section I.B.3). Such particles appear to act as nuclei for growth, and the nucleation process is indicated to be heterogeneous.

(ii) Turbidity Studies.
In order to monitor changes in the number of insoluble particles present in crystallizing dextran solutions and in the mass of these particles, the turbidity, $i_{90}^0$ was measured as a function of time. The values of $i_{90}^0$ observed in the concentrated dextran solutions, and also in the solutions diluted with water, are shown in fig.21 and in fig.22, respectively, for both dextrans. Due to the onset of multiple scatter effects, the turbidity increases could only be observed quantitatively over those time periods indicated in the plots. Turbidity was measured as a galvanometer reading, $i_{90}^0$, when the corresponding glass standard reading was 0.02. While conversion of the readings to absolute turbidity units would thus be possible (Section I.A.1), it is not required, since it is not possible to determine particle molecular weight from the data.
FIG. 21: TURBIDITY INCREASE ASSOCIATED WITH PARTICLE GROWTH DURING DEXTRAN CRYSTALLIZATION FROM SOLUTION
Crystallization conc.: 60% w/w

TURBIDITY OF 10% w/v SOLUTION

CRYSTALLIZATION TIME [sample 17-hours]

sample 7

sample 17

FIG. 22: TURBIDITY INCREASE ASSOCIATED WITH PARTICLE GROWTH DURING DEXTRAN CRYSTALLIZATION FROM SOLUTION
The turbidity of solutions of dextran sample 17 does not increase appreciably over the first 15 hours of crystallization, but after this point, it undergoes a very rapid increase. This behaviour suggests that an induction period exists, and that this is followed by particle growth stage, in keeping with the findings from dissymmetry studies. By way of contrast, turbidity associated with solutions of dextran sample 7 increases continuously from zero time, without an induction period. This observation is also in keeping with conclusions derived from dissymmetry measurements, in which it was considered that continuous growth from small particles took place instantaneously.

In order to examine the quantitative aspects of turbidity development, the observed turbidities, $i_{90^\circ}$, must be corrected for sphere dissymmetry effects. This was accomplished using particle scattering correction factors ($1/p(90^\circ)$, or $i_{10}/i_{90^\circ}$) obtained from tables (91). The corrected turbidities, $i_0$, were plotted as a function of time on a log-log basis, and the results are shown in fig.2.

Considering sample 7, $i_0$, as determined from $i_{90^\circ}$ values measured in the dilute solutions (10% w/v), increases with time according to $t (2.9 \pm 0.2)$. Because of inter-particle interaction effects, it is not considered that quantitative deductions can be derived from the time dependence of turbidity measured in the concentrated (40% w/w) solution. With sample 17, for an initial period of some 17 hours, the increase in $i_0$ with time is practically linear, i.e. dependent on $t^1$. After this point, $i_0$, as determined for the
FIG. 23: INCREASE IN TURBIDITY ASSOCIATED WITH PARTICLE GROWTH DURING DEXTRAN CRYSTALLIZATION FROM SOLUTION
concentrated solution increases in proportion to $t^{(4.1 \pm 0.5)}$, and for the dilute solutions, increases in proportion to $t^{(5.7 \pm 0.3)}$. The latter dependence of turbidity on time is again considered to form the better basis for making further deductions.

Turbidity depends on the number of particles, $n$, present in a solution, and the particle mass, $m$, according to,

$$
\mathcal{Y} \propto nm^2
$$

During dextran crystallization, in time intervals where $m$ is not significantly increasing, but where $n$ is increasing due to development of nuclei or small crystallites, $\mathcal{Y}$ will therefore be proportional to $n$. On the other hand, in time intervals where the predominant process is growth, and the number of growing particles is approximately constant, then $\mathcal{Y}$ will become proportional to $m^2$.

The Avrami equation predicts that the kinetics of diffusion-controlled, spherulitic growth, originating from heterogeneous nuclei, should obey an equation of the form,

$$
m \propto t^{3/2}
$$

where $m$ is the mass of crystalline material formed at time, $t$. (table 1). In a time interval where growth is the predominant process (i.e. $n$ is approximately constant), then,

$$
\mathcal{Y} \propto m^2 \propto t^3
$$

These conditions are believed to apply during crystallization of
dextran sample 7. The observed dependence of turbidity on $t (2.9 \pm 0.2)$ lends support to this proposed kinetic scheme.

Considering sample 17, it has been suggested above that diffusion-controlled, spherulitic growth from homogeneous nuclei could be the crystallization mechanism. For such a mechanism, (table 1) the Avrami equation predicts that,

$$m \propto t^{5/2}, \quad (45)$$

and again, in a region where $n$ can be considered constant,

$$T \propto m^2 \propto t^5 \quad (46)$$

In the case of dextran sample 17, the observed dependence of $T$ on $t (5.7 \pm 0.3)$ suggests that this mechanism might predominate during the latter stages of the crystallization. $n$ may not in fact be constant during this growth phase, since fresh nuclei may appear continuously throughout the course of the crystallization. However, it is known from dissymmetry measurements that $m$ is increasing during this time, and because of the dependence of $T$ on $m^2$, turbidity measurements are more likely to reflect growth rather than changes in the number of growth centres.

Simply because of its form, a growth plot of the form, $T \propto t^5$ will, if plotted in the form $T \propto t$, contain a pseudo-induction period in which at low times, $T$ will not appear to change. Thus, if the $T \propto t^{5.7}$ plot, observed for dextran sample 17 over the 17 - 30 hour period, is extrapolated to lower times, no measurable increase in $T$ should be observed over the early stage of growth.
FIG. 24: DEPOLARISATION FACTOR INCREASE ASSOCIATED WITH PARTICLE GROWTH DURING DEXTRAN CRYSTALLIZATION FROM SOLUTION
However, when the extrapolated $T$ vs. $t^{5.7}$ curve is compared with the experimental data shown in fig. 23, it is evident that there is a significant degree of turbidity development over and above that which would be expected on the basis of the $T$ vs. $t^{5.7}$ relationship. This may be associated with the presence of particles which are responsible for initial turbidity, but if growth of such particles were involved, a dependence of $T$ on a much greater power of $t$ would be expected. It is more likely that the formation of particles of small dimensions takes place at this time, such that the relationship $T \propto n \propto t$ is approximately obeyed.

(iii) Depolarisation Ratio Studies.

The increase in the depolarisation ratio, $\rho_{90}$, observed with the dilute dextran solutions (samples 7 and 17, 10% w/v) is shown in fig. 24 as a function of time of crystallization. In both cases, $\rho_{90}$ is initially low ($\sim 0.02$), pointing to the absence of optically anisotropic species. With sample 7, $\rho_{90}$ increases continuously with time, whereas with sample 17, there is no significant increase for a period of about 16 hours. This increase in depolarisation ratio is associated with an increase in the size of the scattering particles rather than the development of anisotropic species. An induction period before the onset of spherulitic growth to large dimensions is therefore indicated.

It is probable that a relatively narrow particle size distribution exists in these crystallising systems, in which case $\rho_{90}$ will be a periodic function of sphericle particle diameter, $D$ (117); this means that sharp peaks in $\rho_{90}$ will occur at various values of $D$. 

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FIG. 25: DETERMINATION OF DEXTRAN CRYSTALLIZATION RATES BY SOLUTION TURBIDITY MEASUREMENTS
Calculations from theory (115,117) have shown that for $\lambda = 436$ nm, and with refractive index ratio, $m = 1.10$, where $m = n_{\text{particle}}/n_{\text{dispersing medium}}$, such peaks occur at approximate values of $D$ (nm) equal to 0.3, 0.5, 0.7 etc. In the case of the dextran particles, $m$ is also approximately equal to 1.10, and with blue incident light of wavelength 436 nm, a strong increase in $\rho_{90}$ is observed during the latter part of the crystallization. Dissymmetry measurements indicated in figs. 17 and 18 for dextran samples 7 and 17 have shown that particle dimensions at this stage of the crystallization are of the order of 0.2 nm. It is therefore probable that the rapid increase in depolarisation ratio is associated with the fact that particles are approaching the diameter corresponding to that which gives the first peak in the value of $\rho_{90}$, viz. 0.3 nm.

(c) Nephelometer Studies.

In order to investigate the various factors controlling dextran crystallization kinetics, it was necessary to perform a large number of rate determinations under different conditions. It was therefore desirable to adopt a simple and rapid light scattering method for measuring crystallization rate. The nephelometric procedure described in section I.B.2 was investigated as a possibility.

The rate of dextran crystallization from concentrated aqueous solution (samples 7 and 17, 60% w/w) was determined by measuring the increase in turbidity, $(\Delta N_{100})$. The turbidity was measured in dilute solutions prepared from the crystallizing solution at
various intervals of time. The results are shown in fig. 25, in which log (ΔN_{100}) is plotted against log (time). It is evident that for both samples, ΔN_{100} is apparently dependent on a lower power of time than was the turbidity, i_{90°}, as measured in the light scattering photometer. This is due to the fact that when turbidities in the higher range (ΔN_{100} > 500) are measured, the value of ΔN_{100} will be low due to multiple scatter effects. In addition, corrections to account for increasing dissymmetry of scatter with increasing particle size have not been applied, which means that the observed ΔN_{100} will again be lower than the true turbidity. However, accepting that absolute turbidities are not measured in the nephelometric method, on an empirical basis, log (ΔN_{100}) - log (time) plots were observed to be linear over a wide crystallization period. In addition, the relative slope of the plots for dextran samples 7 and 17 (table 24) was similar to that observed when absolute turbidity values were employed (fig. 23). Rather than attempt to reduce the N_{100} values to absolute terms, it was therefore considered adequate to use the observed log (ΔN_{100}) - log (time) plots for the measurement of relative crystallization rates in dextrans.

As a quantitative measure of crystallization rate, it was assumed that over the stage of the crystallization during which ΔN_{100} is increasing rapidly with time, then the following eqn. was obeyed,

$$\Delta N_{100} = kt^m$$

or

$$\log (\Delta N_{100}) = \log k + m \log t$$

The value of k can be taken as a measure of crystallization rate. k, in fact, depends on both nucleation and growth rates, and is thus
a measure of the overall rate of crystallization. For dextran samples 7 and 17, the values of k and m derived from fig. 25 are shown in table 24.

TABLE 24: CRYSTALLIZATION RATE CONSTANTS AND AVRAMI EXPONENTS DERIVED FROM LOGARITHMIC TURBIDITY - TIME PLOTS.

<table>
<thead>
<tr>
<th>Dextran Sample No.</th>
<th>Crystallization Rate Constants, k, (sec.(^{-m}))</th>
<th>Avrami Exponent m</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>((4 \pm 3) \times 10^{-1})</td>
<td>(1.0 \pm 0.1)</td>
</tr>
<tr>
<td>17</td>
<td>((6 \pm 7) \times 10^{-9})</td>
<td>(2.1 \pm 0.1)</td>
</tr>
</tbody>
</table>

(d) Filtration Studies.

As a result of light scattering investigations of dextran crystallization (section I.C.2 (b)), it has been possible to determine how the size of the crystalline particles changes with time (fig. 19). In order to provide more direct evidence in support of these findings, studies relating to the retention of insoluble particles by membrane filters of known pore dimensions, were conducted at various stages throughout the course of the crystallization process.

Dextran was crystallized from concentrated aqueous solution, and the process was halted after various periods of time by dilution of the solution with water. Turbidity \(N_{100}\) measurements carried out on the diluted solutions before and after filtration were used to measure the degree of retention of the crystalline particles by the membrane. In this way, it could be ascertained whether particles present at a particular stage of the crystallization were larger or smaller than the membrane mean pore size. Both dextran
samples 7 and 17 were investigated, the results of the turbidity measurements being given in table 25.

**TABLE 25: TURBIDITIES OF DEXTRAN SOLUTIONS DILUTED AND FILTERED AT VARIOUS STAGES OF CRYSTALLIZATION.**

<table>
<thead>
<tr>
<th>Dextran Sample No.</th>
<th>Crystallization Time, (hrs.)</th>
<th>Membrane Mean Pore Diameter (nm)</th>
<th>Turbidities (N&lt;sub&gt;100&lt;/sub&gt;)</th>
<th>Limits Suggested for Particle Diameter, D (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0.17</td>
<td>50</td>
<td>200</td>
<td>23 D &gt;&gt; 50</td>
</tr>
<tr>
<td>7</td>
<td>0.17</td>
<td>100</td>
<td>200</td>
<td>56 D &gt; 100</td>
</tr>
<tr>
<td>7</td>
<td>0.25</td>
<td>100</td>
<td>200</td>
<td>25 D &gt;&gt; 100</td>
</tr>
<tr>
<td>7</td>
<td>0.50</td>
<td>100</td>
<td>830</td>
<td>20 D &gt;&gt; &gt;100</td>
</tr>
<tr>
<td>7</td>
<td>0.50</td>
<td>200</td>
<td>830</td>
<td>180 100 &lt; D &lt; 200</td>
</tr>
<tr>
<td>7</td>
<td>0.75</td>
<td>200</td>
<td>1090</td>
<td>35 D &gt;&gt; &gt;200</td>
</tr>
<tr>
<td>7</td>
<td>0.75</td>
<td>450</td>
<td>1090</td>
<td>65 200 &lt; D &lt; 450</td>
</tr>
<tr>
<td>7</td>
<td>0.75</td>
<td>800</td>
<td>1090</td>
<td>460 D &lt;&lt; &lt; 800</td>
</tr>
<tr>
<td>17</td>
<td>22</td>
<td>100</td>
<td>190</td>
<td>20 D &gt;&gt; &gt;100</td>
</tr>
<tr>
<td>17</td>
<td>22</td>
<td>200</td>
<td>190</td>
<td>35 D &gt; 200</td>
</tr>
<tr>
<td>17</td>
<td>22</td>
<td>450</td>
<td>190</td>
<td>70 300 &lt; D &lt; 450</td>
</tr>
<tr>
<td>17</td>
<td>49</td>
<td>450</td>
<td>1100</td>
<td>30 D &gt; 450</td>
</tr>
<tr>
<td>17</td>
<td>49</td>
<td>800</td>
<td>1100</td>
<td>40 D &gt; 800</td>
</tr>
</tbody>
</table>

In the case of sample 7, the information derived from the filtration studies supports the growth-time curve shown in fig.19. When sample 17 has been crystallized for 22 hours, the range of values suggested for the particle diameter, D, is also in agreement with that deduced from light scattering data. However, after a 49 hour crystallization period, the filtration studies suggest a larger particle size than would be expected from fig.19 on extrapolating D to higher crystallization times. However, at this point, small amounts of insoluble
gel were present when the concentrated solution was diluted. It is possible that at this stage of the crystallization, dilution gives rise to aggregates rather than to discrete suspended particles, and that these are being retained by the membrane filter. It is also likely that the true retention characteristics of the membrane will be masked under these conditions.

3. NUCLEATION STUDIES.

It has been suggested that crystallization of a dextran which contains a high level of inherent turbidity, proceeds by a heterogeneous nucleation process. In contrast, during crystallization of a dextran with a low level of inherent turbidity, an induction period is necessary before growth can commence, and the nucleation process is considered to be homogeneous. It is therefore reasonable to assume that in the former case, the insoluble particles act as nuclei from which growth can take place instantaneously. In the latter case, the nucleation density is too low for significant spontaneous growth; an induction period is required in which the number of nuclei increases until the dextran can crystallize in a manner similar to that in the former instance. With heterogeneous nucleation, simultaneous growth is possible from a large number of centres; for any given extent of crystallization, the total amount of amorphous polymer transformed will be spread over all these centres. In the case of homogeneous nucleation, in which fewer centres are involved for the same extent of crystallization, the amount of crystalline polymer associated with each centre will be larger.

For dextrans, the extent of crystallization in different samples can be considered similar at the point where solutions of fixed concentration begin to gel. At this point, the dextran sample crystallizing by the homogeneous nucleation mechanism indeed contained larger growth particles than the sample which crystallized by the heterogeneous
nucleation process. In the two dextrans studied, the assumption that the particles responsible for inherent turbidity act as nuclei, is therefore supported by the observed differences in the final size of the growth units.

If the above assumptions relating to heterogeneous nucleation in dextrans are correct, it should be possible either to increase or to decrease the overall rate of crystallization by respectively increasing or decreasing the nucleation density. The objective of the work described in this section was to investigate this possibility by varying the nucleation density in dextrans in four ways, (a) by selecting dextrans of identical molecular constitution, but which, in dilute solution, give rise to different levels of turbidity (b) by deliberately adding nuclei or potential nuclei to solutions, in order to increase nucleation density (c) by storage of dextrans at high relative humidity to promote formation of nuclei, and (d) by removing nuclei through established melting processes, thereby reducing nucleation density.

(a) **Inherent Nucleation Density.**

Since crystallization in polymers generally increases with decreasing molecular weight (37,38,39), the effect of nucleation density on crystallization rate should be exaggerated in the lower molecular weight members of the available dextran range. Two dextrans (samples 2 and 3) both with average molecular weights, $M_w$, of 10,000, were therefore selected for study; nucleation densities were characterised by turbidities ($N_{100}$, 10% w/v solution) of 20 and 200 respectively. With sample 3 of high turbidity, crystallization from 60% w/w solution (22 °C) was too rapid for rate determination to be possible.
Crystallization studies were therefore conducted at a lower dextran concentration (50% w/w) than had been previously used. In each case, rates of crystallization were determined by the nephelometric procedure described in section I.B.2., and the log \( \Delta N_{100} \) - log (time) curves obtained are shown in fig. 25. The data deduced from the plots are given in table 26.

**TABLE 26: RATES OF CRYSTALLIZATION FOR DEXTRANS OF DIFFERENT NUCLEATION DENSITY.**

<table>
<thead>
<tr>
<th>Dextran Sample No.</th>
<th>Nucleation Density (Turbidity, 10% w/v Solution - ( N_{100} ))</th>
<th>Crystallization Rate Parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>20</td>
<td>Rate Constant, ( k ) (sec.(^{-1}))</td>
<td>1.9</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>6 \times 10^{-1}</td>
<td>1.0</td>
</tr>
</tbody>
</table>

It has been observed above with dextran samples 7 and 17, which are similar in molecular constitution, that increasing the nucleation density changes the mechanism of the crystallization process from one which, for the most part, is homogeneously nucleated to one which is heterogeneously nucleated. As a result, the value of the exponent, \( m \), decreased from 2.1 to 1.0 when the nucleation mode changed from homogeneous to heterogeneous. It is probable that with samples 2 and 3, \( m \) decreases from 1.9 to 1.0 for the same reason. Since the exponent, \( m \), changes from one sample to another, it is better to use the rate constant, \( k \), as a measure of relative crystallization rates. It is evident that a 10-fold increase in nucleation density gives rise to a corresponding increase of \( 3 \times 10^4 \) in the value of \( k \). The marked dependence of dextran crystallization rate on the level of inherent
turbidity, i.e. nucleation density, over a wide molecular weight range, is demonstrated below.

(b) Addition of Nuclei.

(i) Foreign Material.

It is possible that nucleation in dextrans could be promoted by traces of fibres, glass, etc., which might contaminate the samples during preparation. If this were so, the addition of foreign material of this kind would enhance crystallization rate. Small amounts of dust, asbestos fibres, and powdered glass were added to a concentrated solution of dextran (sample 17, 60% w/w), which was allowed to crystallize for one hour before dilution and turbidity measurement. No increase in crystallization rate, pointing to increased nucleation density, was observed. Specific nuclei are evidently required to promote the nucleation process. These results are in agreement with those of Barham (7), who found that materials such as powdered chalk, graphite, etc., would not seed the crystallization process. Smoke particles were however reported to be effective in increasing the size of spherulitic growth particles.

(ii) Insoluble Dextran.

In this investigation, nuclei or potential nuclei, in the form of insoluble particles isolated from a rapidly crystallizing dextran, were added to a dextran with a relatively low crystallization rate. Dextran (sample 7) was allowed to crystallize from solution (60% w/w) for one hour, and after diluting this solution to a lower concentration (10% w/v), the insoluble particles were isolated by centrifugation, and washed with water. Concentrated solutions of dextran (sample 17, 60% w/w) containing the insoluble dextran in
various forms, were then prepared, and the rates of crystallization were determined in the usual manner. The results obtained from the log (ΔN_{100}) - log (time) plots, which are shown in fig.25, are given in table 27. It is evident from the lower values of m observed, that in each case, the addition of insoluble dextran particles causes a change in the crystallization mechanism. This change is consistent with a change in the nucleation process from one which is predominantly homogeneous, to one which becomes progressively more heterogeneous with increasing nucleation density. Nucleation also increases overall rate significantly; comparing samples 17 and 17B shows that the magnitude of this increase depends on the number of added particles. Comparison of samples 17B and 7, however, suggests that it is not possible to cause a dextran to crystallize rapidly simply by addition of insoluble dextran nuclei without regard to their nature. It is probable that addition of nuclei of smaller dimensions than were added in sample 17B would be necessary to increase the rate of crystallization of sample 17 to the same order of magnitude as that of sample 7.

(iii) Inherent Nuclei.
One method of introducing effective nucleating species into a dextran which crystallizes at a relatively low rate, is to mix in small quantities of another dextran with a high nucleation density. A dextran which exhibits high inherent turbidity (sample 5, \( \bar{M}_w = 19,300 \), turbidity of 10% w/v solution = 150) was mixed with a dextran (sample 17, \( \bar{M}_w = 41,600 \), turbidity of 10% w/v solution = 30) in the proportions 5%: 95% respectively. The rate of crystallization of the dextran mixture (sample 17D) was examined by the nephelometric procedure, and the results are shown in fig.25. The crystallization rate parameters derived from this plot are shown in table 27.
TABLE 27: Effect of Nucleation with Insoluble Dextran on Rates of Dextran Crystallization from Aqueous Solution (60% w/w).

<table>
<thead>
<tr>
<th>Method of Adding Insoluble Dextran Particles to Nucleate Crystallization of Dextran</th>
<th>Dextran Sample No.</th>
<th>Crystallization Rate Parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rate Constant, $k$ (sec.$^{-1}$)</td>
<td>Avrami Exponent, $m$</td>
</tr>
<tr>
<td>Aqueous particle suspension diluted to give low turbidity ($N_{100} = 40$), and used to prepare dextran solution (sample 17, 60% w/w)</td>
<td>17A</td>
<td>$3 \times 10^{-8}$</td>
<td>2.2</td>
</tr>
<tr>
<td>Aqueous particle suspension diluted to give high turbidity ($N_{100} = 600$), and used to prepare dextran solution (sample 17, 60% w/w)</td>
<td>17B</td>
<td>$9 \times 10^{-6}$</td>
<td>1.8</td>
</tr>
<tr>
<td>Aqueous particle suspension diluted to give high turbidity ($N_{100} = 600$), then boiled, and used to prepare dextran solution (sample 17, 60% w/w)</td>
<td>17C</td>
<td>$2 \times 10^{-6}$</td>
<td>1.9</td>
</tr>
<tr>
<td>Control - no addition of nuclei</td>
<td>17</td>
<td>$6 \times 10^{-9}$</td>
<td>2.1</td>
</tr>
<tr>
<td>Comparison with sample 17B, but no addition of nuclei, i.e. a dextran with high inherent turbidity ($N_{100} = 100$, for 10% w/v solution)</td>
<td>7</td>
<td>$4 \times 10^{-1}$</td>
<td>1.0</td>
</tr>
<tr>
<td>5% of dextran (sample 5) with high inherent turbidity ($N_{100} = 150$, 10% w/v solution) incorporated in dextran sample 17</td>
<td>17D</td>
<td>$2 \times 10^{-4}$</td>
<td>1.6</td>
</tr>
</tbody>
</table>
The increase in rate of crystallization of sample 17 which is associated with the inclusion of 5% of a dextran of high nucleation density, is quite considerable. The increase is greater than that observed when insoluble dextran particles, isolated after a period of growth, were introduced into the same dextran. This confirms that the small insoluble particles which are present in dextrans of high inherent turbidity, are highly effective nucleating agents. A transition from a homogeneous nucleation mechanism in sample 17 to a heterogeneous process in sample 17D is again suggested by the lower value of $m$ observed in the latter case.

The molecular constitution of sample 17 will be altered by addition of the lower molecular weight dextran. However, the effect on $M_w$ is not significant, the calculated value for the mixture being 40,500. The molecular weight distribution is also broadened, but since this normally has the effect of decreasing crystallization rate (44,45), it can be assumed that the increase in rate observed with sample 17D is due to the nucleation effect of the particles introduced from sample 5.

(o) Development of Nuclei.
It is a general observation (4,5,78,81) that exposure of certain amorphous polysaccharides to high relative humidity results in the development of crystallinity. Such polysaccharides frequently contain a relatively large amount of available moisture, which is emitted only under extreme conditions. On humidification, it is assumed that the presence of additional water facilitates the alignment of the chain segments in the solid state. Crystallization
at high humidity has been reported in cellulose esters (124), native dextrans (4,5,36) amylaceous polysaccharides, (4,36,81) and in a number of polysaccharides containing alginate and mucopolysaccharide groups (78). Crystallinity is generally detected by X-ray diffraction methods, and branching of the polymer chains has been shown to retard the crystallization process (5,36). The phenomenon of starch retrogradation is perhaps the best known example; crystallization in the linear amylose fraction takes place in the solid state, and is believed to be the process responsible for the staling of bread (81,82). Humidification has also been employed to convert amorphous acid hydrolysed dextran fractions, similar to those employed in this work, into a partially crystalline state (47). This procedure was therefore examined with a view to increasing the crystallinity of one of the dextrans employed in this work.

Dextran (sample 18) was stored at over 90% relative humidity for two weeks, during which time the moisture content increased from 3.6% to 9.7%. At the same time, the turbidity \( (N_{100}, 10\% \text{ w/v solution}) \) increased from 20 to 38. The rate of crystallization from concentrated aqueous solution (60% w/w) was determined before the initial turbidity had increased, and the \( \log (\Delta N_{100}) - \log \) (time) plot is shown in fig.25. After storage of the dextran at high humidity, crystallization rate was again determined in a similar way, but from a single turbidity measurement. Results of the rate determinations are given in table 28.
TABLE 28: Effects of Humidification on the Inherent Turbidity Rate of Dextran Crystallization.

<table>
<thead>
<tr>
<th>Dextran Sample 18</th>
<th>Inherent Turbidity (N_{100}, 10% \text{ w/v Solution})</th>
<th>Crystallization Rate Constant (\text{sec}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Humidification</td>
<td>20</td>
<td>(6 \times 10^{-9})</td>
</tr>
<tr>
<td>After Humidification</td>
<td>38</td>
<td>(2 \times 10^{-8}) *</td>
</tr>
</tbody>
</table>

* \(m = 2.2\) assumed.

The increase in crystallization rate associated with the increase in inherent turbidity indicates an increase in the nucleation density of the dextran. The magnitude of the increase is similar to that observed when low levels of insoluble dextran were incorporated in sample 17 (sample 17B, table 27).

(d) Removal of Nuclei.

It has been shown that crystalline material which is either originally present in dextrans, or is developed in concentrated solutions, can be partially redissolved both by the action of heat and by the action of certain chemicals (fig.16, tables 20,21,22). Such processes should therefore be effective in reducing the nucleation density of dextrans, and thence in decreasing the overall crystallization rate. To investigate this possibility, rates of crystallization were studied in dextrans in which the nucleation density had been reduced by one or other of the methods identified in section I.B.3.
Several solutions of dextran (sample 8, 10% w/v) were boiled for various periods of time, causing a reduction in turbidity, the magnitude of which is indicated in fig. 26. Dextran was then isolated from each solution by evaporation under vacuum at low temperatures (45 °C.) to a concentration of 30% w/v, followed by freeze-drying. Such processes were previously shown not to affect solution turbidities associated with dextrans. The rates of crystallization of the dried samples were then measured using the nephelometric method. To reduce the experimental effort, rates were determined from turbidity measurements of a single crystallization time, it being assumed that the value of m would remain sufficiently constant and close to that for sample 8 for comparison of rates to be meaningful. The log (ΔN100) - log (time) plot for unmodified sample 8 is shown in fig. 25. The crystallization rate derived from this plot serves as a reference, against which changes in rate brought about by prior heating of the dextran solutions can be compared.

The effect on crystallization rate of pre-heating dextran solutions for various periods of time, thereby reducing initial turbidity, is illustrated in fig. 27. It is clear that reducing the initial turbidity by boiling considerably decreases the overall crystallization rate below that measured for the unheated sample; rate of crystallization decreased with increase in boiling time i.e. with decreasing nucleation density.

These findings indicate that prior boiling of dextran solutions,
FIG. 26: EFFECT OF MELTING IN REDUCING INHERENT NUCLEATION DENSITY IN DEXTRAN SOLUTION
FIG. 27: EFFECT ON DEXTRAN CRYSTALLIZATION RATE OF REDUCTIONS IN NUCLEATION DENSITY INDUCED BY MELTING
a process which reduces the inherent turbidity, is an effective procedure for reducing nucleation density. However, there appear to be limits as to how far the nucleation density can be reduced by this method. These findings are not unexpected in view of the fact that a range of stabilities will exist in the crystalline dextran particles which act as nuclei. As indicated in section I.B.3 (fig.16), this results in a particle melting range, rather than a sharp melting point. It is assumed that when the dextran concentration is 10% w/v, the melting temperature employed in this work, viz. 100 °C., lies within this melting range. Only a portion of the nuclei present will therefore be dispersed by the melting process.

(ii) Action of Chemicals.
To investigate the effect of specific chemicals in reducing initial turbidity, and the resultant effects on crystallization rate, the treatment of a dextran solution with sodium hydroxide prior to crystallization, was studied. The pH of a dilute dextran solution (sample 8, 20% w/v) was increased from 6 to 10, by the addition of sodium hydroxide. After standing for 30 mins., the solution was heated at 50 °C. for 5 mins., cooled, neutralised, and the dextran isolated by freeze drying. The turbidity (N_{100}) of a solution of this dextran (10% w/v) was 25. The rate of crystallization was measured by the nephelometric method, and the result is given in table 29.
TABLE 29: Effect of Added Chemicals on Rate of Crystallization of Dextran (Sample 8).

<table>
<thead>
<tr>
<th>Added Chemical (Concentration)</th>
<th>Time of Addition</th>
<th>Crystallization Rate Constant, k (sec⁻¹ m⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium hydroxide (10⁻⁴ M)</td>
<td>P</td>
<td>9 x 10⁻³</td>
</tr>
<tr>
<td>Urea (8 M)</td>
<td>D</td>
<td>4 x 10⁻⁵</td>
</tr>
<tr>
<td>Sodium hydroxide (1 M)</td>
<td>D</td>
<td>2 x 10⁻⁵</td>
</tr>
<tr>
<td>No addition</td>
<td>D</td>
<td>3 x 10⁻²</td>
</tr>
</tbody>
</table>

The observed reduction in rate associated with pre-treatment of dextran solution with sodium hydroxide under the conditions described, is of the same order as would be anticipated from fig. 27, where heat was employed to reduce initial turbidity. It is evident that a considerable decrease in nucleation density can be brought about by this relatively mild chemical treatment. As indicated in section I.B.3, higher pH levels would be more effective in reducing turbidity. However, because of the occurrence of oxidative degradation when dextrans are subjected to high pH conditions (121), it is possible that the findings might be ambiguous if crystallization studies are conducted on dextrans treated in this way. The rate of crystallization of dextran (sample 8) was in fact measured when urea and sodium hydroxide at concentrations of 8M and 1M respectively, were separately incorporated in the solution (60% w/w) during actual crystallization. Results of rate determinations carried out both in solutions containing the added chemicals, and in the unmodified solution, are given in table 29.
The results suggest that urea, or more effectively, sodium hydroxide, retard the rate of dextran crystallization. However, for the reasons given above, and also due to the fact that the chemicals are present throughout the whole of the crystallization period, the results cannot be wholly attributed to a decrease in nucleation density. However, it is likely that this is the major factor responsible for the observed decrease in rate.

4. INVESTIGATION OF FACTORS CONTROLLING CRYSTALLIZATION KINETICS.

In this section, the objective is to examine molecular and external parameters which affect overall rate of dextran crystallization, and to determine the extent to which kinetics are influenced by these variables. The importance of nucleation density in controlling rate has been demonstrated in the previous section. Since nucleation represents a fundamental stage of the overall crystallization process, it is evident that the kinetics will be influenced by variations in the number and type of nuclei. In order to study the crystallization behaviour of a number of dextrans of different molecular constitution, it is clear that only samples of similar inherent turbidity can be directly compared.

(a) Molecular Parameters.

The molecular parameters in a polymer which can affect crystallization kinetics are (i) degree of chain branching, (ii) molecular weight, and (iii) molecular weight distribution. It has already been mentioned in the introduction that within the range of dextrans being studied, there is no evidence to suggest that variations in the extent of 1→6-linkages exist. The principal molecular parameter investigated in this work was molecular weight, as characterised by
It has been shown that (\(M_w/M_n\)), and hence molecular weight distribution is relatively constant among the dextrans under investigation (table 17).

A brief study of the effects of molecular weight distribution on crystallization kinetics was made possible through the availability of several dextran sub-fractions, the crystallization rate of which could be compared with that of the parent dextran (sample 12).

(i) **Molecular Weight.**

Rates of crystallization from concentrated aqueous solution (60% w/w) have already been measured for several dextrans of different molecular weight (fig.25). Using the nephelometric method, the same parameters were also determined for those remaining dextrans whose molecular characteristics are listed in table 17. The log \((\Delta N_{100})\) - log (time) plots for these samples are shown in figs.26 and 29. A summary of the kinetic data deduced from these plots and also from fig.25 is presented in table 30. It is evident that the range of dextrans studied differed considerably in inherent turbidity. To facilitate comparison of the crystallization rates of dextrans with different molecular weights, the samples were therefore divided into groups of low, medium, and high inherent turbidity, corresponding to \(N_{100}\) values of 20 - 30, 30 - 90, and 90 - 300 respectively.
FIG. 28: DETERMINATION OF DEXTRAN CRYSTALLIZATION RATES BY SOLUTION TURBIDITY MEASUREMENTS
FIG. 29  DETERMINATION OF DEXTRAN CRYSTALLIZATION RATES BY SOLUTION TURBIDITY MEASUREMENTS
### TABLE 30: Summary of Kinetic Data on Dextran Crystallization from Aqueous Solution (60% w/w).

<table>
<thead>
<tr>
<th>Dextran Sample</th>
<th>Inherent Turbidity ($N_{100}, 10% \text{w/v Solution}$)</th>
<th>Crystallization Kinetic Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>$M_w$</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2,800</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>9,600</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>26,000</td>
<td>26</td>
</tr>
<tr>
<td>15</td>
<td>39,600</td>
<td>29</td>
</tr>
<tr>
<td>17</td>
<td>41,600</td>
<td>30</td>
</tr>
<tr>
<td>18</td>
<td>41,600</td>
<td>22</td>
</tr>
<tr>
<td>22</td>
<td>67,500</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
<td>34,600</td>
<td>36</td>
</tr>
<tr>
<td>11</td>
<td>36,000</td>
<td>44</td>
</tr>
<tr>
<td>12</td>
<td>36,100</td>
<td>32</td>
</tr>
<tr>
<td>13</td>
<td>37,200</td>
<td>39</td>
</tr>
<tr>
<td>14</td>
<td>38,000</td>
<td>40</td>
</tr>
<tr>
<td>16</td>
<td>41,200</td>
<td>31</td>
</tr>
<tr>
<td>19</td>
<td>42,000</td>
<td>40</td>
</tr>
<tr>
<td>20</td>
<td>55,000</td>
<td>43</td>
</tr>
<tr>
<td>21</td>
<td>61,400</td>
<td>44</td>
</tr>
<tr>
<td>3*</td>
<td>9,600</td>
<td>200</td>
</tr>
<tr>
<td>4*</td>
<td>17,000</td>
<td>210</td>
</tr>
<tr>
<td>5*</td>
<td>19,300</td>
<td>140</td>
</tr>
<tr>
<td>7</td>
<td>27,700</td>
<td>110</td>
</tr>
<tr>
<td>8</td>
<td>28,000</td>
<td>96</td>
</tr>
<tr>
<td>9</td>
<td>28,000</td>
<td>93</td>
</tr>
<tr>
<td>23</td>
<td>113,000</td>
<td>300</td>
</tr>
</tbody>
</table>

* Estimated from rate determination in 50% w/w solution.
Several low molecular weight dextrans of high inherent turbidity were observed to crystallize too rapidly at the standard concentration (60% w/w) for rates to be measurable. Accordingly, with these dextrans (samples 3, 4, 5), rates were determined at a lower dextran concentration (50% w/w), and the results corrected for the concentration difference. This was made possible by determining crystallization rates in two low molecular weight dextrans, one of high inherent turbidity (sample 7), the other of low inherent turbidity (sample 2), and both with measurable rates at concentrations of 50% w/w and 60% w/w. The log (\(A N_{100}\)) - log (time) plots for these samples at the lower concentration are shown in figs. 25 and 29, along with the plots of the higher concentration which had already been constructed. The ratio of the crystallization rate at 60% w/w to that at 50% w/w was 7.4, for sample 2, and 8.7 for sample 7. Agreement between the two values of the ratio of rates at 60% w/w and 50% w/w is good. It was assumed that the properties of sample 7 (\(N_{100} = 110\), 10% w/v solution) approached those of the rapidly crystallizing dextrans (\(N_{100} = 140-210\), 10% w/v solution) more closely than did sample 2 (\(N_{100} = 20\), 10% w/v solution). The value derived for sample 7 was therefore used to convert rates measured for samples 3, 4, and 5 at the lower concentration (50% w/v) to those at the higher concentration level (60% w/w); the latter are given in table 30.

The value of the exponent, m, which best satisfies eqn. 47 depends on the nature of the predominant nucleation process. Taking dextran samples 7 and 17 as examples, in the former case, it has been deduced that the nucleation process is heterogeneous, and \(m \approx 1\) when rate is determined by the nephelometric method.
In the latter case, the nucleation process has been shown to be predominantly homogeneous in nature, and \( m = 2 \). It might be expected that in some of the dextrans, both heterogeneous and homogeneous nucleation processes could occur simultaneously, in which case, intermediate values of \( m \) would be evident from the kinetics. This, in fact is borne out by the data presented in fig. 30, where \( m \) is plotted as a function of inherent turbidity, \( N_{100} \), (10% w/v solutions). The value of \( N_{100} \) is a measure of the nucleation density of a dextran sample, and the higher \( N_{100} \) is, the more likely it is that a heterogeneous nucleation mechanism will predominate in the kinetics. Fig. 30 thus shows that \( m \) decreases as the heterogeneous nucleation process becomes dominant i.e. as nucleation density increases. In addition, the induction period referred to in section I.C.2, which results from the high value of \( m \) observed for dextrans crystallizing by a homogeneous nucleation process, progressively disappears as the nucleation density increases. A similar dependence of the Avrami exponent on seed crystallinity during crystallization of polyethylene has been reported by Banks et al. (62).

The data observed for sample 2 (\( m = 1, N_{100} = 20 \)) does not appear to agree with this overall picture. It is possible that in accord with its low turbidity, a homogeneous nucleation mechanism does still predominate with this low molecular weight sample, but that the homogeneous nucleation rate is sufficiently rapid for heterogeneous and homogeneous nucleation to be indistinguishable (45). This is a strong probability in view of the extremely high rate at which this dextran is observed to crystallize.
FIG. 30: DEPENDENCE OF EXPONENT IN AVRAMI EQUATION ON NUCLEATION DENSITY OF DEXTRANS
The value of log (crystallization rate constant, $k$,) is shown in fig.31 as a function of $M_w$, for a range of dextrans; these were classified into three groups according to their inherent turbidity. It is evident that over the $M_w$ range 9000 - 40,000, crystallization rate increases as molecular weight decreases, which is in accord with general observations in polymer systems (37,38,39). The dramatic effects of inherent turbidity on rate throughout the whole molecular weight range supports earlier observations on a low molecular weight dextran (table 26). For the range of samples of low inherent turbidity, the very large increase in rate constant between $M_w = 26,000$ and $M_w = 9600$ is associated with a significant change in the value of $m$. In all other cases, however, $m$ is relatively invariable for the samples which are grouped within the turbidity ranges shown.

Above a certain value ($M_w = 40,000$), dextran rate appears to be relatively insensitive to $M_w$. This type of behaviour has been found in a number of systems (37,39,82). In addition, the shape of the normal rate-temperature plot (fig.4) is such that effects of $M_w$ on rate are greatest at the maximum in the curve; closer to $T_g$ or $T_m$, effects will be less pronounced, particularly in the higher molecular weight (lower crystallization rate) regions. It is shown that at dextran concentrations of 60% w/w, the rate maximum appears to lie in the vicinity of 60 °C. Hence, at 22 °C, the temperature used in this work, effects of $M_w$ on rate in the higher molecular weight region are not expected to be very great.

The decrease in dextran crystallization rate at very low molecular
FIG. 31: DEPENDENCE OF RATE OF DEXTRAN CRYSTALLIZATION ON MOLECULAR WEIGHT
weight (sample 1, $\bar{M}_w = 2800$), may be due to the fact that typical polymeric behaviour will not be exhibited until a certain maximum molecular weight is exceeded \((40,42,43)\). The relatively low rate observed for sample 1 may therefore indicate a separate regime of crystallization, representative, for example, of the tetra-, penta- or higher saccharides.

A similar low rate of crystallization in a dextran of molecular weight 2400, relative to that in a 40,000 molecular weight dextran, has been reported by Barham et al. \((7)\). These workers also report that the activation energy for spherulitic growth is lower for the low molecular weight sample than for higher molecular weight dextrans. The new regime of crystallization suggested by the results of this work may in fact be that proposed by Barham, in which the short chains of the low molecular weight dextran do not crystallize in the chain-folded configuration \((41)\) which is typical of spherulitic growth from high molecular weight synthetic polymers. Since no energy is required to fold the chains, a lower activation energy results.

The importance of $\bar{M}_w$ in determining the rate of development of crystallinity in dextrans has been previously recognised by Jeanes et al. \((4)\). Fractions produced by acid hydrolysis of a native dextran were shown to develop crystallinity at a greater rate than the high molecular weight polymer, and the rate increased with increasing hydrolysis time. The rate of crystallization of amylose depends on $\bar{M}_w$ in much the same way as dextran, except that the maximum in rate corresponds to $\bar{M}_w \approx 50,000$ \((81)\). A decrease in rate at very low molecular weight has also been noted \((86)\).
It is probable, at least in the case of dextrans, that the observed increase in overall rate at low molecular weight results from a change in both growth rate and nucleation rate. Evidence has been presented to suggest that the growth process is diffusion controlled. In such circumstances, lowering $\bar{M}_w$ would increase the diffusion coefficient, and reduce the solution viscosity, thereby resulting in a considerable increase in the rate of growth. In addition, heterogeneous nucleation is apparently observed in the case of a low molecular weight dextran (sample 2), which would be expected to crystallize by a homogeneous nucleation mechanism. This suggests that the homogeneous nucleation rate may be sufficiently rapid to make any induction period undetectable. It is possible that nucleation rate may also be diffusion-controlled, in which case the effects of $\bar{M}_w$ on rate will be similar to those on growth rate.

(ii) Molecular Weight Distribution.

The rates of crystallization of three dextran sub-fractions ($S_1$, $S_2$, and $S_3$) were determined by the nephelometric method. In order to calculate $k$, it was assumed that $m = 1$, and values were derived from single turbidity ($N_{100}$) determinations at an appropriate period of time. The sub-fractions were prepared in such a way that the inherent turbidities ($N_{100}$) lay within the range 20 - 30. Inserting the values of $k$ determined for the sub-fractions into fig.31 shows that for a given average molecular weight, the rate of crystallization decreases as the distribution broadens. This behaviour has also been shown to be a feature of synthetic polymers such as polyethylene (45,46).
(b) **External Parameters.**

The two external factors which markedly affect rate of polymer crystallization from solution are, (i) the temperature, and (ii) the solvent concentration. In this section, the effect of temperature on dextran crystallization rate is investigated at two concentrations where the rate is readily measurable. At the same time, information on the effect of solvent concentration on rate over a range of temperatures is also obtained.

(i) **Temperature.**

The rate of crystallization of dextran (sample 10) was measured nephelometrically at two concentrations, (50% w/w and 60% w/w). In each case rate was also determined over a range of temperatures varying from 10 °C. to 120 °C. The value 1.9 for the Avrami exponent, m, measured during dextran crystallization from 60% w/w solution at 22 °C., was assumed to apply to all temperature and concentration conditions. The value of k was calculated from single turbidity \( N_{100} \) measurements on dextran solutions diluted to 10% w/v after appropriate periods of time. Results are presented in fig.32 in which crystallization rate constant, k, is shown as a function of temperature, T, for crystallization at both dextran concentrations.

The overall shape of the k vs. T plot observed for crystallization from 50% w/w solution is consistent with a crystallization process, and a maximum in rate is evident in the range 60 - 80 °C. At the higher concentration (60% w/w), because of increased crystallization rates and the formation of gels, it was not possible to
FIG. 32: TEMPERATURE DEPENDENCE OF RATE OF DEXTRAN CRYSTALLIZATION FROM AQUEOUS SOLUTION.
observe a similar maximum, although the rate does increase up to a temperature of 40 °C. As indicated below, the maximum should be displaced to higher temperatures as the dextran concentration increases. The fact that a maximum in rate is observed at the lower concentration does however conclusively demonstrate that crystallization rather than, for example, a chemical cross-linking process, is responsible for insolubilisation in dextrans.

The data enables probable ranges for \( T_M \) and \( T_G \) to be defined for the polymer, under conditions where the solvent is present to give a concentration of 60% w/w. \( T_G \) is indicated to be of the order of 0 °C; \( T_M \) is less well defined, but a value between 150 - 200 °C. would not be inconsistent with the data, and also with the frequently observed difference of 100 - 150 °C. between \( T_M \) and \( T_G \) (44).

(ii) Solvent Concentration.

In studying the effects of solvent on rate of crystallization, rates were measurable only over a relatively narrow dextran concentration range. Previous studies (samples 2 and 7) at 22 °C. have shown that if the dextran concentration is increased from 50% w/w to 60% w/w the crystallization rate constant increases by a factor of about 8-fold. The results shown in fig.32, where a factor of 11 is observed between rate constants at the same two concentrations and at 22 °C., are in good agreement with this finding. At higher temperatures, the difference between the rates at the two concentrations increases, while at lower temperatures, the difference decreases. These findings can be interpreted on the basis that the principal effect of solvent on polymer crystallization rate...
arises from the depression of the melting point. This means that
at any temperature, the degree of supercooling is greater in the
case of the more concentrated solution. To compare rates at the
same degree of supercooling, it is therefore necessary to compare
crystallization rate from the 50% w/w solution at a given temper-
ature, e.g. 22 °C., with that from the 60% w/w solution at some
higher temperature. Comparing rates under these conditions, there
is good indication from fig.32 that rate of dextran crystallization
from 60% w/w solution is still significantly greater than that from
50% w/w solution.

The results of Mandelkern et al. (44) suggests that at least for
some polymer systems, crystallization rate is independent of solvent
concentration at the same degree of supercooling. This would suggest
rate control by a process of secondary nucleation rather than by
diffusion. However, in the case of dextran crystallization from
concentrated solution, diffusion control of kinetics is considered
a strong possibility i.e. mass transport to the growing front will
be diffusion limited. At the same degree of supercooling, rate
would therefore be expected to increase with increasing concen-
tration, until such time as mass transport increases associated with
increases in concentration gradient, Δc, are outweighed by trans-
port rate decreases resulting from increasing solution viscosity.
The results in fig.32 suggest that up to a dextran concentration
of 60% w/w, the maximum value in rate at a given degree of super-
cooling has not been reached.

It is also evident from fig.32 that at room temperature, the
dextrans must exist in the glassy, or supercooled amorphous state.
It is not possible to heat the polymer through the temperature
region in which crystallization can occur, and then supercool the melt to induce crystallization, because the polymer decomposes before its melting point is reached. However, by dissolving the polymer in water, the $T_G - T_M$ range, in which crystallization can occur, is displaced to lower temperatures. In this way, the melting point of the dextran is lowered sufficiently to allow crystallization to be observed in the temperature range $0 - 100 \, ^\circ C$, where the polymer is stable. It is interesting to note that in order to isolate the product from solution in the supercooled amorphous state, it must pass through a temperature-solvent environment in which dextran can crystallize at a measurable rate. The conditions of evaporation and drying used to isolate the dextran from solution are therefore extremely important in determining the inherent turbidity of the dextran i.e. the amount of crystalline material present in the isolated product.

Similar findings concerning the effects of concentration and temperature on crystallization rate have been reported for amylose solutions. Whistler and Johnson (86), for example, report rates of retrogradation to be highly temperature dependent. For a 0.85% amylose solution, rate is very rapid at 25 $^\circ C$, and decreases as the temperature is lowered. Above 60 - 70 $^\circ C$, the amylose solutions remain stable and do not retrograde. A strong dependence of crystallization rate on amylose concentration has also been noted by Loewus et al. (87). Rate was found to increase approximately with the square of the amylose concentration, but the kinetics were not simply second order. An induction period, not unlike that observed in dextran crystallization studies described above, was
reported. This may have been associated with a homogeneous process.
The major difference between the crystallization behaviour of the
two polysaccharides is that amylose crystallization proceeds to
completion at low concentrations (>1%); on the other hand, the
dextrans studied in this work will only crystallize to a small
extent and at much higher concentration levels. This is most
probably due to the low but significant branch content of the
dextran. The tendency for adjacent molecules to associate will
be lower than that for the linear amylose chains. The crystall-
ization behaviour of a chemically-synthesized, unbranched dextran
($M_w \sim 40,000$) in fact resembles that of amylose more closely than
that of the branched dextrans (5% non-1\rightarrow6 linkages) being
studied in this work (125). It is also possible that the absence
in dextrans of the primary 6-hydroxyl group, which forms particu-
larly strong hydrogen bonds in cellulose and amylose may diminish
the free energy change promoting crystallization.
5. CONCLUSIONS.

During dextran crystallization from concentrated aqueous solution, spherical crystalline particles grow to dimensions of the order of 200 nm before the solution begins to gel. The plot of particle diameter against time is not linear, but over a part of the growth phase, the dimensions increase in proportion to $t^{1/2}$, suggesting diffusion control of kinetics. Filtration studies using membranes of graded pore size support particle diameter-time relationships deduced from light scattering studies, and confirm that particle growth continues up to the point of gelation.

Two different mechanisms of dextran crystallization are possible depending on the nucleation density of the dextran. When the nucleation density is low, crystalline particle growth originates predominantly from homogeneous nuclei. In this case, an induction period exists before significant growth is observed. Turbidity studies show that the amount of crystalline material produced depends approximately on $t^{5/2}$, in accordance with the Avrami analysis for diffusion-controlled spherulitic growth at a low extent of crystallization. When the nucleation density is high, growth proceeds instantaneously from heterogeneous nuclei. The amount of crystalline polymer formed increases approximately in accordance with $t^{3/2}$, as predicted from theory for a diffusion-controlled, spherulitic growth process originating from heterogeneous nuclei.

The rate of dextran crystallization from aqueous solution depends markedly on the nucleation density. Rate can be increased,
(1) by addition of nuclei, such as those of the type that are initially present in some of the dextrans, (ii) by increasing the nucleation density of a dextran by storage at high humidity, (iii) by adding insoluble dextran particles to the solution (seeding). Conversely, crystallization rate can be decreased by reducing the nucleation density. This can be accomplished either by heating a solution of the dextran, i.e. melting the crystalline nuclei, or by treating a dextran solution with hydrogen-bond-breaking chemicals, such as urea or sodium hydroxide.

For dextrans of similar degree of branching and similar nucleation density, rate of crystallization from aqueous solution increases as the average molecular weight decreases, down to a value of $M_w \approx 10,000$. Lower molecular weight dextrans ($M_w \approx 3000$) crystallize less rapidly, which may indicate that the molecular weight is not sufficiently high for typical polymeric behaviour to be exhibited. Crystallization rate is relatively insensitive to molecular weight above a value of $M_w \approx 40,000$. For any average molecular weight, rate also decreases as the molecular weight distribution broadens.

When dextran is crystallized from concentrated solution (50% w/w), $T_{\text{max.}}$ there is a rate at a temperature in the range $60^\circ - 80^\circ C$. This behaviour is typical of crystallization in polymer systems. At room temperatures, dextrans which are soluble in cold water must therefore exist in the supercooled amorphous (glassy) state. In order to isolate such dextrans from solution, they must be subjected to concentration-temperature conditions under which crystallization can take place.
At the same degree of supercooling, rate of dextran crystallization increases with increasing polymer concentration. This observation supports the assumption that growth is diffusion-limited, rather than being controlled by secondary nucleation.

A parallel exists between the crystallization behaviour of dextran and that of the linear polysaccharide, amylose, which is present in starch. The effects of molecular weight, temperature, and concentration on rate of amylose crystallization from solution are similar to those observed for dextran.
1. INTRODUCTION.

In the previous section, crystallization studies were conducted exclusively in a single solvent, viz. water. However, kinetic studies in a mixed solvent-non-solvent system are of considerable interest, since non-solvent is frequently added to aqueous dextran solutions for the purposes of fractional precipitation. Such studies are the objective of the work described in this section.

In order to obtain an effective fractionation, it must be ensured that on adding non-solvent to an aqueous dextran solution until the cloud-point is reached, the phase transformation observed is to a liquid-liquid phase rather than to a crystalline phase (31). This is normally checked by raising the temperature by a few degrees, when a liquid-liquid dispersion will become clear, whereas a liquid-crystalline polymer dispersion will not. After liquid-liquid separation, the polymer-rich phase must be allowed to settle for some time before separation of the two liquid layers can be effected. In the polymer-rich phase, dextran will be dissolved at high concentration in a solvent-non-solvent mixture. If the conditions in this phase are favourable towards crystallization, it may appear that a liquid-crystal transition has occurred, whereas in actual fact, two consecutive processes, liquid-liquid separation, followed by rapid crystallization of the polymer-rich phase, may have taken place.

A study of the kinetics of dextran crystallization from solvent-
non-solvent mixture, and a comparison of crystallization rates with those previously measured for aqueous solutions under similar conditions, will enable the effect of non-solvent on rate to be determined. In addition, an investigation of the factors controlling rate during crystallization from solvent-non-solvent mixtures will reveal the extent to which crystalline dextran can be produced under typical fractionation conditions. The dextrans studied in this work were highly fractionated by repeated partial precipitation using ethanol as non-solvent (20). If conditions during fractionation are such that crystallization is particularly rapid, it is possible that the crystalline material responsible for inherent turbidity is introduced into the product at this stage of the manufacturing process.

To study rates of crystallization from aqueous ethanol solution, aqueous solutions (10% w/v) were prepared, and ethanol added at a constant temperature, viz. 25 °C. No crystallization was observed up to the point of liquid-liquid phase separation, when the solution became turbid. It was demonstrated that the process taking place was liquid-liquid separation, rather than crystallization, simply by warming the solution a few degrees; the turbidity was observed to disperse completely. After the solution had been allowed to stand at 25 °C, for some time, two liquid layers formed, corresponding to polymer-rich and solvent-rich phases. In the polymer-rich phase, dextran is present at a relatively high concentration, the magnitude of which depends on the amount of ethanol added. This phase was initially clear, but developed turbidity over a period of time (days), and eventually gelled. It is evident that crystallization is taking place. By
following the rate of turbidity development in the dextran-rich phase, the effect on crystallization rate of parameters such as dextran concentration, temperature, and initial turbidity (nucleation density) could be investigated.

2. MEASUREMENT OF CRYSTALLIZATION RATES.

Sufficient ethanol was added to an aqueous solution of dextran (sample 18, 25 °C.) until phase separation occurred. A further amount of ethanol was added to give a dextran concentration of 36% w/v in the polymer-rich phase, at which point the ethanol concentration was 35% w/w of the total solvent. Several aqueous solutions of the same dextran were treated in this way, and the polymer-rich phase separated from the supernatant liquid after various periods of time. In each case, the extent of crystallization was determined by diluting the dextran-rich phase to a lower concentration (1% w/v), and measuring the turbidity in the nephelometer.

The log (ΔN_{100}) - log (time) plot observed when dextran crystallized from aqueous-ethanol solution under the above conditions, is indicated in fig.33. The value for m of 2.2, which was determined from the plot, is similar to that observed during dextran crystallization from aqueous solution (table 30). An induction period of some 20 hours is also apparent, which is reflected in the low value of 6 x 10^{-10} sec.^{-m}, determined for the rate constant, k. Using a factor of 10 to convert the observed rate constant to the same units as were used in the study of aqueous solutions (N_{100} at 10% w/v), it is evident that crystallization rate from a 36% w/v aqueous-ethanol solution is comparable to that from
FIG. 33: RATE OF DEXTRAN CRYSTALLIZATION FROM AQUEOUS-ETHANOL SOLUTION
a 60% w/w (~ 65% w/v) aqueous solution. Bearing in mind that rate increases very rapidly with increase in concentration, a marked dependence of crystallization rate on the presence of non-solvent in a solvent-non-solvent mixture, is apparent.

Inherent turbidity, i.e. nucleation density, has a marked effect on rate of dextran crystallization from aqueous solution (section I.C.3). The dependence of rate of crystallization from aqueous-ethanol solution on nucleation density was also briefly investigated. The inherent turbidity ($N_{100}$, 10% w/v solution) of a dextran (sample 18) was increased from 20 to 38 by storage at high relative humidity. The rate of crystallization from aqueous-ethanol solution was measured after this change had taken place, the rate determination being carried out under conditions similar to those described above. The rate constant was determined from a single measurement of turbidity, after a crystallization period of 48 hours. The value of $2 \times 10^{-9}$ sec.$^{-1}$ for $k$ is some 3 times greater than the corresponding value determined for the same dextran, but with approximately half the initial turbidity. The order of magnitude of the effect of nucleation density on rate is comparable to that observed when the same dextran was crystallized from aqueous solution (table 28).

To ensure that the insoluble dextran particles formed on crystallization from aqueous-ethanol solution were of a similar nature to those produced on crystallization from aqueous solution, the light scattering properties of particles present in a sample of polymer-rich phase were examined in the SOFICA photometer. In this
aqueous-ethanol solution, dextran (sample 18) had been allowed to crystallize for 24 hours at 25 °C. At low dextran concentration, (1% w/v), the measured dissymmetry was 17.7 (λ = 436 nm), which from fig. 15 would indicate a spherical particle diameter of 200 nm. This finding is in keeping with the fact that clarification was not possible using a membrane filter of 800 nm pore diameter. Thus, there would appear to be no significant change in the nature of the growth particles formed due to crystallization from aqueous-ethanol solution, relative to those developed on crystallization from an aqueous solvent environment. In addition, under the particular concentration and temperature conditions defined, the rate of growth is similar in the two solvent environments, as would be expected from the nephelometric studies described above.

3. **INVESTIGATION OF FACTORS CONTROLLING CRYSTALLIZATION KINETICS.**
The effects on crystallization rate of molecular and external parameters has been investigated in the case of crystallization from aqueous dextran solution. Since it is unlikely that effects of molecular properties on rate would be significantly different on crystallization from aqueous-ethanol environment, further studies along these lines were not pursued. However, the magnitude of the effects of dextran concentration and of temperature on rate are almost certain to be different. This is due to the fact that at any given dextran concentration, melting point is not lowered to as great an extent by addition of the poor solvent (aqueous-ethanol) as it is by addition of water alone (fig.5).
Concentrated dextran solutions in which the solvent consisted of an aqueous-ethanol mixture, were produced as polymer-rich phase using the precipitation methods described in section I.D.l. The ethanol:water ratio was varied in order to change the dextran concentration in the polymer-rich phase, and the conditions under which the rate studies were conducted are given in table 31. The table shows that dextran was crystallized from several aqueous-ethanol solutions, with dextran concentrations in the range 30 - 53% w/v. Measurable crystallization rates could be observed at these concentrations. The corresponding range for the dextran content of the polymer-rich phase, as a fraction of the amount originally present, was 50 - 95%.

Since high molecular weight dextran precipitates first at the low ethanol:water ratios, as more ethanol is introduced, the average molecular weight of the dextran in the polymer-rich phase is decreasing, and the molecular weight distribution is becoming broader. However, in view of the fact that crystallization rate is not particularly sensitive to changes in these molecular parameters above $M_w \geq 40,000$, it is unlikely that the effect on kinetics will be great. It was necessary to vary the ethanol concentration only over the range 34% w/w to 40% w/w (% ethanol in total solvent) in order to bring about the required range of dextran concentrations in the polymer-rich phase. While the solvent composition is thus not strictly constant, the changes in solvent composition, in affecting melting point, will have a much lesser effect on crystallization rate than the associated changes in the concentration of dextran, i.e. in the amount of total solvent present.
Rates were determined when dextran was crystallized from a range of aqueous-ethanol solutions. These were produced as precipitated phase when varying amounts of ethanol were added to an aqueous solution of dextran (sample 18) giving the dextran concentrations indicated in table 31. Rate determinations were based on turbidity measurements, carried out on dilute dextran solutions (1% w/v), prepared from the polymer-rich phase after a suitable crystallization time. The variation of rate constant, ke, with dextran concentration is indicated in fig.34. The results indicate that rate increases with dextran concentration, in accordance with previous findings relating to crystallization from aqueous solution.

The increased rate of dextran crystallization from the more concentrated solutions could be due to one or both of two reasons, (i)
FIG. 34: EFFECT OF DEXTRAN CONCENTRATION ON RATE OF CRYSTALLIZATION FROM AQUEOUS-ETHANOL SOLUTION
diffusion control of kinetics, mass transport being concentration dependent, or (ii) elevation of the melting point, giving a greater degree of supercooling. Since with aqueous solutions, at the same degree of supercooling, rate still appears to increase with increase in dextran concentration, it is probable that a similar situation exists in the case of the solvent mixture, i.e. diffusion processes may control the crystallization rate.

(b) Temperature.
In this investigation, several aqueous-ethanol dextran solutions of constant concentration were prepared by precipitation as polymer-rich phase at 25 °C. After allowing 24 hours for the precipitates to settle, the supernatant liquids were removed and the precipitates were stored at a range of temperatures. At each temperature, the rate of crystallization was determined by measuring the turbidity of dilute dextran solutions (1% w/v), prepared from the polymer-rich phases after a suitable period of crystallization. The crystallization rate constants determined over the temperature range 20 - 40 °C. are indicated in table 32.
<table>
<thead>
<tr>
<th>Temperature of Crystallization *</th>
<th>Crystallization Rate Constant **, k (sec.(^{-m})) \times 10^{10} (m = 2.2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>35</td>
<td>40</td>
</tr>
<tr>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

* After precipitation of dextran-rich phase (sample 18) at 25 °C. over a 24 hour period.

** Based on turbidity \(N_{100}\) measurements on 1% w/v dextran solutions.

The results show that when the dextran concentration is 36% w/v in aqueous-ethanol, crystallization rate increases with increasing temperature. The rate constants (except that at 25 °C.) are not absolute, in that the initial stage of the crystallization would, in each case, have taken place at 25 °C. However, the established trend indicates that the position of the maximum in the rate-temperature plot is above 50 - 60 °C., under the particular conditions of the experiment, viz. 36% w/v dextran concentration, 35% w/w ethanol in total solvent. The maximum rate of crystallization from aqueous solution is in the same temperature region when the dextran concentration is 65% w/v. This confirms that the effect of the aqueous-ethanol on rate is caused by an elevation of the melting point of the dextran, above that which
exists in an aqueous solution of the same dextran concentration. By reducing the power of the solvent, both the melting point and the maximum in crystallization rate, corresponding to any given polymer concentration, are displaced to higher temperatures. These effects are illustrated graphically at the beginning of section I.F., once the process of melting in crystalline dextran has been discussed in greater detail.

Observations by other workers relating to crystallization of dextran from aqueous-ethanol solutions can now be readily explained on the basis of the investigations performed in this work. For example, the development of crystallinity in native dextrans when in contact with aqueous-ethanol has been noted by Jeanes and co-workers (4). It is apparent from these reports that dextrans isolated by drying were found to contain considerable amounts of crystallinity. In addition, the rate of crystallization increased with increase in the ethanol:water ratio. These observations are consistent with the findings of this work, in which it has been established that rapid dextran crystallization rates are observed under conditions of fractional precipitation with ethanol. Jeanes et al. (4) also observed that products of acid-hydrolysis of dextran developed crystallinity during fractional precipitation using 60% w/w aqueous-ethanol. The lowest molecular weight fractions crystallized particularly rapidly, which is in accordance with the work reported above, relating to effects of molecular weight on crystallization rate. The higher molecular weight fractions, which could be isolated without significant development
of turbidity, could be induced to crystallize after a further period of contact with aqueous-ethanol. These findings are now explicable in terms of the effects of the non-solvent, ethanol, in raising the melting point of the dextran; crystallization rate is thereby increased over that in purely aqueous solution. In addition, the rapid crystallization rate at the high dextran concentrations which exist in the precipitated phases would further contribute to the production of crystalline material under the experimental conditions used by Jeanes (4).

The rate of crystallization of amylose from aqueous solution has been shown to be strongly dependent on the conditions of precipitation from solution, using non-solvents such as ethanol or butanol (85). This suggests that partial crystallinity had developed during contact with the solvent-non-solvent mixture. On subsequently re-dissolving the product in water, the particulate crystalline material which would be present would nucleate the crystallization process and increase rate.

4. CONCLUSIONS.

When the non-solvent, ethanol, is added to a dilute aqueous dextran solution, the processes of liquid-liquid separation and crystallization occur consecutively. The polymer-rich phase formed on liquid-liquid separation contains dextran in aqueous-ethanol solution at a relatively high concentration; under such conditions, dextran crystallizes readily from solution at a measurable rate.

The rate of crystallization of dextran from aqueous-ethanol solution
is affected by the same parameters which control rate of crystallization from aqueous solution. Rate increases with increase in nucleation density, and decreases with increase in the level of total solvent. Rate also increases with increase in temperature in the range 20° - 40 °C.

The crystallization rate of dextran from aqueous-ethanol solution is greater than that from aqueous solution under the same conditions of temperature and polymer concentration. This is due to a lowering of the solvent power, which causes the melting point of the polymer to be raised. The associated increase in the degree of supercooling results in an increase in crystallization rate.

In the manufacture of dextrans for clinical use, the polymer is fractionated by repeated partial precipitation with a non-solvent, such as ethanol. Conditions exist at this stage of the manufacturing process which are favourable towards the development of crystalline dextran. This is the most probable explanation for the presence of small quantities of crystalline particles in dextran products. It is these particles which are responsible for turbidity in dextran solutions.

Hitherto unexplained reports relating to the development of crystallinity both in dextran and in amylose, during prolonged contact with aqueous-ethanol, can also be rationalised as a result of the kinetic studies performed in this work.
1. INTRODUCTION.

It has been shown in section I.C.2 that dextran crystallization can proceed by different nucleation mechanisms, but it was not possible to show that differences also existed in the nature of the crystalline end-products. However, an investigation of melting processes, which conversely to those of crystallization, involve the creation of a disordered state from an ordered one, can often provide information as to the nature of the crystalline structures being destroyed. In this section, the objective was therefore to study melting of crystalline dextran present both in aqueous and in aqueous-ethanol solution.

Initially, relatively simple nephelometric methods were employed to provide basic information on the melting process.

More detailed light scattering procedures were then used to provide additional information, relating to the nature of the crystalline entities which were present before and after partial melting had been carried out. The melting behaviour both of crystalline dextran which is initially present, and also of that which is deliberately produced by crystallization from solution, was investigated by these methods.

Crystalline structures on polymers are characterised by a melting range, rather than by a sharp melting point, due to the fact that the structures possess a range of stabilities. Thus, at any given temperature, melting processes may not be complete, i.e. a
fraction of the crystalline species present may be resistant to melting. An objective of this work was to determine whether such a situation existed in the case of dextran. If so, an additional aim would be to identify the nature of the crystalline dextran particles which were resistant to melting under specific conditions. It was further intended that the degree of control which could be exercised over the stability of crystalline particles, and hence on their melting behaviour, by conditions existing during the crystallization process, should also be investigated.

The process of melting in crystalline polymers is dependent on the amount and nature of solvents present. An objective to this work was therefore to study the effects of solvent level and solvent type on melting in crystalline dextran, using a range of aqueous and aqueous-ethanol solutions. It was then intended to interpret the findings in terms of the established effects of solvent on melting behaviour in polymer systems.

2. MELTING IN AQUEOUS SOLUTION.
(a) Nephelometer Studies.
(i) Inherent Crystalline Material.
It has been established in previous studies that the crystalline particles which are responsible for inherent turbidity in dextrans can be partially melted by boiling a suspension in dilute (10% w/v) dextran solution. This was illustrated in fig. 26, from which it is evident that a major part of the crystalline material in the dextran (sample 8) can be melted within a 15 minute period. A definite melting rate exists, which is consistent with the behaviour of
crystalline polymer systems. The persistence of a small amount of turbidity after boiling the solution \( N_{100} = 18 \) indicates that the crystalline dextran particles pass a range of stabilities. This also is typical of crystalline polymers, in which the stability of the crystal form is frequently related to the conditions of crystallization \( (45,71,72,73) \).

A number of solutions of other dextrans (samples 9, 19, 17) were boiled for periods up to 30 minutes, and the decrease in turbidity determined; the results are shown in fig. 35. A small amount of turbidity persists in dextrans containing both low and high initial levels of crystalline material. In addition, the amount of persistent turbidity does not appear to be related to the amount of crystalline material present before melting. This suggests that a range of types of crystalline particles were produced during manufacture of the dextrans. A small fraction of these crystalline structures may have a high melting point, and resists dispersion under conditions during which the bulk of the crystalline material will melt. At any melting temperature, the relative proportions of the heat-stable and non-heat-stable crystalline particles would depend on the crystallization conditions which existed during manufacture of the dextran.

Several of the dextran solutions which had been boiled for 30 minutes were treated with sodium hydroxide. A further reduction in turbidity was observed in each case, the results being given in table 33. It is evident that a proportion of the crystalline species which resist melting under boiling conditions are susceptible to dispersion by the action of sodium hydroxide. Dextran solutions exhibiting both high and low residual turbidities after boiling, responded to this treatment in much the same way. It is clear that a small
FIG. 35: MELTING, IN DILUTE AQUEOUS SOLUTION, OF CRYSTALLINE MATERIAL INHERENTLY PRESENT IN VARIOUS DEXTRANS.
but definite amount of very stable crystalline material exists, which cannot be dispersed by any of the procedures uncovered in this work. A range of crystalline particles, differing widely in melting point, is apparently present in dextrans. As mentioned above, these particles most likely arise due to the existence of conditions favourable for crystallization during dextran manufacture.

More direct evidence for the existence of a melting range in crystalline dextran structures has been provided by the data given in fig. 16. In this work, a dextran solution (sample 6, 10\% w/v) was heated for 15 minutes at various temperatures, and the reduction in turbidity determined. Melting is observed to commence in the region of 60 °C, and is not complete up to a temperature of 120 °C; this indicates a broad range of stability in the crystalline particles responsible for inherent turbidity. Comparing fig. 16 with fig. 35, it is also apparent that at 100 °C, the efficiency of the melting process is enhanced by boiling. This is presumably due to the increased thermal agitation of the boiled solution.

Solutions of a dextran (sample 5, $\bar{M}_w = 19,300, 10\% w/v$), of higher inherent turbidity ($N_{100} = 140$) than sample 6, were also subjected to a similar melting procedure. The results, which are shown in fig. 16 for comparison, again show that the major proportion of the crystalline particles are dispersed at temperatures above 60 °C, but that complete melting cannot be achieved within the temperature range imposed by the solvent.
TABLE 33: Effect of Sodium Hydroxide on the Turbidity of Boiled Dextran Solutions (10% w/v).

<table>
<thead>
<tr>
<th>Dextran Sample No.</th>
<th>Turbidity ($N_{100}$)</th>
<th>Before Addition of Sodium Hydroxide</th>
<th>After Addition of Sodium Hydroxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>18</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>20</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>10</td>
<td>29</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>17</td>
<td>16</td>
<td></td>
<td>18</td>
</tr>
</tbody>
</table>
(ii) Developed Crystalline Material.

Previous nephelometric studies (table 22) have shown that large quantities of crystalline material, formed by crystallizing dextran from concentrated solution, can be almost totally dispersed by boiling an aqueous suspension. Further studies were conducted to determine the effects of boiling on various levels of turbidity developed in concentrated solutions of dextran (samples 7 and 17). Results are shown in table 34. After boiling dilute dextran solutions with low levels of developed turbidity, the residual turbidity was only slightly greater than the residual inherent turbidity. However, when the developed turbidity was higher, i.e. crystallization time was longer, the residual turbidity was observed to increase. Nevertheless, the fraction of the total turbidity which remains after boiling is still much lower in the case of developed turbidity than in the case of inherent turbidity.

TABLE 34: Effect of Boiling on Turbidity Inherently Present and Developed in Dextran Solutions.

<table>
<thead>
<tr>
<th>Dextran Sample No.</th>
<th>Turbidities (N₁₀₀, 10% w/v Solution)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inherent</td>
</tr>
<tr>
<td></td>
<td>Before Boiling</td>
</tr>
<tr>
<td>7</td>
<td>110</td>
</tr>
<tr>
<td>17</td>
<td>30</td>
</tr>
</tbody>
</table>

* Sample 7 - 15 minutes, sample 17 - 16 hours

** Sample 7 - 60 minutes, sample 17 - 30 hours
When the level of developed turbidity is low, the amount which persists on boiling the solution appears to be related to the amount of inherent turbidity which persist under the same conditions. It is therefore possible that the heat-stable crystalline particles, which are responsible for persistent developed turbidity, are similar to the heat-stable particles which are initially present. The crystalline particles initially present, which resist melting under the conditions used, may, in effect, be stable nuclei or "seeds". The process of growth from these nuclei to crystalline particles of larger dimensions, is apparently almost totally reversible under the melting conditions employed. The melting process results in the reappearance of the original stable nuclei, together with a small amount of growth product, possibly derived from such nuclei. The melting of growth particles produced from less stable nuclei may result in dispersion of the total crystalline entity, including the nucleus.

The fact that in dextran sample 17, for example, the crystallization process has been found to be homogeneously nucleated, does not invalidate these suppositions. In this dextran, a small number of stable nuclei do exist, from which growth may develop. This is indicated by light scattering observations, pointing to growth in the induction period before significant growth from homogeneous nuclei commences. The observed melting behaviour of this dextran therefore suggests that homogeneously-nucleated crystalline growth particles may not be resistant to melting. On the other hand, the nuclei associated with heterogeneously-nucleated growth particles appear to persist under similar melting conditions, although most
of the crystalline material grown on such nuclei will melt. In other words, crystallization and melting appear to follow the same pathways, but in reverse.

When the level of developed turbidity is high (table 34) i.e. the crystallization time is longer, a much larger amount of turbidity remains after boiling the solutions. The growth particles are evidently more stable, and necessary conditions for the development of such crystalline species are discussed below.

(b) Detailed Light Scattering Studies.
In earlier studies, the rate of crystallization of dextran from concentrated solution (60% w/w) was determined by carrying out light scattering measurements on dilute solutions (10% w/v) prepared from the concentrate. In order to study the melting behaviour of the developed crystalline material, several of the dilute solutions were heated to 100 °C., maintained at this temperature for 15 minutes, cooled, and the light scattering parameters remeasured. The values of turbidity and dissymmetry are indicated in fig.36 for both dextran sample 7 of high inherent turbidity and for sample 17 of low inherent turbidity.

Considering sample 17, the small decrease in inherent turbidity observed on melting is in keeping with results of the nephelometric studies. While the turbidity developed as a result of crystallization can be almost totally eliminated by heating, the small amount which resists dispersion by melting increases with time. This observation identifies a condition which is favourable
FIG. 36: DEPENDENCE ON CRYSTALLIZATION TIME OF (a) TURBIDITY, (b) DISSYMMETRY, ASSOCIATED WITH CRYSTALLINE DEXTRAN PARTICLES WHICH ARE RESISTANT TO MELTING
towards the development of persistent turbidity, viz. prolonged crystallization from concentrated aqueous solution. Irrespective of the nature of the crystalline species which resist dispersion, the inference is that only those particles which are allowed to develop over a relatively long period of time resist melting. This is in fact a common situation in crystallizing polymer systems (44,45,72,73). The slower the rate of crystallization, the more perfect is the crystal form, and the higher its melting point becomes. It has been suggested above that the crystalline particles which resist dispersion are stable nuclei and their growth products. In view of the fact that the observed turbidity increase is small, the particles which remain after melting of the shell of crystalline material developed round the nucleus, cannot be significantly greater than the size of the nucleus itself. It is possible that a process of strong adhesion of the polymer to the nucleus is involved, whereby the effective size of the nucleus is increased after melting. The longer the period of crystallization, the greater is the opportunity for optimum orientation of the polymer chains, leading to the formation of stable structures which will resist dispersion under the melting conditions employed.

The dissymmetry of scatter associated with the crystalline particles initially present in dextran sample 17, increases slightly under melting conditions, suggesting preferential dispersion of the smaller nuclei present. However, as the period of crystallization increases, the particles which resist dispersion tend to assume larger dimensions. This indicates that the longer the
period during which conditions favourable for crystallization exist, the greater is the tendency for large stable growth particles to form. Such growth particles are nevertheless very much smaller than the crystalline particles which exist prior to melting.

With sample 7, the melting behaviour, as studied by light scattering methods, is very similar to that of sample 17. As indicated from the nepheolmetric studies, the decrease in inherent turbidity on melting is much more marked than in sample 17, and the residual level is higher. A larger content of stable nuclei is indicated. On melting crystalline particles developed on crystallization from concentrated dextran solution, the majority are dispersed but, as with sample 17, a small residual turbidity, apparently related to residual inherent turbidity, persists. The level of this persistent turbidity again increases with time of crystallization.

The dissymmetry of particles responsible for inherent turbidity in sample 7 also increases under melting conditions, indicating that the particles resistant to melting are the larger stable nuclei. Again, as the period of crystallization increases, those particles which resist melting tend towards higher average dimensions. The same explanation as was advanced in the case of sample 17 would appear to apply.

Although the absolute rate of crystallization was much greater in the case of sample 7 than with sample 17, stable growth products were still formed. It is thus apparent that it is not the absolute crystallization rate which controls subsequent crystalline
particle stability under melting conditions. Most probably, it is the rate of crystallization from stable heterogeneous nuclei, relative to that from smaller, less stable nuclei, or from homogeneous nuclei, which is important in determining the amount of residual turbidity, i.e., the amount of crystalline material which resists melting under the stated conditions.

(c) Dependence of Melting on Dextran Concentration.
It has been suggested above that differences exist in the stability of crystalline particles produced by heterogeneously nucleated growth processes, and that these differences depend on the stability of the nuclei. If this is so, it is also probable that similar differences might exist between the stability of particles produced by homogeneous, as opposed to heterogeneous, nucleation processes. This does not necessarily follow from the light scattering studies described above; the higher residual turbidity on melting observed with sample 7 (predominantly heterogeneous nucleation), compared with sample 17 (predominantly homogeneous nucleation), could be due simply to different levels of stable heterogeneous nuclei. In order to provide further information on this topic, the melting behaviour of the same two dextrans was studied under a range of conditions selected to demonstrate differences in the stability of crystalline material developed by different nucleation mechanisms.

Since solvent lowers the melting point of a polymer, if the melting behaviour is studied as a function of dextran concentration, at a constant temperature, then melting will only be observed below a
particular concentration. Thus, at the dextran concentration where turbidity undergoes a rapid decrease on boiling the solution, the melting point of the suspended crystalline particles will be 100 °C. If, for the two dextrans, (samples 7 and 17), differences exist between the concentrations at which the bulk of the developed crystalline material undergoes melting, the crystalline dextran melting at the lower concentration must possess the higher melting range.

Crystalline material was produced in solutions of dextran (samples 7 and 17, 60% w/w) for periods of time such that both gave comparable turbidities. The suspensions of the crystalline material were diluted to various lower dextran concentration levels, and boiled for a 30 minute period. The efficacy of the melting procedure was determined by measuring the turbidity of the boiled suspension at a constant dextran concentration (10% w/v). Residual turbidity after boiling was plotted as a function of dextran concentration, results being shown in fig.37. In each case, melting was not observed until the dextran concentration had decreased to a particular value, viz. 41% w/v for sample 17, and 35% w/v for sample 7. It is evident that the crystalline material developed in sample 7 by a predominantly heterogeneous process, is more resistant to melting than that in sample 17, produced by a predominantly homogeneous nucleation mechanism. The dependence of melting point on dextran concentration observed in each case is typical of solvent effects on melting in polymer systems.

One hypothesis which would explain these findings, based on the suggestions of Price and Turnbull (66,67) for melt-crystallized
FIG. 37: DEPENDENCE OF MELTING RANGE OF CRYSTALLINE DEXTRAN PARTICLES ON SOLVENT CONDITIONS
systems, is that the heterogeneous nuclei may contain crevices into which the concentrated dextran solution will penetrate. Crystallization will proceed in these regions in the normal way, but on subsequently attempting to melt the total structure, melting will be resisted. This is attributable to adhesion of the polymer to the stable nucleus, which has the effect of raising the melting point.

3. MELTING IN AQUEOUS-ETHANOL SOLUTION.
In studies of the effect of crystallization conditions on the melting behaviour of crystalline dextran, time of crystallization and inherent crystalline particle content, i.e. seed nucleation density, have been identified as factors affecting the melting range. Other crystallization conditions which could also affect melting behaviour include the concentration of dextran, and the temperature. The role played by these variables in determining the melting properties of crystalline dextran, suspended in aqueous-ethanol dextran solution, was therefore investigated.

To study the melting of crystalline dextran in aqueous-ethanol solution, turbid solutions prepared by the procedures described in section I.D.1 were employed. These solutions, containing suspended crystalline dextran particles, were produced as polymer-rich phase by precipitation of dextran from aqueous solution by addition of ethanol. Melting was studied in dextran crystallized from solution under various concentration and temperature conditions, and from a dextran in which the inherent turbidity had been increased by storage at high humidity. In investigating each
variable, two melting procedures were examined, (i) melting in aqueous-ethanol dextran solution: the concentrated aqueous-ethanol solution from which the dextran was crystallized was diluted to a lower concentration (10% w/v) with water, and then refluxed for 30 minutes, (ii) melting in aqueous dextran solution: ethanol was evaporated from the concentrated aqueous-ethanol solution, after it had been diluted to a lower concentration (15% w/v) with water. During this time, the dextran concentration increased to approx. 30% w/v. The total evaporation process lasted 30 minutes, the bulk of the ethanol being removed during the initial 10 minute period. The amount of crystalline material which resisted melting under these two sets of conditions was measured nephelometrically, as turbidity at a standard dextran concentration of 10% w/v.

(a) Dependence of Melting on Crystallization History.

(i) Time of Crystallization.

Samples of dextran which had been crystallized at 25 °C. from aqueous-ethanol solution over various periods of time for the purpose of rate determination (see fig.33), were melted by the procedures described above. Residual turbidities after melting the bulk of the crystalline material are shown in fig.38 as a function of crystallization time. It is evident that the longer the crystallization period becomes, the greater is the amount of crystalline material which resists dispersion under the melting conditions employed. This supports earlier findings on the melting behaviour of crystalline dextran in aqueous solution, and it is suggested that the formation of relatively large stable nucleating structures is involved. These are considered to originate from the slow growth of stable nuclei which are
initially present. It was evident from dissymmetry measurements on the boiled solutions that the stable crystalline species which resisted melting were not large particles, grown slowly by a homogeneous nucleation process to give more perfect crystal structures. Only a slight increase in the size of the particles over those initially present was indicated.

The melting process was less effective in aqueous-ethanol solution than that performed in aqueous solution of the same dextran concentration. This is due principally to the fact that ethanol raises the melting point of the polymer at any total solvent level, although there will also be a small effect due to the lower boiling temperature of the aqueous-ethanol solutions. This effect is reproduced consistently throughout further melting studies described below.

A definite rate of formation of stable crystalline particles thus exists, which is much lower than the overall rate of crystallization. The effect on this rate, of crystallization conditions such as temperature, dextran concentration, and inherent turbidity, will now be considered.

(ii) Temperature of Crystallization.

The polymer-rich phase produced by adding ethanol to an aqueous dextran solution was allowed to settle for 24 hours at 25 °C., and the supernatant solvent-rich phase removed. Samples of the aqueous-ethanol solution (36% w/v) dextran, 35% w/w ethanol in total solvent) were subsequently stored at several temperatures in the range
20° - 40 °C., during which time the formation of crystalline particles was observed (see table 32). The melting behaviour of such solutions under the two sets of conditions defined above, was examined as a function of storage temperature, the residual turbidities after melting being shown in fig.38. It is clear that an increasing crystallization temperature favours the formation of stable crystalline particles.

The larger amounts of heat-stable crystalline material which are produced at higher temperatures, can be attributed to the increase in the amount of stable material present before melting (see table 32). This will arise from the increase in rate of crystallization from stable nuclei which will occur as the temperature is raised. It is evident that increases in the amount of heat-stable crystalline material at higher crystallization temperatures must outweigh any reductions in the amount of this material, which might be anticipated from the formation of less stable, more imperfect forms at high crystallization rates.

(iii) Inherent Nucleation Density.
Dextran (sample 18) was crystallized from aqueous-ethanol solution by the procedure described above, both before and after the inherent turbidity had been increased by storage at high humidity. The residual turbidities after melting of the bulk of the crystalline material developed before and after humidification of the dextran, are as shown in table 35. The humidification procedure also increased the amount of inherent crystalline material which resisted melting on boiling a dilute dextran solution (10% w/v).
Before melting

$\text{TURBIDITY (N_{100} x 10^{-2})}$

$\text{CRYSTALLIZATION TIME (hours)}$

$\text{CRYSTALLIZATION TEMP (°C)}$

$\text{CRYSTALLIZATION CONCENTRATION (\% w/v)}$

**FIG. 38**: EFFECTS OF THE TIME, TEMPERATURE, AND DEXTRAN CONCENTRATION, ON THE DEVELOPMENT OF CRYSTALLINE PARTICLES WHICH RESIST MELTING IN DILUTE SOLUTION

DEXTRAN SAMPLE 18. MELTING CONDITIONS:

A-E - AQUEOUS-ETHANOL SOLUTION

A - AQUEOUS SOLUTION

<table>
<thead>
<tr>
<th>Dextran Solution Turbidities (N_100, 10% w/v Dextran Solution)</th>
<th>Dextran (Sample 16) after Humidification</th>
<th>Dextran (Sample 18) after Humidification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inherent turbidity</td>
<td>20</td>
<td>38</td>
</tr>
<tr>
<td>Inherent turbidity, after boiling aqueous solution (10% w/v)</td>
<td>18</td>
<td>30</td>
</tr>
<tr>
<td>Turbidity* developed in aqueous-ethanol dextran solution (36% w/v, 25 °C, 48 hours)</td>
<td>170</td>
<td>430</td>
</tr>
<tr>
<td>Residual developed turbidity, after boiling aqueous-ethanol dextran solution (15-30% w/v)</td>
<td>65</td>
<td>175</td>
</tr>
<tr>
<td>Residual developed turbidity after boiling aqueous dextran solution (10% w/v)</td>
<td>62</td>
<td>97</td>
</tr>
</tbody>
</table>

* Measured in 1% w/v solution.
The results show that the amount of heat-stable material developed on crystallization depends on the amount of heat-stable crystalline material (i.e. stable nuclei) initially present. This conclusion was reached previously in relation to heat-stable crystalline dextran which had been developed in aqueous solution.

(iv) Dextran Concentration during Crystallization.
Dextran (sample 16) was crystallized at 25 °C. from a range of aqueous-ethanol solutions of different dextran concentration. Initially a relatively short period of crystallization and a low crystallization temperature were selected, to ensure that heat-stable crystalline particles would not be formed due to the time and temperature factors which have been identified as being important in controlling levels of persistent turbidity. After melting by the two boiling procedures described above, the residual turbidities measured in each case were as shown in fig.38 for each of the crystallization conditions, i.e. dextran concentrations, examined. The results with the more effective melting procedure (boiling an aqueous solution) show that as the total amount of crystalline material present before melting increases, due to crystallization at higher dextran concentrations (see fig.32), there is no tendency for greater amounts of heat-stable material to develop. The evidence suggests that relatively long crystallization times, which allow specific structures initiated to stable nuclei to develop, are necessary for the development of heat stable growth particles. It has been shown above that a definite but low rate of formation of stable crystalline entities does exist, which is increased at higher temperatures. However, under the crystallization conditions of this experiment, the amount of heat-stable
crystalline material developed was not sufficiently great for
the effects of dextran concentration during crystallization,
on rate of production of stable material, to be determined.

Different results were obtained when a similar study was
conducted, but with a longer crystallization period of 48
hours before melting. As indicated in fig.38, the amount of
heat-stable turbidity increased with increasing dextran con-
centration. This indicates that dextran concentration during
crystallization, like crystallization temperature, can affect
the rate of development of heat stable crystalline particles.

All of the factors affecting the overall rate of crystallization
in dextran can thus also affect the rate of development of the
fraction which is resistant to melting under given conditions.
As mentioned above, the heat-stable material is believed to
result from growth initiated by stable heterogeneous nuclei. It
is not therefore surprising that its rate of formation is in-
fluenced by those same parameters generally affecting rate in
heterogeneously- nucleated crystallization processes.

(b) Dependence of Melting on Solvent Composition.
It has been inferred from the melting behaviour of crystalline
dextran under two sets of conditions, viz. in dilute aqueous
solution, and in dilute aqueous-ethanol solution, that the
presence of ethanol in the solvent raises the melting range.
The melting behaviour of dextran (sample 17) crystallized from
aqueous solution over a range of dextran concentrations has
already been examined (see fig.37). A similar study was con-
ducted using aqueous-ethanol as solvent, in order to demonstrate directly the effects of ethanol on melting.

Dextran (sample 17) was crystallized from an aqueous-ethanol solution, produced as polymer-rich phase from an aqueous solution by precipitation with ethanol. The concentrated dextran solution was separated from supernatant after a suitable period of time, and several dilute solutions at a range of concentrations were prepared with water. Each of these solutions was boiled under reflux for 30 minutes, and the amount of crystalline material which resisted dispersion was determined by measuring turbidity on standard dilute solutions (10% w/v). The turbidity remaining after this melting procedure is shown in fig. 37 as a function of the dextran concentration in the boiled solution.

The results show that in the case of the aqueous-ethanol solutions, melting of the bulk of the developed crystalline particles is not observed until the dextran concentration reaches 30% w/v, whereas in the aqueous solution case, melting takes place at the higher dextran concentration of 46% w/v. This shows that the effect of ethanol is to raise the melting point, such that at any temperature, lower dextran concentrations are required in order to melt the bulk of the crystalline material.

4. CONCLUSIONS.

A fraction of the crystalline material initially present in dextrans can be melted by heating or boiling a dilute solution to 100 °C. A definite melting rate exists, and a fifteen minute heating period is necessary to ensure that this fraction has been completely melted. The bulk of the heat dispersable fraction can be melted in dilute dextran solution (10% w/v) at
temperatures in the region of 60 °C., but further small amounts can be melted at progressively higher temperatures, until at 100 °C., a small heat-stable fraction persists. A proportion of the crystalline material which does not melt at 100 °C. can be destroyed by the action of sodium hydroxide, which suggests that it is simply crystalline dextran of a highly stable form.

A range of heat-stabilities therefore exists among the various types of crystalline particles responsible for inherent turbidity in dextrans. The stable crystalline particles, melting at the high temperature end of the melting range, are the larger of the crystalline species initially present in dextrans. These are among those particles which have been shown to be effective as heterogeneous nuclei during crystallization of dextran from aqueous solution.

Under all crystallization conditions, a large proportion of dextran crystallized from concentrated aqueous solution can be melted by diluting the solution with water, and boiling. Although the amount of crystalline dextran which resists melting under these conditions increases with time of crystallization, the rate of development of stable material is much lower than the overall crystallization rate. The rate of development of stable material, and hence the amount of such material present at any given time, increases with increasing crystallization temperature, and with increasing dextran concentration during crystallization.

The amount of heat-stable crystalline material developed in
Dextran solutions is always slightly greater than the amount of heat-stable crystalline material inherently present. This would imply that crystallization and melting follow similar pathways, but in reverse. The heat-stable crystalline particles are most probably large stable nuclei and their growth products. A slow crystallization rate is necessary if stable growth products are to form, i.e., relatively long crystallization times are required in order to form large amounts of heat-stable crystalline structures.

The larger proportion of developed crystalline material is of large particle size (≈ 200 nm). Such particles do not resist melting at 100 °C. in dilute dextran solution. However, the stability of such material does appear to depend on the mechanism of crystallization, in that particles, the growth of which is initiated by small heterogeneous nuclei, melt at higher temperatures than homogeneously nucleated crystalline growth particles.

A range of stabilities thus also exists among the various types of growth particle formed on crystallizing dextran from aqueous solution. The stability depends on the mechanism and conditions of the crystallization process responsible for their formation. It is possible that the stability of the growth particles arises as a result of crystallization of the dextran from solution within crevices of the large nuclei. On melting of large growth particles formed from such structures, the particle may revert to a slightly larger stable nucleus state, due to adhesion of the polymer in the area of the crevices.
The presence of a good solvent, such as water, affects the melting range of the crystalline dextran particles; the melting point of the bulk of the particles decreases as the solvent concentration increases. This means that, at any temperature, crystalline dextran will only melt when a particular solvent level is exceeded. As the same dextran concentration, the presence of ethanol in the solvent elevates the melting range of the crystalline material over that in aqueous solution. At any temperature, when non-solvent is present, a lower dextran concentration is therefore required to allow the crystalline dextran to melt.
I.F. THE IMPLICATIONS OF CRYSTALLIZATION AND MELTING IN THE PREPARATION OF DEXTRANS.

I. INTRODUCTION.

In previous sections, the factors affecting rate of dextran crystallization from solution have been studied in detail. The conditions which control melting processes in crystalline dextran have also been determined. One objective in this section is to indicate how this information may be employed to identify particular stages of the dextran preparation process at which the formation and dispersion of crystalline dextran may occur. The findings will apply equally to laboratory preparation of dextrans for research purposes, or the commercial manufacture of dextrans for clinical use.

Following a review of the work described in the preceding sections, relating to the crystallization and melting behaviour in dextrans under different conditions of temperature, solvent concentration and solvent composition, the various stages in the dextran preparation process are described. The objective of this is to consider the conditions existing at each of these stages, in terms of their contribution towards either increasing or decreasing the crystalline particle content of the final dextran product by the respective processes of crystallization and melting.

The existence in dextrans of crystalline dextran particles with varying degrees of resistance to melting has been established in the studies described in section I.E. In this section, the aim is to discuss the possible origin of these particles, and to identify those stages of the preparation process which lead to
their formation. The intention is also to suggest methods of controlling turbidity formation during and storage of dextrans.

2. REVIEW OF CRYSTALLIZATION AND MELTING BEHAVIOUR OF DEXTRANS.

As a result of information derived from studies of crystallization and melting in dextrans, it is possible to outline the general form of the crystallization rate-temperature plots under various solvent conditions. While it may be possible to obtain a more complete picture from further studies, it is felt that the information gained from this work is of value for the purposes of identifying methods of control over crystallization and melting processes occurring at various stages of dextran manufacture.

The information derived from this work, which is relevant to the definition of the plot of rate of crystallization from aqueous solution as a function of temperature, is as follows:

(i) when the dextran concentration is 65% w/v, a maximum in crystallization rate is observed at a temperature in the range 60 °C - 80 °C.

(ii) the melting temperature of a high proportion of crystalline material present in a dextran solution of concentration, 10% w/v, is in the range 50 °C - 60 °C.

(iii) the melting temperature of crystalline dextran present in a dextran solution of concentration, 40% w/v, is 100 °C.

(iv) for the same degree of supercooling, rate of crystallization decreases with increasing solvent concentration; at a constant temperature, viz. 22 °C, rate also decreases with increasing solvent concentration.
Making an assumption that the difference between the melting point, $T_M$, and the glass point, $T_G$, is of the order of 150 - 200 °C., which is typical for a number of polymers (126), the crystallization rate-temperature plots at various dextran concentrations would be expected to take the form indicated in fig.39 (b).

The following information derived from this work is relevant to the definition of the form of similar plots, relating to the temperature dependence of rate of crystallization from aqueous-ethanol solution (35% w/w ethanol in total solvent):-

(i) the melting temperature of a solution of concentration, 30% w/v, is of the order of 100 °C.

(ii) a maximum in the rate of crystallization occurs above a temperature of 40 °C. when the dextran concentration is 36% w/v.

(iii) in aqueous-ethanol solution, rate of crystallization is increased by a factor of several hundred over that in aqueous solution.

(iv) rate of crystallization at 25 °C. increases with decreasing solvent concentration.

The crystallization rate-temperature plots for this particular solvent composition would therefore be expected to be of the form shown in fig.39 (a).

Comparison of figs.39 (a) and 39 (b) shows that at any total solvent level, the effect of the ethanol is to raise the melting point, thereby also displacing $T_G$ and the temperature of maximum crystallization rate to higher temperature regions. The effect of solvent on rate of crystallization and on melting point is particularly evident in fig.39 (a).
FIG. 39: EFFECTS OF CONCENTRATION AND TEMPERATURE ON DEXTRAN CRYSTALLIZATION AND MELTING IN AQUEOUS AND AQUEOUS-ETHANOL SOLUTIONS
The semi-quantitative information which can be derived from figs. 39 (a) and (b) may be utilised to identify conditions of temperature, dextran concentration, and solvent composition which would particularly favour the production of crystalline material during the preparation of dextrans.

3. THE DEXTRAN MANUFACTURING PROCESS.
The dextrans used in these studies were manufactured by a process basically identical with that which would be used in the laboratory, and which consisted of the following essential stages:

(a) Fermentation, using Leuconostoc mesenteroides, NRRL B512, acting on a sucrose substrate, to produce dextran of molecular weight in the range 30 - 50 million.
(b) Isolation of the dextran from the fermentation broth by precipitation with ethanol.
(c) Controlled high-temperature acid hydrolysis, to reduce the weight-average molecular weight of the dextran to a value less than 150,000.
(d) Multi-stage fractionation by repeated partial precipitation with ethanol, to produce the narrow molecular weight distributions required for pharmaceutical purposes.
(e) Ethanol removal from the solution containing the fractionated dextran, by distillation.
(f) Concentration of aqueous solution, and drying, to give a dextran powder containing 5 - 10% moisture.

For pharmaceutical use, the dried products are dissolved in an aqueous solution (e.g. 6% w/v dextran, \(M_w = 60,000\), 10% w/v dextran, \(M_w = 40,000\)) containing sodium chloride (0.9% w/v), or glucose (5% w/v). The mixture is autoclaved, and stored in a state
of readiness for intravenous injection.

It is evident that conditions favourable to crystallization and melting may exist in several of the preparative stages. It is these conditions which must control the content of crystalline material which is present in the dried products and in solutions reconstituted therefrom. This material is responsible for the turbidity referred to as "inherent" turbidity in this work.

4. **PREPARATION CONDITIONS CONTROLLING CRYSTALLINE PARTICLE CONTENT.**

(a) **Fermentation Stage.**

The ability of dextrans to crystallize depends very strongly on the branch content of the polymer chains; highly branched molecules will not readily crystallize, whereas more linear dextrans are capable of undergoing rapid crystallization under appropriate conditions. The branch content of the polymer is controlled primarily by the bacterial strain utilised in the fermentation. The particular strain, *Leuconostoc Mesenteroides* NRRL B512 used to manufacture the dextrans studied in this work, gives rise to relatively linear molecules with a low proportion of non-1→6-linkages. Conditions during the fermentation process are therefore rigorously controlled in order to ensure variations in the branch content of the chains do not occur (20). Such variations could significantly affect the crystallization behaviour of the dextran.

(b) **Precipitation/Hydrolysis Stages.**

The first stage of manufacture during which crystalline dextran may actually be formed, is in the precipitation of native dextran from the fermentation broth. The formation of precipitates containing
considerable turbidity occur at this point only if the precipitates are stored for long periods (20). However, since the hydrolysis process is carried out at high temperature in moderately dilute solution, the bulk of the crystalline material formed at this point will be subsequently melted.

(c) Fractionation Stage.

During fractionation of the hydrolysis product by ethanol precipitation methods, there are considerable opportunities for the development of crystallinity. A multi-stage, partial precipitation procedure is necessary to ensure that the final product is of sufficiently narrow molecular weight distribution. This means that dextran is present in concentrated aqueous-ethanol solution for a lengthy total period of time, conditions under which the extent of crystallization may be considerable. Crystalline material formed during the early precipitation stages, will act as nuclei for crystallization during later stages of fractionation. In this way, the amount of crystalline material produced will increase, with increasing number of precipitation stages in the fractionation process. It is evident that time delays during precipitation, settling, and storage of precipitates at high dextran concentration and high temperature, must be avoided in order to minimise the formation of turbidity during fractionation. This is particularly true in the later stages of the fractionation process, when nucleation density will have increased, and will play a highly critical role in the development of crystalline material.
Crystallization during fractional precipitation can have serious effects on the efficiency of the fractionation process (31). If conditions are such that the crystallization rate is particularly high when the droplets of polymer-rich phase separate out from the homogeneous solution, crystallization can occur in the droplets before they can coalesce and settle to form a precipitate. Settling, in the normal sense, is not possible, and a granular precipitate, which is not readily separable from the supernatant liquid is formed (20).

(a) **Solvent Removal Stages.**

While considerable amounts of insoluble crystalline dextran can be formed during the fractionation process, the two stages of manufacture which follow fractionation serve to eliminate a large proportion of the insoluble material. The crystalline dextran will be melted by the action of heat during the process of ethanol removal by distillation, and also during subsequent evaporation processes, in which the dextran concentration is increased to a level at which the drying process may be commenced. The investigations of melting in crystalline dextran, which were undertaken in this work, indicate that a suitable period of time during which the solvent concentration is maintained above a certain critical level, (see fig.37) will be necessary for melting to take place. Melting is preferentially carried out in the absence of ethanol. Removal of crystalline material at this stage will also be accelerated by extended boiling of a dilute aqueous dextran solution containing the suspended crystalline particles.

Since no further conditions conducive to the dispersion of cry-
When crystalline dextran exist in the manufacturing process, it is evident that crystalline material which resists melting under these conditions will be present in the final product. The inherent content of stable crystalline material in the dextrans studied in this work is thus controlled by the conditions of crystallization existing during the fractionation process.

(e) Drying Stage.

It has already been mentioned (section 1.0.4) that in order to isolate dextran from solution in the supercooled state, the solution must pass through concentration and temperature conditions under which crystallization can take place. At low temperature, crystallization rate would be sufficiently low that provided the residence time of the solution under conditions favourable for crystallization, was short, no further crystalline material would be formed. In support of this contention, it is observed that the crystalline particle content of dextran is not increased by freeze drying concentrated dextran solutions. However, if drying is conducted at higher temperatures, rate of crystallization will be appreciable as the solution passes through high concentration states. If the residence time of the solution in these high concentration high temperature states is long, additional crystalline material will be introduced into the final product. However, it is unlikely that crystalline material formed under these conditions will resist melting (e.g. during autoclaving), unless extremely long drying times are involved.
If the solution being dried already contains seed crystalline material, the rate of crystallization during drying will be enhanced. Dextran solutions containing a definite level of crystalline material after the evaporation stage will therefore be likely to develop further amounts on drying. However, the nature of the crystalline material produced at these two stages is likely to be different; only crystalline material developed during fractionation will be resistant to melting at 100 °C. in dilute dextran solution. Two different types of crystalline material, one type being readily dispersed under these melting conditions, the other being stable, have in fact been observed in dextrans with high contents of crystalline material (section I.E.2). The existence of these different types of crystalline material can now be explained in terms of their formation under different conditions, at two separate stages in the dextran manufacturing process.

(f) Storage of Solutions.

During storage of dilute dextran solutions (10% w/v) for clinical use as plasma extenders, the formation of insoluble particles ("flakes") has been occasionally observed under particular conditions (1,2). Although such particles are known to consist of crystalline dextran, the mechanism of formation has hitherto been unknown. The findings of this work indicate that while crystallization is the process responsible for their formation, this process does not occur at measurable rates in the bulk solution. Flake formation is believed to arise primarily as a result of evaporation effects. Temperature fluctuations during storage
result in evaporation of solvent from the surface of the storage vessel, followed by condensation onto those walls of the vessel which are not in contact with liquid. Solvent may also evaporate from droplets of solution adhering to the walls of the vessel above the liquid surface. A skin of concentrated dextran solution therefore forms on top of the dilute solution, or on the surface of the droplets, and conditions within this skin are favourable for crystallization. Such surface skins eventually solidify and break up on agitation to give crystalline dextran, or turbidity. Non-fluctuating temperature conditions are therefore important for the long term storage of dextran solutions if the formation of flakes or turbidity is to be avoided. It also follows that the formation of surface skins of open vessels should be avoided during manufacture of dextrans, to eliminate the possibility of further crystalline material being introduced into the product.

During the preparation of linear carbohydrates, conditions existing at stages where non-solvent precipitation is involved, have already been shown to be important in controlling the content of crystalline material in the final product (4,36). Experiences reported during the preparation of amyllose, (81) and verified for dextran in this study, confirm that the drying time during final isolation of the product from solution, will determine the amount of crystalline material which develops.

Furthermore, with amyllose, it is reported that rate of retrogradation increases with drying time (85). The reason for this is now apparent. From investigations on the crystallization
behaviour of dextran, it is evident that prolonged drying would result in the production of crystalline nuclei capable of enhancing the rate of subsequent crystallization processes. The longer the drying time, the more nuclei would be produced, and the higher the rate of crystallization (retrogradation) would become when the dried amylose product was redissolved in aqueous solution.

5. CONCLUSIONS.

During manufacture or laboratory preparation of dextrans, several stages exist during which conditions are favourable towards the formation or dispersal of crystalline material. The principal stage during which heat-stable crystalline particles are formed is the fractional precipitation process. Only heat-stable crystalline species developed at this stage will resist melting during subsequent distillation and evaporation processes, and eventually appear in the final product. Additional crystalline material may also be introduced during drying operations, and thence appear in the final product. However, this material will not resist subsequent melting in dilute dextran solution at 121 °C during autoclaving procedures.

A proportion of the crystalline dextran particles responsible for so-called "inherent" turbidity in dextran solutions, are heat-stable when suspended in dilute dextran solution at 100 °C. These particles are produced at the fractionation stage of the manufacturing process. The remainder of the crystalline particles, which can be dispersed by the action of heat, are formed during the drying stage.
The development of crystalline material ("flakes") in dilute dextran solutions under particular storage conditions is due to acceleration of crystallization rate in surface skins containing dextran at a higher concentration than in the bulk solution. Skin formation is caused by surface evaporation effects, which are accelerated by uneven temperature conditions during storage.
GENERAL CONCLUSIONS.

Crystallization is responsible for the formation of insoluble dextran which causes turbidity in aqueous solutions of the polymer. Dextrans normally contain small amounts of crystalline material, since conditions favourable to crystallization exist during their manufacture.

Dextran can be readily crystallized from concentrated aqueous solution. The crystalline particles formed are spherical in shape, and the rate of growth appears to be diffusion controlled over part of the crystallization period. The nucleation density of the dextran, as measured by initial turbidity, determines whether the process nucleating growth is predominantly homogeneous or predominantly heterogeneous. Nucleation density can be increased by deliberate addition or formation of nuclei, and can be decreased by melting of nuclei through the action of heat or of hydrogen-bond breaking chemicals.

The rate of dextran crystallization from aqueous solution is affected by the following parameters:

1. Nucleation density: rate increases with increasing nucleation density.
2. Molecular weight: rate decreases with increasing weight average molecular weight, being relatively constant above $M_w = 40,000$.
3. Molecular weight distribution: rate decreases as the molecular weight distribution broadens.
(4) Temperature: rate attains a maximum at a particular temperature which depends on the amount of solvent present, and decreases at the temperature rises, tending towards the melting point, or falls, tending towards the glass point.

(5) Solvent concentration: for the same degree of supercooling, rate increases with decreasing solvent concentration.

(6) Solvent Composition: rate increases as the non-solvent content in a solvent-non-solvent mixture increases.

The behaviour of dextran under these conditions is very similar to that of amylose, the linear polysaccharide which is responsible for the process of retrogradation in starch, and also to that of synthetic polymers, generally.

Crystalline dextran particles exhibit a melting range, indicating species with different stabilities to be present. The particles which resist melting in dilute dextran solution at 100 °C. are stable nuclei and their growth products. The rate of development of heat-stable crystalline material is much lower than the overall rate of crystallization, but increases with increasing nucleation density, temperature and dextran concentration in a similar manner to the overall rate. The melting range of crystalline dextran decreases as the total solvent concentration increases, and increases as the non-solvent: good solvent ratio increases. The melting behaviour of crystalline dextran is thus very similar to that found generally in polymer systems.

Although crystalline material may be introduced into dextrans at fractional precipitation and drying stages of the manufacturing
process, only a small proportion of the material produced during the fractionation stage will resist melting in dilute dextran solution at 100 °C. By careful control of conditions at the precipitation and drying stages, it should be possible to minimise the amount of crystalline material developed in dextran.

Turbidity development and flake formation in dilute dextran solutions during storage is due to crystallization in surface skins containing dextran at high concentration. These skins are formed as a result of surface evaporation effects which are promoted by uneven storage temperatures.
EXPERIMENTAL SECTION.

1. DETERMINATION OF THE SPECIFIC ROTATION OF DEXTRAN.

Dextran (samples 7, 12, 17, and 18, approx. 2.0 g.) were dried over phosphorus pentoxide in a vacuum desiccator (0.5 mm. Hg). Drying periods of 2 - 4 days at 22 °C. were required to achieve a constant weight. An accurately weighed sample of each dextran (approx. 2.0 g.) was shaken mechanically with distilled water (60 ml.) for 30 minutes; the solutions were then made up to 100 ml. with water in a volumetric flask. After standing for 24 hours, portions of each solution (15 ml.) were transferred to a 2 dm. polarimeter tube. The optical rotation of the solutions was determined using a Hilger polarimeter, which was located in a room thermostatted to 20 °C ± 1 °C. Light of wavelength 589.3 nm, emitted by a sodium vapour lamp, i.e. the yellow sodium-D spectral line, was used for the measurements.

For each dextran, the specific rotation at 20 °C., for light of wavelength 589.3 nm, \([\alpha]_{D}^{20^\circ}\), was calculated from the equation,

\[
[\alpha]_{D}^{20^\circ} = \frac{100}{c} \alpha \quad \text{--------- (49)}
\]

\(\alpha\) is the measured optical rotation (degrees), c is the solute concentration (g./dl.) and l is the cell path length (dm.)

The mean value determined for the specific rotation of the four dextrans was + 198.5° ± 0.3°. This is in good agreement with literature values (127), relating to dextrans of the same type as those studied in this work.
2. DETERMINATION OF DEXTRAN MOISTURE CONTENT.

The dextran polymers were received as free-flowing powders containing 5 - 10% moisture. The exact moisture content was determined to enable solutions of specific concentration to be prepared. Accurately weighed amounts of each dextran (5.5 g.) were shaken mechanically with water (35 ml.) for 30 minutes; the solutions were then made up with water to 50 ml. in volumetric flasks. In each case, the dextran content of the solution was determined by polarimetry (2 dm. cell, [\alpha]_D^{20°} = +198.5°). The moisture content of the dextrans was calculated from the known amounts of dextran present in the solution, relative to the weight of powder dissolved. Results are given in table 36.
TABLE 36: Moisture Contents and Inherent Turbidities (10% w/v Solution) of Dextrans.

<table>
<thead>
<tr>
<th>Dextran Sample No.</th>
<th>Moisture Content (%)</th>
<th>Turbidity of 10% w/v Solution ($N_{100}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.1</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>5.6</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>4.2</td>
<td>200</td>
</tr>
<tr>
<td>4</td>
<td>6.4</td>
<td>210</td>
</tr>
<tr>
<td>5</td>
<td>5.5</td>
<td>140</td>
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<tr>
<td>6</td>
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<td>26</td>
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<tr>
<td>7</td>
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<td>8</td>
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<td>96</td>
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<td>9</td>
<td>3.8</td>
<td>93</td>
</tr>
<tr>
<td>10</td>
<td>6.2</td>
<td>36</td>
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<tr>
<td>11</td>
<td>3.3</td>
<td>44</td>
</tr>
<tr>
<td>12</td>
<td>9.8</td>
<td>32</td>
</tr>
<tr>
<td>13</td>
<td>12.2</td>
<td>39</td>
</tr>
<tr>
<td>14</td>
<td>11.8</td>
<td>40</td>
</tr>
<tr>
<td>15</td>
<td>8.9</td>
<td>29</td>
</tr>
<tr>
<td>16</td>
<td>5.8</td>
<td>31</td>
</tr>
<tr>
<td>17</td>
<td>9.7</td>
<td>30</td>
</tr>
<tr>
<td>18</td>
<td>3.6</td>
<td>22</td>
</tr>
<tr>
<td>19</td>
<td>7.7</td>
<td>40</td>
</tr>
<tr>
<td>20</td>
<td>8.9</td>
<td>43</td>
</tr>
<tr>
<td>21</td>
<td>9.7</td>
<td>44</td>
</tr>
<tr>
<td>22</td>
<td>4.0</td>
<td>25</td>
</tr>
<tr>
<td>23</td>
<td>9.4</td>
<td>300</td>
</tr>
</tbody>
</table>
3. LIGHT SCATTERING INVESTIGATIONS IN DEXTRAN SOLUTIONS.

(a) SOFICA Light Scattering Photometer Studies.

(i) Description of Instrument.

The light source in the SOFICA instrument is a water cooled, high pressure, mercury vapour lamp (500 watts). The wavelength of the light is selected by one of two interchangable filters. Maximum transmission of either blue (\( \lambda = 436 \text{ nm} \)) or green (\( \lambda = 546 \text{ nm} \)) lines in the mercury spectrum can be obtained. The light then passes through a polariser assembly, allowing resolution into horizontally or vertically polarised components, as required. The intensity of the nearly parallel beam, of cross-section 2 mm. x 10 mm., is adjusted by passage through a deformable parallelogram slit. Finally the incident light passes through a vat of benzene, before reaching the glass sample cell located in the centre of the vat. The benzene was replaced periodically by filtering fresh solvent (B.D.H. Reagent for Light Scattering Studies) through a Millipore filter (0.45 \( \mu \text{m} \) pore diameter) directly into the vat.

The scattered light intensity can be measured by rotating a photomultiplier tube (type RCA IP 28) to give well defined angles to the transmitted beam in the range 30° - 150°. The signal received by a reference photomultiplier, located at 90° to the incident beam, automatically adjusts the high voltage supply to the measuring photomultiplier to compensate for variations in the intensity of the incident light. The intensity of scattered light is measured in arbitrary units on a galvanometer scale; to convert
to absolute terms, the instrument must be calibrated by the procedures described below, using a medium of known scattering power.

(ii) Scattering Cell Preparation.
The light scattering cells (no. 40051) consist of glass cylinders, diameter 28.5 mm, minimum capacity 15 ml., which are mounted in stainless steel holders fitted with lids. Before use, the cells were immersed in chromic acid for 24 hours, and rinsed in distilled water which had been filtered through a Millipore membrane (0.45 μm mean pore diameter). Without allowing the cells to dry, they were mounted, in their holders, inside a filter flask. A stream of acetone was introduced by filtering under vacuum through a Flotronics membrane (0.2 μm mean pore diameter). The filtered acetone was allowed to fall into the cells, and overflow for a 5 minute period, thereby removing the last traces of dust from the walls. The lids were carefully placed on the cells, which were then inverted to allow the bulk of the acetone to escape from the space between the glass and the holder. Finally, the last traces of acetone were removed from the cells by oven drying at 50 °C.

(iii) Alignment Check.
A solution of fluorescein (10 mg./l.) was filtered through a Millipore membrane (0.22 μm mean pore diameter) directly into a clean light scattering cell. Placing a blue filter (max. trans. λ = 436 nm) in the path of the unpolarised incident beam, and a green filter (max. trans. λ = 546 nm) in the path of the scattered beam, the angular dependence of scatter was measured in arbitrary units, over the range 30° to 150°. The results (table 2) were used to
show that the geometry of the optical system was correct, and that the light beam passed through the centre of the cell.

(iv) Calibration.

Colloidal Silica Dispersions: Colloidal silica, (Syton 2X, Monsanto Chemicals Ltd.) was supplied as a concentrated aqueous dispersion (30%). To stabilise the colloid on dilution, sodium chloride was added to give a constant concentration (0.05 M) in each of the dilute silica dispersions.

Silica dispersions (50 ml.) were prepared, with concentrations in the range 0.5 - 3% w/w, each being filtered directly into the light scattering cell through a Millipore filter (0.45 μm mean pore diameter). In each case, the incident light intensity at wavelengths of 436 nm and 546 nm, was adjusted using the variable slit, to give a galvanometer reading of 0.1 with the glass standard in place. The intensity of scatter at 90°, 45°, and 135° was then measured under the same conditions, for each dispersion and at both wavelengths. The results of these measurements are shown in table 37.

Using a pipette, the dispersions were transferred from the light scattering cell into a silica spectrophotometer cell (path length 4 cm.). Absorbances were measured over the wavelength range 400 - 600 nm in a Hilger Uvispek instrument, using a sodium chloride solution (0.05 M) as reference. Results are shown in table 38 and fig.6.
<table>
<thead>
<tr>
<th>Silica Concentration (% w/w)</th>
<th>Wavelength of Incident Light (nm)</th>
<th>Measured Scattered Intensity* (i 90)</th>
<th>Turbidity, T (cm⁻¹)</th>
<th>Log₁₀ (T/1₉₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>436</td>
<td>41.2</td>
<td>0.058</td>
<td>-2.850</td>
</tr>
<tr>
<td>0.50</td>
<td>546</td>
<td>52.2</td>
<td>0.024</td>
<td>-3.337</td>
</tr>
<tr>
<td>0.75</td>
<td>436</td>
<td>60.4</td>
<td>0.036</td>
<td>-2.841</td>
</tr>
<tr>
<td>0.75</td>
<td>546</td>
<td>74.8</td>
<td>0.036</td>
<td>-3.318</td>
</tr>
<tr>
<td>1.0</td>
<td>436</td>
<td>77.5</td>
<td>0.116</td>
<td>-2.815</td>
</tr>
<tr>
<td>1.0</td>
<td>546</td>
<td>97.2</td>
<td>0.048</td>
<td>-3.306</td>
</tr>
<tr>
<td>2.0</td>
<td>436</td>
<td>121</td>
<td>0.215</td>
<td>-2.750</td>
</tr>
<tr>
<td>2.0</td>
<td>546</td>
<td>164</td>
<td>0.092</td>
<td>-3.250</td>
</tr>
<tr>
<td>3.0</td>
<td>436</td>
<td>141</td>
<td>0.276</td>
<td>-2.708</td>
</tr>
<tr>
<td>3.0</td>
<td>546</td>
<td>194</td>
<td>0.120</td>
<td>-3.208</td>
</tr>
</tbody>
</table>

* Galvanometer reading - glass standard settings equivalent to those in table 4.

# From spectrophotometric measurements.
TABLE 38: Spectrophotometric Turbidity Measurements on Silica Suspensions.

<table>
<thead>
<tr>
<th>Incident Light Wavelength (nm)</th>
<th>0.5% Silica</th>
<th>0.75% Silica</th>
<th>1.0% Silica</th>
<th>2.0% Silica</th>
<th>3.0% Silica</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( A )</td>
<td>( \gamma ) (cm(^{-1}))</td>
<td>( A )</td>
<td>( \gamma ) (cm(^{-1}))</td>
<td>( A )</td>
</tr>
<tr>
<td>400</td>
<td>0.144</td>
<td>0.083</td>
<td>0.214</td>
<td>0.123</td>
<td>0.290</td>
</tr>
<tr>
<td>425</td>
<td>0.113</td>
<td>0.065</td>
<td>0.170</td>
<td>0.098</td>
<td>0.226</td>
</tr>
<tr>
<td>450</td>
<td>0.089</td>
<td>0.051</td>
<td>0.134</td>
<td>0.077</td>
<td>0.179</td>
</tr>
<tr>
<td>475</td>
<td>0.072</td>
<td>0.042</td>
<td>0.108</td>
<td>0.062</td>
<td>0.143</td>
</tr>
<tr>
<td>500</td>
<td>0.060</td>
<td>0.035</td>
<td>0.088</td>
<td>0.051</td>
<td>0.118</td>
</tr>
<tr>
<td>525</td>
<td>0.049</td>
<td>0.028</td>
<td>0.072</td>
<td>0.041</td>
<td>0.099</td>
</tr>
<tr>
<td>550</td>
<td>0.039</td>
<td>0.022</td>
<td>0.061</td>
<td>0.035</td>
<td>0.082</td>
</tr>
<tr>
<td>575</td>
<td>0.034</td>
<td>0.020</td>
<td>0.052</td>
<td>0.030</td>
<td>0.069</td>
</tr>
<tr>
<td>600</td>
<td>0.028</td>
<td>0.016</td>
<td>0.044</td>
<td>0.025</td>
<td>0.057</td>
</tr>
</tbody>
</table>

\( A \) = Absorbance (4 cm. cell)

\( \gamma = 2.303 \frac{A}{d} = 0.576A \)
Benzene: Benzene (B.D.H. Reagent for Molecular Weight Determinations) was filtered into the light scattering cell through a Millipore membrane, (0.45 μm mean pore diameter) which had been conditioned by prior soaking in benzene for 24 hours. Using the variable slit control, the intensity of scatter from benzene at 90° was adjusted to give a galvanometer reading of 0.50, with incident light at both wavelengths. The measuring cell was replaced by the glass standard, when readings of 0.56 and 0.46 were obtained for incident wavelengths of 436 nm and 546 nm respectively.

(v) Preparation of Solutions.
Stock solutions of dextran were prepared for molecular weight determination, by dissolving the polymer (approx. 2g.) in distilled water (100 ml.), and allowing the solution to stand for 24 hours before use. To permit extrapolation to zero concentration, a minimum of six dilute solutions were prepared from each stock solution, to give dextran concentrations in the range 0.002 - 0.02 g./ml.

For molecular weight determination, clarification of dextran solutions prior to carrying out light scattering measurements, was effected using a Millipore filter adaptor fitted to a syringe (capacity 20 ml.). While in the preliminary investigations, membranes of mean pore diameter 0.10, 0.22, and 0.45 μm were employed, the 0.10 μm mean pore diameter membrane was used in subsequent routine investigations. A quantity of water (50 ml.) was passed through the filters before use; membranes were changed daily. The solutions were filtered directly into the clean cells by means of a small hole drilled in the metal lid. Prior to filtration, each solution
was autoclaved in a pressure cooker, at a pressure of 1 bar (121 °C.), for a 30 minute period.

The concentrations of all solutions were determined by transferring the solutions from the light scattering cells to a polarimeter tube (2 dm.). Dextran concentrations were calculated from the rotation measured by means of a Hilger polarimeter, using a specific rotation value of +198° (eqn.49).

During crystallization studies, solutions were filtered using a Millipore pressure-filter device, pressurised to 2 bar with nitrogen gas. Membrane discs (4.7 cm. diameter), with mean pore diameter 0.8 μm were employed unless otherwise stated, and a new membrane was used for each solution.

Filtrations were normally carried out at a dextran concentration of 10% w/v. In the instances in which more concentrated dextran solutions were filtered (40% w/w and 60% w/w), the membrane permeates were collected in the light scattering cell, and degassed by placing in a dessicator and applying vacuum (20 inches Hg.).

(vi) Light Scattering Measurements.
For molecular weight determinations, with the glass standard in the light beam, the intensity of the unpolarised incident light was adjusted to give a galvanometer reading corresponding to scatter at 90°, of 0.56 with blue light (λ = 436 nm), or 0.46 with green light (λ = 546 nm). The polymer solution was placed in the light beam, and the galvanometer readings corresponding to scattered intensity at
90°, 45°, and 145° were measured. The 90° reading, on which turbidity is based, was then re-checked against the standard, to ensure that no drifting in the setting has occurred.

In crystallization and melting studies, with the glass standard in position, the intensity of the unpolarised incident light was adjusted to give a galvanometer reading corresponding to scatter at 90°, of 0.02 with blue light (\(\lambda = 436\) nm). The polymer solution was placed in the light beam, and the galvanometer readings corresponding to scattered intensity at 90°, 45°, and 135°, were measured. In certain instances, the depolarisation ratio was measured by observing the 90° scattered intensity when the incident light was polarised both horizontally and vertically.

(b) **Nephelometer Studies.**

In the nephelometer (Evans Electroselenium Ltd.), light scattered from a beam which passes vertically through the base of a test-tube (\(\frac{5}{8}\) inch diameter), is collected by a reflector surface surrounding the tube. It is then directed back on to the surface of an annular photocell mounted round the test-tube. The reflector is shaped in such a way that light reflected from the meniscus of the liquid is excluded. Readings of turbidity, relative to a scratched perspex rod standard, are presented on the linear scale of an external galvanometer (EEL Unigalvo Type 20).

The galvanometer was set to zero when the nephelometer photocell was inactivated. With the standard in position, the sensitivity control was then adjusted to give a full-scale reading of 100.
Dextran solution (15 ml.) was placed in one of a series of matched test-tubes. This was then located in the nephelometer, and the turbidity observed on the galvanometer scale. The instrument calibration was then re-checked using the perspex rod standard.

In cases where the dextran solutions were extremely turbid, the sensitivity control was reduced such that the perspex standard gave a galvanometer reading of 50, 25, 10, or 5. The turbidity reading for dextran solutions on the new scale was multiplied by the appropriate factor. This allowed a turbidity scale extending up to 2000 to be employed, in which all values were related to the original standard.

The validity of the method was checked using a range of silica dispersions (Syton 2X, Monsanto Chemicals Ltd.), similar to those employed for calibration in the light scattering photometer. Dispersions with concentrations in the range 0.02 - 3.0% w/w, were employed, in which nephelometric turbidities both smaller and greater than 100, as determined by the above procedure, were observed. The absorbance corresponding to these turbidities was determined in a Hilger Uvispek Spectrophotometer (cells with path length 4 cm.) at a wavelength of 400 nm. Results are shown in fig.13, indicating approximate linearity in the absolute turbidity-nephelometer reading plot.

(c) Differential Refractometry.

The refractive index increment of dextran in aqueous solution was determined using a differential refractometer of the type
manufactured by Polymer Consultants Ltd. In this instrument, light from a mercury vapour lamp (125 watts) passes successively through a condenser, filter, divided glass cell, and an aperture, thence into a telescope fitted with a calibrated eye-piece. The eye-piece, in which a vertical image of the slit is observed, has a horizontal scale at right angles to the light beam.

Water was placed on both sides of the divided cell, and after allowing 15 minutes to attain thermal equilibrium, the position of the slit image was measured on the scale using the telescope to focus the image, and a calibrated drum on the eye-piece to move the cross-wires. Twelve readings were taken with light of wavelengths 436 nm and 546 nm, incident on each side of the divided cell; the highest and lowest readings were discarded, and the remaining ten averaged to give mean values for left-side and right-side reading. For the particular cell setting, the blank was calculated as the difference between the mean left-side and right-side readings, \( \Delta d_{OR} \) and \( \Delta d_{UL} \),

\[
\Delta d_{d_0} = d_{OR} - d_{UL} \quad \text{(50)}
\]

The refractometer was calibrated with aqueous solutions of sucrose, the solution being placed on the right-hand side of the cell, and water on the left-hand side. The shift, \( \Delta d_S \) of the slit image was determined as described above, \( d_{SR} \) and \( d_{SL} \) being right and left-hand readings,

\[
\Delta d_S = d_{SR} - d_{SL} \quad \text{(51)}
\]

The total shift of the image for the solution, relative to that for water, is given by,

\[
\Delta d = \Delta d_S - \Delta d_0 = (d_{SR} - d_{SL}) - (d_{OR} - d_{UL}) \quad \text{(52)}
\]
The difference in refractive index between solution and solvent, \( dn \), is proportional to the shift, \( \Delta d \) (eqn.31). Using both blue and green light (\( \lambda = 436 \text{ nm} \) and \( \lambda = 546 \text{ nm} \)), the value of \( \Delta d \) was determined using three accurately prepared sucrose solutions. The average value of the calibration constant, \( K \), was then calculated at each sucrose concentration, using literature values for the sucrose refractive index increment, \( (dn/dc) \). Results are shown in table 39.

Solutions of dextran (sample 12) were examined in the same way. A solution was placed in the right-hand side of the cell and water in the left-hand compartment, and \( \Delta d \) was determined. Dextran concentrations were determined by polarimetry (2dm. cell), using a value of +198° for the specific rotation of dextran. Values of \( \Delta d \) were measured for three dextran solutions at each wavelength, and the average value of \( (dn/dc) \) was calculated from the known calibration constants and dextran concentrations. Details of the results are shown in table 40.
TABLE 39: Differential Refractometer Calibration Data.

<table>
<thead>
<tr>
<th>Wavelength of Incident Light (nm)</th>
<th>Sucrose Concentration (% w/v)</th>
<th>Shift of Slit Image (Δd)</th>
<th>Calculated Refractive Index Difference dn x 10^3</th>
<th>Calibration Constant, K x 10^4</th>
</tr>
</thead>
<tbody>
<tr>
<td>436</td>
<td>3.046</td>
<td>4.916</td>
<td>4.410</td>
<td>8.98</td>
</tr>
<tr>
<td>436</td>
<td>2.000</td>
<td>3.221</td>
<td>2.897</td>
<td>8.99</td>
</tr>
<tr>
<td>436</td>
<td>1.004</td>
<td>1.582</td>
<td>1.455</td>
<td>9.20</td>
</tr>
<tr>
<td>546</td>
<td>3.046</td>
<td>4.852</td>
<td>4.355</td>
<td>8.96</td>
</tr>
<tr>
<td>546</td>
<td>2.000</td>
<td>3.161</td>
<td>2.860</td>
<td>9.05</td>
</tr>
<tr>
<td>546</td>
<td>1.004</td>
<td>1.554</td>
<td>1.435</td>
<td>9.24</td>
</tr>
</tbody>
</table>

* Calculated from literature values for the refractive index increment of sucrose.
TABLE 40: Dextran Refractive Index Data.

<table>
<thead>
<tr>
<th>Wavelength of Incident Light (nm)</th>
<th>Dextran Concentration (g.dl.(^{-1}))</th>
<th>Shift of Slit Image (Δd)</th>
<th>Refractive Index Difference (dn x 10(^3))</th>
<th>Refractive Index Increment (dn/dc) (dl.g.(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>436</td>
<td>1.621</td>
<td>2.743</td>
<td>2.484</td>
<td>0.153</td>
</tr>
<tr>
<td>436</td>
<td>0.999</td>
<td>1.658</td>
<td>1.502</td>
<td>0.150</td>
</tr>
<tr>
<td>436</td>
<td>0.485</td>
<td>0.812</td>
<td>0.736</td>
<td>0.151</td>
</tr>
<tr>
<td>546</td>
<td>1.621</td>
<td>2.690</td>
<td>2.445</td>
<td>0.151</td>
</tr>
<tr>
<td>546</td>
<td>0.999</td>
<td>1.632</td>
<td>1.483</td>
<td>0.149</td>
</tr>
<tr>
<td>546</td>
<td>0.485</td>
<td>0.795</td>
<td>0.723</td>
<td>0.149</td>
</tr>
</tbody>
</table>

4. DEXTRAN VISCOSITY MEASUREMENTS.

(a) Intrinsic Viscosity Determination.

Viscosity measurements were conducted using a modified, number 3, suspended level viscometer, reference no. BS/IP/SL(S)/3, as supplied by Messrs. Poulton, Selfe and Lee. Details of this viscometer were as follows:-

- Capillary diameter = 0.6 mm.
- Length of capillary = 80 mm.
- Distance between measuring points = 32 mm. above and below bulb
- Volume of bulb = 5.6 ml.
- Volume of reservoir = 25 ml.

The viscometer was mounted vertically in a water bath thermostatted to 25.00 ± 0.01 °C. At this temperature, the flow time for water was 111.1 secs.
Before use, the viscometer was cleaned for 24 hours in chromic acid solution; the acid was then drained out, and the viscometer rinsed with distilled water which had been filtered through a Millipore membrane of mean pore diameter 0.22 µm. Finally, the viscometer was rinsed with acetone, which had been filtered to the same standards (Flotronics membrane, 0.2 µm). After drying the viscometer in an oven at 60 °C., final traces of acetone were removed by blowing through the tubes with filtered air.

To measure intrinsic viscosities, solutions were prepared by dissolving dextran (approx. 0.45 g.) in distilled water (25 ml.). They were allowed to stand for 24 hours before use. Using a syringe fitted with a filter holder, solution (approx. 10 ml.) was filtered through a Millipore membrane (mean pore diameter 0.45 µm), directly into the viscometer. Distilled water (approx. 30 ml.) was passed through each of the membrane filters before use. When thermal equilibrium had been attained (15 minutes), solution was sucked into the upper bulb of the viscometer using a pipette filter, and the flow time between the two marks measured. In each case, six reading of flow time, constant to ± 0.05 second, were obtained.

In order to enable accurate extrapolation to zero concentration, flow times were determined for a minimum of five further solutions. These were prepared from the stock solution, with dilution factors of up to 10-fold, giving dextran concentrations in the range 2.0 - 0.2 g./dl. Dilution was carried out either by filtering additional quantities of distilled water directly into the viscometer, and mixing, or by replacing all or part of the solution in the viscometer.
by a filtered solution of lower concentration. Finally, the water flow time was measured, enabling relative and specific viscosities to be calculated at each of the dextran concentrations.

The concentration of all solutions was measured by transferring the solutions from the viscometer to a polarimeter tube (2dm.). The dextran concentration was determined from the observed rotation by assuming a value of +198° for the specific rotation. The viscosity number and inherent viscosity was calculated at each concentration from the values of concentration and specific viscosity. A typical plot illustrating the method of extrapolating viscosity numbers and inherent viscosities to zero concentration, is shown in fig.10. When viscosity was determined from single flow times, the dextran concentration employed was 0.5 g./dl. The measured inherent viscosity (eqn.36) at this concentration was approximated to the intrinsic viscosity of the dextran.

(b) Shear Dependence of Viscosity.

The viscosity of solutions of dextrans with concentrations up to 30 g./dl. was examined in a Ferranti rotating coaxial cylinder viscometer, at shear rates in the range 50 - 500 sec⁻¹. The solutions (250 ml.) were placed in beakers which were situated in a water bath thermostatted to (25.0 ± 0.1)°C. The coaxial cylinders of the viscometer were immersed in the solution, and shear rate varied by altering the size of the cylinder and the speed of rotation.
5. **END GROUP ANALYSIS OF DEXTRANS.**

(a) **Reagents.**

All the reagents employed were supplied by B.D.H., and Analar grade was used wherever possible. The following stock solutions were prepared:

(i) Aqueous sodium metaperiodate (0.5 M)

(ii) Aqueous sodium metabisulphite (10% w/v)

(iii) Chromotropic acid, sodium salt, (500 mg.) was dissolved in water (50 ml.), and the solution made up to 250 ml. with sulphuric acid (12.5M).

(iv) Saturated aqueous solution of thiourea was diluted with an equal volume of water.

(b) **Procedure.**

Dextrans (1 - 8 g.) were dissolved in water, and the solutions made up to 100 ml. The exact dextran concentration was determined by polarimetry (2dm.cell), assuming a value of +198° for the specific rotation of dextran.

Sodium borohydride (61 mg., 1.61m.mole) in water (2 ml.) was added to the above dextran solutions (3 ml.), and after 70 hours reduction at 20 °C., the excess of borohydride was destroyed with a small excess of glacial acetic acid (0.12 ml., 2.1m.mole). The calculated amount of acetic acid is 0.092 ml., according to the equation,

\[ 2\text{NaBH}_4 + 2\text{HOAc} = 2\text{NaOAc} + \text{B}_2\text{H}_6 + 2\text{H}_2 \]
The reduced solution (pH 5.5) was oxidised with 0.5M sodium metaperiodate solution (5 ml., 2.5 m.mole) at 20 °C. for 25 hours in the dark. An aliquot (1 ml.) of this solution was then added to sodium metabisulphite solution (2 ml.) and water (2 ml.) in a Quickfit MF 24/3 test tube. A closed length of 24/32" Visking dialysis tubing, containing water (5 ml.) was immersed in the solution, and the system allowed to equilibrate in the stoppered test-tube for 20 hours, with occasional shaking.

The dialysis tubing was removed from the test-tube, and aliquots (1 ml.) of the dialysate were added to chromotropic acid reagent (10 ml.) in test-tubes covered with watch glasses. The tubes were immersed in a boiling water bath for 30 minutes, cooled rapidly to 20 °C., thiourea solution (1 ml.) added, and the solutions thoroughly mixed. A red colour developed, and the absorbances of the solutions were measured at 570 nm using a Hilger Uvispek spectrophotometer. Measurements were carried out in 1 cm. cells, using water as reference liquid. The formaldehyde content of the analysed solution is then equivalent to 1/100 of the original dextran present, 1 mole of dextran yielding 1 mole formaldehyde according to the scheme shown in fig.12.

Control solutions containing dextran were prepared by treating sodium borohydride (61 mg.) in water (2 ml.) with glacial acetic acid (0.12 ml.), and adding the above dextran solutions (3 ml.). The control solutions were then oxidised with periodate, reduced with bisulphite, dialysed, and the colours developed simultaneously with the reduced polysaccharide.
Formaldehyde concentrations were determined from the plot of absorbance versus formaldehyde concentration, which was obtained by oxidising mannitol under the same conditions, as follows. Standard mannitol solutions (5 ml.) containing 1.5 - 15 mole, were oxidised with sodium periodate solution (0.5 N, 5 ml., 2.5 m.mole) at 20 °C. for 4 hours in the dark. Aliquots of each solution (1 ml.) were added to sodium metabisulphite (2 ml.) and water (7 ml.). Aliquots of the resulting solution (1 ml.) were treated with chromotropic acid reagent (10 ml.) and assayed as described above. The formaldehyde content of the analysed solution is thus equivalent to 1/50 of the original mannitol, 1 mole mannitol yielding 2 moles formaldehyde on oxidation.

The plot of absorbance against formaldehyde concentration was linear over the range of concentrations studied (fig.40). Typical analytical results are indicated in table 41.
FIG. 40: COLORIMETRIC DETERMINATION OF FORMALDEHYDE WITH CHROMOTROPIC ACID REAGENT
### TABLE 41: Typical End-Group Analytical Data on Dextrans.

<table>
<thead>
<tr>
<th>Dextran Sample No.</th>
<th>12</th>
<th>20*</th>
<th>20*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran Concentration (% w/v)</td>
<td>5.226</td>
<td>7.768</td>
<td>7.768</td>
</tr>
<tr>
<td>Absorbance of Reduced Dextran Solution**</td>
<td>0.085</td>
<td>0.162</td>
<td>0.174</td>
</tr>
<tr>
<td></td>
<td>0.084</td>
<td>0.150</td>
<td>0.182</td>
</tr>
<tr>
<td>Absorbance of Non-Reduced Dextran Solution</td>
<td>0.014</td>
<td>0.087</td>
<td>0.112</td>
</tr>
<tr>
<td>Corrected Mean Absorbance</td>
<td>0.070</td>
<td>0.069</td>
<td>0.066</td>
</tr>
<tr>
<td>Formaldehyde Content of Solution (μ mole)</td>
<td>0.055</td>
<td>0.052</td>
<td>0.050</td>
</tr>
<tr>
<td>Weight of Dextran Equivalent to Liberated Formaldehyde (μg.)</td>
<td>1570</td>
<td>2330</td>
<td>2330</td>
</tr>
<tr>
<td>Number-Average Molecular Weight, Mn</td>
<td>28,000</td>
<td>45,000</td>
<td>47,000</td>
</tr>
</tbody>
</table>

* Two determinations on same dextran, with different batches of chromotropic acid reagent.

** Two separate samples reduced with sodium borohydride, and carried through the analytical procedure.

Absorbance Measurements: 570 nm, 1 cm. cells, reference water.
6. FILTRATION STUDIES OF DEXTRAN SOLUTIONS.

Static filtration studies were carried out in a Millipore pressure filter holder (volume 100 ml.), which was pressurised to 2 bar during filtration, using nitrogen gas. A range of cellulose ester Millipore filters, diameter 4.7 cm, with mean pore diameter of 0.05, 0.1, 0.22, 0.45 and 0.80 μm were utilised. The 0.05 and 0.10 μm membranes were asymmetric, and required that the active side containing the skin-layer, be placed in contact with the upstream solution. Turbid dextran solution (approx. 100 ml.) was introduced to the metal holder, pressure was applied, and the first 20 ml. of permeate collected for turbidity measurement. Studies were normally conducted at a dextran concentration of 10% w/w.

Dynamic filtration studies were conducted in the equipment which is shown diagrammatically in fig. 41. The dextran solution to be filtered (2% w/v) was placed in a reservoir (capacity 2 litres), and pumped, under pressure, through a series of filtration cells arranged in series. Any number of these cells may be used at one time. Pumping was effected by means of an electrically-driven, Centurion 100 metering pump (Metering Pumps Ltd.) with variable output up to 22.5 l./hr., against a maximum working pressure of 62 bar. Pressure and pressure differential across the filtration cells was measured by means of two pressure gauges (Baudenburg Co. Ltd., 0 - 110 bar), located close to upstream and downstream sides of the cells. Pump pulsations were dampened by incorporating an on-line hydraulic accumulator (Greer-Mercier, 10 in.³ capacity) in the circuit, and pressure was controlled by means of a spring-actuated pressure relief valve (Hale-Hamilton Valves Ltd., 4.8 bar max. working pressure). Tap water recirculation
FIG. 41: MEMBRANE FILTRATION EQUIPMENT - FLOW SCHEME
through the on-line heat exchanger (water condenser) enabled a constant operating temperature to be maintained.

The construction of the stainless-steel filtration cells (max. pressure 200 bar) is indicated in fig.41. These cells hold membrane discs of diameter 4.7 cm. High linear flow rates across the membrane surface are obtained by forcing the solution to flow through narrow channels of semi-circular cross-section (3 mm. diameter), arranged in the form of a spiral baffle. The upstream side of the membrane is in contact with the spiral baffle, and forms the diameter of the semi-circular flow channel. Solution enters tangentially to the circumference of the membrane and leaves the cell at the spiral centre. The membrane (active area 11.5 cm.$^2$ per cell) is supported mechanically by a sintered stainless-steel disc, through which the permeate is withdrawn and collected in a small-bore tube. Sealing between high and low pressure compartments of the cells is effected by a double O-ring system, as shown in fig.41. A filter paper disc, placed between the membrane and the porous metal, acts as a cushion to protect the membrane, when high pressures are employed.

In the work on dextran filtration, the pump was operated at maximum output, and a pressure of 2 bar. Dextran solution was recirculated continuously through the cells until the requisite amount of permeate (20 ml.) had been collected. The same series of Millipore membranes which had been used in the static-filtration studies, were employed in this work.
FIG. 42: CROSS-SECTION OF MEMBRANE FILTRATION CELL

- fluid inlet
- sintered metal disc
- spiral baffle
- permeate
- fluid outlet
- filter paper
- membrane
- O-ring seals

Note: "LI" seems to be a typographical error and should be ignored.
7. IDENTIFICATION OF INSOLUBLE MATERIAL IN DEXTRAN.

A solution of dextran (sample 7, 60% w/w, 25 g.) was allowed to stand for one hour at 25 °C. After diluting the solution to a lower concentration (10% w/v), the insoluble material was isolated and washed free of soluble dextran by repeated centrifugation (4000 r.p.m., 30 mins.) and water washing. The washing procedure involved decantation of the supernatant liquid, and replacement with an equal volume of water. The insoluble product (1.15 g.) was finally isolated by centrifugation and drying (50 °C). A second sample of insoluble dextran (1.13 g.) was prepared by allowing the insolubilisation to proceed for two hours at 25 °C., when a gel was formed. The gel was redispersed in water as small particles, being washed free of soluble dextran and isolated as before.

The dextran contents of small samples of these insoluble materials (0.2 g.), as determined by the extended drying procedure described above, were 90.5% and 87.8% in the products isolated before and after gelation, respectively.

The two insoluble products (weight equivalent to 0.50 g. dry weight, based on above dextran contents) were redissolved in boiling water (~70 ml.) and the volume made up to 100 ml. in a volumetric flask, to give a 0.5% w/v solution. Viscosity and optical rotation measurements were conducted on each of the solutions.
8. STUDIES OF RATES OF DEXTRAN CRYSTALLIZATION FROM AQUEOUS SOLUTION.

(a) **Measurement of Inherent Turbidity.**

Dextran (weight equivalent to 5.00 g. dry weight, based on moisture contents determined in table 36) was shaken mechanically with water (~35 ml.) for 30 minutes, and the volume made up to 50 ml. in a volumetric flask to give a 10% w/v solution. The inherent turbidities of the dextrans were measured by the methods given in section 3 (b), and the results are given in table 36.

(b) **Light Scattering Measurements using SOFICA Instrument.**

In the light scattering studies on concentrated solutions, water (33.3 ml.) was added to dextran (sample 17, weight equivalent to 50.0 g. dry weight, based on dextran content of 90.3%), to give a solution of concentration, 60% w/w. Water (75 ml.) was also added to dextran (sample 7, weight equivalent to 50.0 g. dry weight, and based on dextran content of 91.3%), to give a dextran concentration of 40% w/w.

In the light scattering studies on dilute solutions (10% w/v), preparation procedures employed were identical to those used in the nephelometric studies below (section 3 (c)), viz. crystallization of dextran from solution (60% w/w) for various periods of time, followed by dilution with water. Methods of preparing the solutions for light scattering and of conducting the measurements have already been described.

(c) **Rate Determination by Nephelometric Methods.**

Using a microburette, water (3.33 ml.) was added to dextran (weight equivalent to 5.00 g. dry weight, based on the dextran
contents given in table 36), to give a total weight of 8.33 g. The dextran concentration is then 60\% \text{ w/w}. The mixture was stirred for 5 minutes with a glass rod, and the viscous solution, with rod, allowed to stand in a thermostatted room (22 °C. ± 1°C.) for various periods of time. The whole mixture was then diluted, transferred to a volumetric flask, and made up to 50 ml. with water. The turbidity of the resultant solution (10\% \text{ w/v}) was measured in the nephelometer after the air bubbles had been removed by applying vacuum to the nephelometer tube. Where turbidity developed too rapidly at a dextran concentration of 60\% \text{ w/w}, a lower concentration (50\% \text{ w/w}), obtained by adding water (5.00 ml.) to a sample of dextran (equivalent to 5.00 g. dry weight), was employed. As before, the solution was then diluted to a lower concentration (10\% \text{ w/v}) for turbidity measurement. Depending on the particular dextran being studied, the total period of time required for crystallization up to the point of gelation ranged from 1 to 50 hours. To measure rate of crystallization, the above procedure was repeated at intervals throughout the appropriate period of time.

The effect of temperature on rate of crystallization was investigated by preparing two sets of concentrated aqueous dextran solutions (sample 10, 60\% \text{ w/w} and 50\% \text{ w/w}) at various temperatures in the range 22 ° - 100 °C. The solutions were then stored in a thermostatted water bath (± 0.5 °C.) at the required temperature, and the turbidity measured after cooling, and dilution to 10\% \text{ w/v}. A 60\% \text{ w/w} solution was also stored for one hour under steam pressure (1 bar, 121 °C.) and the turbidity measured on cooling and dilution to 10\% \text{ w/v}, as before.
(d) Effects of Nucleation Density on Dextran Crystallization Rates.

(i) Addition of Insoluble Dextran.

A sample of the insoluble dextran (0.1 g.), isolated by the procedures described above, was dispersed in water (approx. 100 ml.) to give a suspension with nephelometric turbidity reading ($N_{100}$) of 40. This turbid suspension was employed in place of water to prepare a concentrated dextran solution (sample 17A, 60% w/w), and the rate of crystallization was determined by the standard procedure described in section 8 (c). A second experiment was also conducted in which insoluble dextran (0.1 g.) was dispersed in water (approx. 10 ml.) to give a suspension with a nephelometric turbidity reading ($N_{100}$) of 600. This suspension was used in place of water to prepare a concentrated dextran solution (sample 17B, 60% w/w) and the rate of crystallization was determined as before. In a third experiment, an aqueous suspension with a nephelometric turbidity ($N_{100}$) of 600 was again prepared. The suspension was however boiled for 30 minutes reducing the turbidity reading ($N_{100}$) to 55 before being employed in place of water to prepare a concentrated dextran solution (sample 17C, 60% w/w). The effect on crystallization rate was again determined by the procedure described in section 8 (c).

(ii) Mixing of Dextrans.

Dextran (sample 17, 5.24 g.) and dextran (sample 5, 0.26 g.) were mixed, and water (3.33 ml.) added from a microburette to give a total weight of 8.33 g. The total dextran concentration was then 60% w/w, and the rate of crystallization was determined in the manner described in section 8 (c).
(iii) **Humidification of Dextran.**

Dextran (sample 18, 100 g.) was stored in an open container inside a sealed wooden box, the base of which was kept moist. The humidity inside the box ranged from 90 - 95%. Over a period of two weeks, the moisture content of the dextran increased from 3.6% to 9.7%, and the inherent turbidity (N₁₀₀, 10% w/v solution) increased from 20 to 38. Rates of dextran crystallization were determined before and after humidification by the methods described in section 8 (c).

(iv) **Boiling of Dextran Solutions.**

Solutions of dextran (sample 8, 10% w/v, 50 ml.), which had been previously boiled for periods of 0, 2, 4, 10, 15, 25 and 40 minutes, were employed in this experiment. Each solution was transferred to a tared round-bottom flask, the volume was reduced to approx. 25 ml. by rotary film evaporation under vacuum at 45 °C., and the residual solution was freeze-dried. The rate of crystallization from solution (60% w/w) was then determined by the standard nephelometric procedure (section 8 (c)). A solution of the same dextran (10% w/v), which had not been heated, was carried through the isolation procedure and reconstituted at the same concentration without change in turbidity. This indicated that the vacuum evaporation and freeze drying processes did not substantially affect the crystalline particle content of the dextran.

9. **STUDIES OF RATES OF DEXTRAN CRYSTALLIZATION FROM AQUEOUS-ETHANOL SOLUTION.**

(a) **Precipitation of Dextran-Rich Phase with Ethanol.**

Precipitations were carried out in a 3-neck round-bottom flask
(250 ml.), fitted at the bottom with a cone which led to a drain tap. The flask was fitted with a link-stirrer and a burette for adding ethanol, and was immersed up to the neck in a water bath thermostatted to 25.0 °C ± 0.1 °C.

A solution of dextran (sample 18, 10% w/v, 100 ml.) was brought to thermal equilibrium in the flask, and ethanol (Boroughs Absolute Alcohol) was added continuously, with stirring, over a 30 minute period, until the solution became cloudy. Further amounts of ethanol were added to precipitate increasing proportions of the total amount of dextran present (table 31). In each case, stirring was continued for a further 30 minutes after the requisite amount of ethanol had been added. The droplets of precipitated phase were then allowed to settle for 24 hours. A homogeneous, concentrated, dextran-rich phase formed during this period.

These standard precipitation conditions were used throughout this work. The dextran-rich phase was removed from the flask, and its volume measured before dilution with water (400 ml.). The ethanol was removed by boiling for 30 minutes, and the solution made up to a known volume (500 ml.) with water. The dextran concentration was then determined by polarimetry. Having measured its volume, the supernatant liquid was also boiled for 30 minutes to remove ethanol, the solution was made up to a known volume (100 ml.) with water, and the dextran concentration was determined by polarimetry. The amount of added ethanol necessary to produce various concentrations of dextran in the polymer-rich phase
was thus established for the particular precipitation conditions employed (see table 31).

(b) Rate Determination by Nephelometric Methods.

To study rate of dextran crystallization from the precipitated aqueous-ethanol solution, the precipitation was carried out as described above. Sufficient ethanol (70 ml.) was added to give a dextran concentration of 36% w/v in the precipitated phase. Several precipitations were carried out in which the precipitated liquid at 25 °C. was allowed to remain in contact with the supernatant for various periods of time, up to 100 hours. The concentrated dextran solution was then separated from supernatant, diluted to 1% w/v with water, and the turbidity measured in the nephelometer.

In studying the effects of dextran concentration on rate of crystallization, various amounts of ethanol were added under the above conditions, and in each case the precipitated liquid was separated from supernatant after settling for 24 hours at 25 °C. Having determined the dextran concentrations in the precipitated liquids, each was diluted to 1% w/v with water, and the turbidity was measured in the nephelometer. The whole procedure was then repeated with a longer settling period of 48 hours before diluting the dextran-rich phase to 1% w/v, and measuring the turbidity.

In studying the effects of temperature on crystallization rate, the dextran-rich phase was precipitated at 25 °C. over a 24 hour
period to give a dextran concentration of 36% w/v in the aqueous-ethanol solution. The precipitated phase was then separated from supernatant and stored for a further 24 hours at temperatures in the range 20 °- 40 °C. After this 24 hour period, the concentrated solution was diluted to 1% w/v with water, and the turbidity measured in the nephelometer. The whole procedure was repeated at each storage temperature.

10. MELTING STUDIES IN DEXTRAN SOLUTIONS.

(a) Heating at Various Temperatures.

Dextrans (samples 5 and 6, weight equivalent to 50.0 g. dry weight, based on dextran contents of 94.5% and 96.7% respectively) were each shaken mechanically with water (~35 ml.) for 30 minutes, and the solutions made up to 500 ml. in a volumetric flask. Aliquots (~20 ml.) of these dextran solutions (10% w/v) were introduced into a nephelometer tube, which was placed in a water bath thermostatted to the required temperature. After exactly 15 minutes, the tube was removed, cooled rapidly, and the turbidity measured in the nephelometer. The temperature range covered was 50 °- 100 °C. The effect of heating at a temperature above 100 °C. was determined by autoclaving a more concentrated solution of the dextran (approx. 17% w/v) under steam pressure (1 bar), estimated temperature, 121 °C., for 10 minutes. The turbidity was measured after cooling, and dilution to the standard concentration (10% w/v).

(b) Boiling for Various Times.

(i) Crystalline Material Inherently Present in Aqueous Solution.

Solutions of several dextrans (samples 8, 9, 10, 17, 10% w/v,
500 ml.) were prepared as in the above heating studies (section 10 (a)). Aliquots of each solution (50 ml.) were introduced into conical flasks (100 ml. capacity), and while stirring, each aliquot was rapidly heated to boiling point on a hot-plate. The aliquots were then boiled under reflux for various periods of time in the range 2 - 40 minutes, cooled in a bath of cold water, and their turbidities measured in the nephelometer. Turbidity measurements were also performed on the solutions which had not been heated, and on those which had been heated to boiling point, but not boiled. Precautions were taken to ensure that heating and cooling cycles were identical in each case.

(ii) Crystalline Material Inherently Present, and Developed in Aqueous Solution (SOFICA Studies).

In the light scattering studies on melting, carried out using the SOFICA instrument, the solutions employed were those of two dextrans (samples 7 and 17, 10% w/v), produced by dilution of concentrated solutions (60% w/w), which had been allowed to crystallize for various periods of time. The method of preparation, crystallization, and dilution of these solutions, and of carrying out light scattering measurements in the SOFICA photometer has already been described (section 3 (a)). After light scattering measurements had been carried out, the dilute solutions (10% w/v), in the light scattering cells, were placed in a boiling water bath for 30 minutes, cooled in a bath of cold water, and replaced in the SOFICA photometer for light scattering measurements.
(iii) Crystalline Material Developed in Aqueous Solution.
Crystallization was induced in concentrated solutions of several dextrans (samples 7, 8, 17, 20, 60% w/w, 8.33 g.) by standing at 22 °C. for appropriate periods of time. The solutions were then diluted with water to give a final volume of 50 ml. The diluted solutions were each boiled under reflux for 5 minutes, cooled in a water bath, and their turbidity measured in the nephelometer.

(iv) Crystalline Material Developed in Aqueous-Ethanol Solution.
In the nephelometric studies of melting in aqueous-ethanol solutions, the procedures described above, relating to precipitation of dextran-rich phase with ethanol, followed by crystallization under various conditions of time, concentration, temperature and inherent turbidity, were used to prepare the solutions. In each case, crystalline dextran in a dextran-rich aqueous-ethanol dextran solution of known concentration was melted in two ways: -

(i) the concentrated solution was diluted to 10% w/v with water, and boiled under reflux for 30 minutes.

(ii) the concentrated solution was diluted to 15% w/v with water, and the ethanol evaporated by boiling. Water was added to prevent the volume from decreasing by more than 50%, such that the dextran concentration was maintained at a concentration below 30% w/v. The solution was then diluted to 10% w/v with water.

After these melting procedures had been carried out, the residual turbidity present in the 10% w/v solutions was determined in the nephelometer (see fig. 36).
In studies on the melting behaviour of crystalline dextran under different conditions of solvent concentration and composition, the following solutions were employed. Methods of preparation have already been described, and the following crystallization conditions used:

(i) sample 7: 60% w/w aqueous solution, crystallization time, 30 minutes at 22°C.

(ii) sample 17: 60% w/w aqueous solution, crystallization time, 40 hours at 22°C.

(iii) sample 17: 36% w/v aqueous-ethanol solution produced as dextran-rich phase by precipitation of dextran from aqueous solution (10% w/v) with ethanol at 25°C, and allowing 24 hours settling before separating precipitated phase from supernatant.

In each case, the concentrated solutions were diluted with water to give several solutions (5% w/v to 45% w/v), each of which was boiled under reflux for 30 minutes. After adjustment of the dextran concentration to 10% w/v, generally by dilution with water, the residual turbidity after boiling was determined in the nephelometer. However, for the 5% w/v solutions, concentration to 10% w/v for the purposes of turbidity measurement, was effected by rotary film evaporation under vacuum, at 45°C.

11. EFFECTS OF CHEMICAL ADDITION TO DEXTRAN SOLUTIONS.

(a) During Preparation of 10% w/v Solution.

(i) Urea.

Dextran (sample 8, 5.2 g.), urea (24.0 g.) and water (10 ml.) were
shaken mechanically for 30 minutes, and the solution made up to 50 ml. in a volumetric flask. The turbidity of the resulting 10% w/v dextran solution was immediately measured in the nephelometer.

(ii) Sodium Hydroxide.
Dextran sample 8 (5.2 g.), sodium hydroxide (2.0 g.) and water (35 ml.) were shaken mechanically for 30 minutes, and the solution made up to 50 ml. in a volumetric flask. Slight yellowing of the solution was observed. The turbidity was immediately measured in the nephelometer.

(b) After Preparation of 10% w/v Solution.
A large volume (250 ml.) of dilute aqueous dextran solution (sample 8, 16.7% w/v) was prepared, various chemicals were added to aliquots of this solution (15 ml.) in the amounts shown below. After chemical addition, the volume was made up to 25 ml. with water in a volumetric flask, to give a dextran concentration of 10% w/v, and the following concentrations of added chemical agents:

<table>
<thead>
<tr>
<th>(i)</th>
<th>urea</th>
<th>(12.0 g., 8 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ii)</td>
<td>sodium hydroxide</td>
<td>(2.0 g., 1 M)</td>
</tr>
<tr>
<td>(iii)</td>
<td>sodium chloride</td>
<td>(7.0 g., 4.8 M)</td>
</tr>
<tr>
<td>(iv)</td>
<td>glycine</td>
<td>(4.0 g., 2.1 M)</td>
</tr>
<tr>
<td>(v)</td>
<td>glucose</td>
<td>(5.0 g., 1.0 M)</td>
</tr>
<tr>
<td>(vi)</td>
<td>hydrochloric acid</td>
<td>(2.5 ml. (10M), 1.0 M)</td>
</tr>
</tbody>
</table>

The turbidity of the 10% w/v solutions was measured in the nephelometer, results being given in table 20.
(c) **After Boiling of 10% w/v Solution.**

Dextran solutions (samples 8, 9, 10, 17, 10% w/v, 25 ml.), which had each been boiled for 30 minutes, were treated with sodium hydroxide (1.0 g.) and the turbidity measured in the nephelometer. Turbidity determinations before and after chemical addition are given in table 33.

(d) **Before Development of Crystalline Dextran.**

The pH of a dextran solution (sample 8, 20% w/v, 20 ml.) was increased from 5.3 to 10.3 by addition of sodium hydroxide solution (1M), and the solution allowed to stand for 30 minutes at 22 °C. The solution was heated to 50 °C. for 5 minutes, cooled, and the pH readjusted to 5.3 by the addition of hydrochloric acid (1M). After isolating the dextran by freeze drying, a concentrated solution (60% w/w) was prepared by the procedures described above, and allowed to stand for 1 hour at 22 °C. The turbidity of the solution was measured in the nephelometer after dilution with water to a dextran concentration of 10% w/v.

(e) **During Preparation of 60% w/w Solution.**

Urea solution (6M, 3.15 ml.) was mixed with dextran (sample 8, 5.18 g.), and the resultant dextran solution (60% w/w) allowed to stand for 1 hour at 22 °C. The solution was made up to 50 ml. with water in a volumetric flask, and the turbidity of the dilute dextran solution (10% w/v) measured in the nephelometer. The procedure was repeated using sodium hydroxide solution (1M, 3.15 ml.) in place of the urea solution.
After Development of Crystalline Dextran.

A dextran gel (4.3 g.) was prepared by allowing a concentrated dextran solution (sample 8, 8.33 g., 60% w/w) to stand for 24 hours. The gel was cut into small pieces and shaken with an aqueous urea solution (8M, 10 ml.) for 1 hour. The procedure was repeated, using sodium hydroxide solution (1M, 20 ml.) in place of urea solution.
PART II

MASS TRANSPORT STUDIES DURING MEMBRANE FILTRATION

OF 6-AMINOPENICILLANIC ACID SOLUTIONS
In part I of this thesis, filtration studies using graded-pore membranes were employed to determine the size of insoluble particles present in dextran solutions. Partial retention of dextran was observed even with membranes of mean pore diameter considerably greater than the size of the dextran molecules (table 19). This effect is associated with the formation of a boundary layer of particles which are retained on the membrane surface. Several references to a similar phenomenon, sometimes referred to as "dynamic membrane formation", appear in the literature (128 - 132). Similar effects were observed by the author during attempts to fractionate dextran using ultrafiltration membranes. In a fraction of broad molecular weight distribution, it was found that transmission of the lower molecular weight components was retarded by those of higher molecular weight.

Since it would appear difficult to interpret the results in anything other than empirical terms, further investigations into boundary layer formation during filtration of dextran solutions were not undertaken. However, during quite separate investigations into the feasibility of using membranes for the concentration and purification of the penicillin intermediate 6-aminopenicillanic acid (6-APA), an opportunity arose for putting semi-empirical findings, relating to effects of boundary layer formation on mass transport, to practical use.
In the studies of 6-APA concentration and purification, it was observed that traces of retained enzymic protein contaminants interfered with the normal transport of water and 6-APA across cellulose acetate reverse osmosis and ultrafiltration membranes. The protein content of 6-APA is particularly difficult to determine, because of interference by the 6-APA with conventional analytical methods. The possibility arose that if a quantitative and reproducible correlation could be established between mass transport rates and protein concentrations, then transport rate measurements might be used to determine protein levels in 6-APA.

The objectives of the work described in part II were therefore as follows:

(i) to quantify the effects of trace levels of enzymic protein on the transport rates of water and 6-APA across cellulose acetate reverse osmosis and ultrafiltration membranes.

(ii) to investigate the possibility of using transport rate measurements to determine protein concentration in 6-APA solutions.

(iii) to propose a mechanism to explain the observed effects of protein on mass transport rates.

1. MEMBRANE SEPARATION PROCESSES.

(a) Osmosis and Reverse Osmosis.

The phenomenon of osmosis has been studied for over two centuries, and its profound importance in biological systems recognized (133). Osmosis is the solvent transport process which occurs when a solution is separated from its solvent by a membrane impermeable to solute. If a hydrostatic head is allowed to develop on the
solution side of the membrane, the solvent flow will be reduced to zero, and the pressure at which this is observed is the osmotic pressure of the solution, \( \Pi \). The osmotic pressure of the solution is the additional pressure necessary to raise the chemical potential of the solvent in the solution phase to equal that in the solvent phase (134).

If a pressure exceeding the osmotic pressure is applied to the solution side of the semi-permeable membrane, reversal of solvent flow becomes possible, and the process is termed "reverse osmosis" (135). Considerable pressures are required to operate the reverse osmosis process, not only to overcome the osmotic pressure, but also to force solvent through the membrane at a practical rate. Since semi-permeable membranes possess little mechanical strength of their own, they therefore require to be mounted on porous supports during use.

Although the reverse osmosis process for removing dissolved salts from water has been known for over forty years (136), it was not until the 1960's that a practical process for separating ions and small molecules from water was developed. The first important discovery, due to Reid and co-workers (137) in 1959, was that homogeneous cellulose acetate films were outstandingly effective in being able to retain sodium chloride. However, the water transport rate was too low for the separation process to have a practical value, except when the polymer was prepared in the form of hollow-fibres (138), which exhibit an extremely high membrane surface area to volume ratio.
The second important advance was made by Loeb and Sourirajan in 1963 (139). Making use of the earlier work of Dobry (140) in the 1930's, these workers added an aqueous magnesium perchlorate solution to a solution of cellulose acetate in acetone. When the mixture was cast into film form, and the cast film was immersed in water, a highly swollen structure was produced. When this water swollen, initially porous film was annealed in water at about 80 °C., it developed desalination properties. More significantly, it showed a 100-fold increase in water transport rate over that of a homogeneous cellulose acetate film. This discovery enabled reverse osmosis to be adopted as a commercial separation process, and a large number of applications in desalination (141), in food product processing (142), and in effluent treatment (143), have now been developed.

An important factor to be considered in understanding the significance of these empirical discoveries, is the effect of the Loeb casting procedure on membrane transport rate. It is now known that the important feature of cellulose acetate membranes prepared in this way, is the presence of a very thin layer of homogeneous polymer on one surface of the membrane (144). The remainder of the membrane sub-structure is porous and does not limit mass transport significantly. For a homogeneous film, flux is inversely proportional to thickness. The original cellulose acetate films studied by Reid (137) were both homogeneous and 100 μm in thickness. On the other hand, the structure of films prepared by the Loeb-Sourirajan method (139) is asymmetric, and the homogeneous surface layer which is responsible for solute
retention is only 0.1 - 0.2 μm thick. It is therefore evident why the asymmetric membranes have much higher water transport rates than homogeneous films of the same overall thickness.

The mechanism of ion retention by cellulose acetate desalination membranes is controversial. The main issue centres around whether the active part of the membrane is homogeneous or porous i.e. whether retention occurs due to the insolubility of the ionic species in the polymer, while water is transported by diffusion (145,146), or whether ions are retained due to a type of sieving mechanism, and the water is transported by viscous pore flow (147, 148). However, the argument reduces to the definition of a "pore", and whether the water molecules in the water swollen polymer form clusters of sufficient size to form a structure which is distinct from a homogeneous water-swollen polymer.

When an aqueous solution is pressurised in contact with a reverse osmosis membrane, such that the pressure difference across the membrane is ΔP, the water transport rates, J_w, is given by (145,148),

\[ J_w = A \left( \Delta P - \Delta II \right) \]  

where A = water permeability coefficient, 
and ΔII = osmotic pressure difference across the membrane. No water flow is observed until Δ P exceeds ΔII.
In membranes in which solute transport occurs by diffusion, the solute transport rate, \( J_s \), is given by \((145,146)\),

\[
J_s = B \Delta c
\]

where \( \Delta c \) = solute concentration difference across the membrane.

Defining solute retention, \( R \), and transmission, \( T \), by,

\[
R = 1 - T = 1 - \left( \frac{c_s^2}{c_s^1} \right)
\]

where \( c_s^1, c_s^2 \) are solute concentrations in upstream and downstream solution respectively, then,

\[
R = 1 - \frac{J_s}{J_w c_s^1}
\]

Since \( J_s \) is effectively independent of pressure, whereas \( J_w \) is strongly pressure dependent if \( \Delta P \gg \Delta \Pi \), it is evident that \( R \) increases with increasing pressure. High pressures, considerably in excess of \( \Delta \Pi \), are therefore necessary to achieve maximum solute retention in diffusive type reverse osmosis membranes.

(b) Ultrafiltration.

Ultrafiltration may be defined as the process whereby solvent and small molecules or ions are removed from macromolecular solutions, by transport through a porous membrane under the action of a pressure gradient. An arbitrary distinction between reverse osmosis and ultrafiltration can be made on the basis of the pressure required to operate the process. The permeation process may be termed reverse osmosis when the operating pressure exceeds 6 bar, and ultrafiltration when the trans-membrane pressure difference lies below this value. The required operating pressure is of course related to the osmotic pressure of the retained solution, and thence to the
molecular weight of the membrane impermeable solutes. Since ultrafiltration membranes are porous, their selectivity is based on molecular size. Most ultrafiltration membranes have pore diameters in the range 1 - 100 nm (149,150).

While the process of ultrafiltration has been known since the late 1800's, the first systematic studies did not take place till the turn of the century. The early membranes were based on cellulose and its derivatives (collodion and cellophane), and over the period 1900 - 1940, an extensive range of methods were developed for preparing membranes of graded pore dimensions. The greatest contribution came from the studies of Elford and Ferry in the 1930's; their work was concerned with the use of collodion membranes for protein ultrafiltration, and for concentration of bacterial and viral suspensions. A review of this work and of earlier studies of collodion membranes is given by Ferry (151).

Although ultrafiltration was employed extensively in the laboratory up to the 1960's, commercial scale operation did not become practical up to this point. This was partly because the cellulosic membranes were generally employed in static or "dead-end" filtration systems, and water transport rate tended to decrease rapidly, and partly because of the poor resistance of the polymers to chemical and microbiological attack. However, with the advent of the high flux cellulose acetate membrane in 1963, interest in commercial ultrafiltration was aroused and several porous asymmetric structures were developed (150). Indeed, the Loeb-type cellulose acetate membrane is porous in the non-annealed state, and may be used to
Dewater and demineralise macromolecular solutions (152, 153, 154). A range of asymmetric polymeric membranes based on non-cellulosic polymers with superior chemical stability can also be prepared (155).

In porous ultrafiltration membranes, solute is carried convectively with solvent, through pores in the membrane which are large enough to allow solute molecules to pass. The solute transport rate is given by (156),

\[ J_s = c_s \left(1 - \alpha\right) J_w \]  

In this case \(1 - \alpha\) represents the fraction of the total solvent flux carried by non-retentive pores, and the solute retention, \(R\), is equal to \(\alpha\).

Solvent transport rate is directly proportional to trans-membrane pressure difference generally only when pure solvent is being ultrafiltered. If macromolecules are present, transport rate becomes virtually pressure independent at quite low pressures, due to severe concentration polarisation effects. Increased pressure, which causes a more rapid transport rate towards the membrane, simply increases the boundary layer resistance such that the overall increase in transport rate across the membrane is zero (156).

(c) **Membrane-Solution Interfacial Effects.**

While the high-flow asymmetric membrane is an essential component of the present-day membrane filtration process, the method of using the membrane is of equal importance. The development of such membranes has brought to light a phenomenon which was of much
lesser practical importance in operating the earlier types of membranes with lower solvent transport rates. This phenomenon is known as concentration polarisation (157), which in simple terms is the build-up of retained solutes at the membrane-solution interface. During filtration, molecular diffusion of solute from the membrane surface into the bulk solution, opposes convective transport of solute towards the membrane. In the steady state, a concentration gradient is established in the proximity of the membrane-solution interface, such that the solute concentration at the membrane surface, $c_s^\infty$, exceeds that in the bulk solution, $c_s^\infty$.

Since concentration polarisation is affected by the rate of diffusion of retained solutes from the membrane surface to the bulk solution, the solute build-up is much greater for macromolecules with low diffusion coefficients than for low molecular weight species. A further significant difference between the behaviour of micromolecular and macromolecular solutions is that above particular concentrations which are characteristic of the solute, many macromolecular solutions cease to behave as fluids, and display gel-like properties. The boundary layer can therefore become a pseudo-solid film or a highly viscous liquid, displaying completely different properties from the bulk solution (158).

Concentration polarisation effects normally diminish the efficiency of membrane separation processes. In reverse osmosis, due to the increased boundary layer concentration, the effective osmotic pressure difference across the membrane is increased, and water transport rate is reduced (eqn.53). At the same time, solute
transport rate is increased (eqn. 54), and the solute retention factor decreases (eqn. 56). In ultrafiltration, gel-like boundary layers diminish the transport rates of water and of smaller molecules, across the membrane. Because of these effects, it is clear that in any membrane separation process, it will be necessary to take steps to minimise the degree of concentration polarisation. This is frequently achieved by passing the pressurised solution over the membrane surface under turbulent flow conditions. Such conditions are employed in the majority of membrane filtration systems (159).

During membrane filtration of solutions under turbulent flow conditions, the solute concentration in the boundary layer can be related to the bulk solution concentration by a relationship of the form (157, 158),

\[
\frac{c^w_s}{c^b_s} = e^{(J_w/k)}
\]  

(58)

where \( J_w \) is the transport rate of water, and \( k \) is a mass transport coefficient. 

\( k \) is a function of solution viscosity and density, solute diffusion coefficient, flow velocity across the membrane, and the hydraulic mean diameter of the flow channel. This equation shows that the concentration polarisation ratio, \((c^w_s/c^b_s)\), increases exponentially with increasing water transport rate. \( c^w_s \) will become constant if the retained solute gels or precipitates, and forms a permanent barrier to mass transport.
While membrane-solution interfacial effects normally impair the efficiency of membrane separation processes, in certain circumstances, it is beneficial to deliberately add specific materials to the solution to be filtered. For example, surfactants have been added to sodium chloride solutions to improve the retention properties of cellulose acetate desalination membranes, by formation of an interfacial film (160). Likewise, dynamic membranes with desalination properties can be formed on porous supports (0.1 - 5 μm pore diameters) by introducing membrane forming additives into the solution (128 - 132).

2. APPLICATIONS OF MEMBRANE PROCESSES IN PENICILLIN PRODUCTION.
Reverse osmosis and ultrafiltration are separation processes which can be operated at low temperature without change of phase. They are therefore ideally suited to the concentration and purification of aqueous solutions containing heat-sensitive species, such as antibiotics, hormones, enzymes, and other proteinaceous materials of biological origin (161, 162, 163). Antibiotics of the penicillin family (I), and intermediates involved in their preparation, are inherently stable in aqueous solution (164), and therefore fall into this category. Thus, during the manufacture of semi-synthetic penicillins, several stages exist where membrane separation processes can be applied to advantage.

Semi-synthetic penicillins, of which ampicillin (α-aminobenzylpenicillin, (II) is perhaps the commonest, are prepared from the naturally occurring benzylpenicillin (III), which is produced by moulds of the genus *Penicillium*. The first step involves enzymatic
I. Penicillin

\[
R - \text{CO} - \text{NH} - \text{CH} - \text{CH} - \text{COOH}
\]

or \[
R - \text{CO} - \text{NH} - \varnothing - \text{COOH}
\]

II. Ampicillin (\(\alpha\)-aminobenzylpenicillin)

\[
\text{NH}_2 - \text{CH} - \text{CO} - \text{NH} - \varnothing - \text{COOH}
\]

III. Benzylpenicillin

\[
\text{H}_2\text{N} - \varnothing - \text{COOH}
\]

IV. 6-Aminopenicillanic Acid

V. Penicilloyl-protein conjugate

\[
R - \text{CO} - \text{NH} - \text{CH} - \text{CH} - \text{COOH}
\]

\[
\text{CO} - \text{NH} - \text{CH} - \text{COOH}
\]

Protein

VI. Penicilloic Acid (Penicic Acid)

\[
\text{H}_2\text{N} - \text{CH} - \text{CH} - \text{COOH}
\]

\[
\text{COOH} - \text{NH} - \text{CH} - \text{COOH}
\]
cleavage of the amide linkage in benzylpenicillin, using a bacterial (E. Coli) amidase (165):

\[
\text{Benzylpenicillin (K}^+\text{ salt)} \xrightarrow{\text{E. coli}} \text{phenylacetic acid (Na}^+\text{/K}^+\text{ salt)} + \text{6-aminopenicillanic acid (Na}^+\text{/K}^+\text{ salt)}
\]

During the fermentation process, phenylacetic acid (PAA), and the penicillin nucleus, 6-aminopenicillanic acid (6-APA) (IV), are produced in stoichiometric amounts as sodium and potassium salts. The 6-APA concentration is typically 2% w/v. After suspended cell debris is removed by filtration, traces (~100 mg./litre) of enzymic, or cell-wall protein remain in solution. This protein is considered to be bound to 6-APA, and is present as a penicilloyl-protein conjugate (V) (166).

To isolate the 6-APA intermediate, the 6-APA/PAA solution is concentrated until the 6-APA concentration is in the range 10-20% w/v. Being an amino-acid, 6-APA has minimum solubility at its isoelectric point (pH 4.3), and therefore precipitates from the concentrate on acidification. The acidification and precipitation is carried out in the presence of a suitable solvent, such as methyl isobutyl ketone, into which the phenylacetic acid is extracted. The 6-APA can then be separated from the aqueous mother-liquors by filtration. Since protein contaminants co-precipitate with 6-APA on acidification, the 6-APA is not purified to any significant extent at this stage, or on any subsequent recrystallization stages (165).
Ampicillin is synthesised from 6-APA and phenylglycine by a multi-stage process. Protein contaminants present in 6-APA are carried through the synthesis, and their presence in the penicillin, even in trace amounts, can have serious consequences. Immunogenic reactions to penicillins occur in 5 - 10% of patients undergoing treatment with this antibiotic (167). Although the incidence of fatal reactions is low, there is always a potential danger for hypersensitive persons. Following the discovery of powerful antigenic contaminants (penicillin-protein conjugates) in 6-APA and penicillins (168-170), further work indicated that these proteinaceous materials might be the major cause of hypersensitivity (171,172). Although the ability of various 6-APA breakdown products and derivatives, e.g. 6-APA polymers (173,174), to elicit an immunological response is uncertain, macromolecular protein undoubtedly results in the production of high antibody levels. As indicated above, the protein is probably a heterogeneous mixture derived from the enzyme employed in the cleavage of benzylpenicillin; it is suggested that the molecular weight of penicillin -protein conjugates has to be at least 12,000 in order to give a detectable response (165).

The molecular separations to which membrane processes apply are,

(i)  6-APA concentration, i.e. retention of 6-APA, transmission of water, by reverse osmosis membranes.

(ii) 6-APA purification, i.e. retention of enzymic protein, transmission of 6-APA and water, by ultrafiltration membranes.

The feasibility of using reverse osmosis and ultrafiltration processes for the concentration and purification of 6-APA has been
demonstrated by the author. Details of this work will be published at a later date. In this section, the objective is to consider only those aspects of the work relating to the effects of retained protein on the transport rates of 6-APA and water across the membranes.

3. APPLICATION OF MEMBRANES TO THE DETERMINATION OF TRACES OF PROTEIN IN PENICILLINS.

Minute traces of biologically-active proteinaceous material present in 6-APA and penicillins (10 - 100 ppm.) are not detectable by classical analytical methods. This is because the amino-acid 6-APA, and other chemically similar species, such as 6-APA polymers, interfere with the analytical procedures. Antigenic material is detectable by quantitatively measuring the immunological response when 6-APA is injected into the bloodstream of animals; since the larger part of the antigenic material is proteinaceous, this test gives a semi-quantitative indication of the protein content of the 6-APA sample (175). However, the method is extremely expensive, and takes several weeks to give a meaningful result; moreover, a measure of the absolute protein level is not obtained.

A vastly superior analytical procedure is based on ion-exchange chromatographic separation of the amino-acids of the protein (165). The bulk of the 6-APA is removed from a sample by exhaustive dialysis, leaving as retentate a macromolecular residue with all components having a molecular weight in excess of 5000. This residue is then hydrolysed, and the resultant amino-acids are separated chromatographically, and determined by colorimetric methods. Summing the weights of the individual amino-acids, with the exception of
penicillamine, glycine, etc., which are derived or potentially derived from 6-APA itself, or from 6-APA polymers, enables the absolute protein content of the sample to be determined. However, while this method is satisfactory, the total analysis time is five days.

Membrane filters have been employed for the rapid micro-assay of protein in the presence of substances which interfere with normal colorimetric or spectrophometric methods of analysis. The usual procedure involves quantitative binding of the protein to the membrane, followed by dye staining of the protein (176). The dye which is retained on the membrane can then be eluted, the amount of bound dye being directly proportional to the quantity of protein applied to the membrane. Some success has been obtained when this method is applied to enzyme protein in 6-APA, but the binding of peptides and polypeptides complicates the determination (165).

During membrane filtration of proteinaceous solutions, protein may influence the transport properties of the membrane, both for solvent and for smaller permeable solute species. The interaction of protein with cellulose acetate membranes was therefore studied in an attempt to correlate the protein content of solutions with associated effects on transport rates of solvent and solutes. The objective was to investigate the applicability of transport rate measurements to the rapid, quantitative determination of enzymic protein in 6-APA.
RESULTS AND DISCUSSION.

II.A. INVESTIGATIONS INTO EFFECTS OF PROTEIN CONTAMINANTS ON MASS TRANSPORT RATES.

1. INTRODUCTION.

The amino-acid, 6-aminopenicillanic acid, which is used in the manufacture of semi-synthetic penicillins, is produced by a fermentation process. As a result, it normally contains traces of enzymic (amidase) protein, which is derived from the bacterial cells (E.Coli) used in the fermentation. During investigations into the feasibility of concentrating and purifying solutions of this amino-acid by reverse osmosis and ultrafiltration, it was observed that the protein contaminants interfered with the normal transport of 6-APA and water. In this section, the objective is to quantify these general observations, and to propose reasons for their occurrence. It is also intended to compare the filtration behaviour of the 6-APA-protein systems with that of other comparable systems, in which high molecular weight additives are deliberately incorporated to improve the efficiency of the membrane separation process.

2. REVERSE OSMOSIS STUDIES.

In this work, a range of type I cellulose acetate reverse osmosis membranes were employed (177). The membranes were characterised initially using sodium chloride solution (0.4% w/v). Membranes with a range of sodium chloride retentions were obtained by varying the temperature of heat treatment in the final stage of preparation (177). Water and sodium chloride permeabilities, and sodium
chloride retentions were determined when this solution was processed (100 bar, 20 °C.). Several 6-APA solutions (2% w/v), containing traces of enzymic protein from the fermentation (4 mg./l.), and several synthetic solutions of pure 6-APA (2% w/v), were then subjected to reverse osmosis (100 bar, 20 °C.) using these membranes. The permeabilities of water and 6-APA, and the 6-APA retentions, all relative to the analogous parameters for sodium chloride, were determined for a range of membranes; results are shown in figs. 43, 44 and 45.

It is evident that while the water permeability of the membrane is not measurably affected by the presence of the protein, the 6-APA permeability, and hence 6-APA retention, is markedly reduced. This is attributable to the formation of a proteinaceous layer on the membrane surface. The effect appears to be analogous to that reported by Kesting and co-workers (160, 178), in which the addition of traces of certain polymers, e.g. poly(vinylmethylether), to saline solutions which are being concentrated by cellulose acetate reverse osmosis membranes, brings about a significant improvement in solute retention, with only a slight deterioration in water transport rate. The efficiency of the reverse osmosis separation process is thereby increased.

The exact mechanism whereby improved retention properties are conferred on the primary membrane is uncertain. Kesting and co-workers (178) favour the argument that adsorption of the additive on the surface is responsible. This is based on extensive studies of surfactant addition, in which a high hydrophilic/hydrophobic
FIG. 43: RELATIVE WATER PERMEABILITIES DURING MEMBRANE FILTRATION OF 6-APA AND SODIUM CHLORIDE SOLUTIONS
FIG. 44: RELATIVE 6-APA AND SODIUM CHLORIDE PERMEABILITIES DURING MEMBRANE FILTRATION OF AQUEOUS SOLUTIONS
Fig. 45: Relative 6-APA and Sodium Chloride Retentions during Membrane Filtration of Aqueous Solution
ratio has been shown to favour the formation of membranes with a high water permeability, and low sodium chloride permeability. It is suggested that the amount of bound water increases in the presence of surfactants. Water can associate with these hydrophilic sites, and can thereby be transported across the interface; ionic species can not be dissolved by the bound water, and are therefore retained. It also follows from this argument that an ability to form hydrogen bonds with water is a necessary requirement for a feed additive.

In the case of the 6-APA-protein solutions, it seems probable that the protein will be adsorbed on to the cellulosic membrane surface (176,179). In this case the same argument as proposed by Kesting et al. (178) could apply, viz. the protein will immobilise water in the area of the interface, and only water, and not ions, can be transferred through this layer by a site-to-site diffusion-type mechanism (180). However, proteinaceous films formed on less retentive cellulose acetate membranes are apparently porous (153,158), since the rapid transport of relatively large solute molecules is permitted. Since it is considered unlikely that the proteinaceous films formed in this instance are any different, a diffusion-type transport mechanism in these protein films does not seem a likely possibility.

The existence of a small number of relatively large pores has been demonstrated in cellulose acetate desalination membranes (181,182). From measurements of the pressure dependency of solute flux, i.e. the extent of viscous flow of saline solution in pores, Michaels et al. (183) deduce that the action of an additive such as poly (vinylmethyl ether) is simply one of pore blockage. However, the
idea of internal pore-blocking in membranes of this type does not agree with observations of the effect of protein on the characteristics of similar, but more porous, cellulose acetate membranes (153,158). Although such membranes contain pores of dimensions more suited for pore-blocking by protein, the fact that intrinsic membrane characteristics can be rapidly restored on cleaning, suggests that only surface effects are involved (183). From the simple picture of an asymmetric pore structure, it is also apparent that molecules capable of entering the pores are likely to be transported, rather than cause pore blockage (118).

It is possible that the surface adsorption may lead to blockage of pore entrances, and that it is this, rather than internal blockage which takes place. For instance, in many instances of dynamic membrane formation (126 - 132), the pore diameter of the supports is much larger than the molecular size of the retained, membrane-forming additive. It is probable that the initial effect is one of adsorption, which leads to a build-up of additive at the upstream solution-support interface. The fact that solute retention is observed must mean that the retentive layer is close to the interface, since concentration polarisation would otherwise reduce the retention to zero. This is confirmed by the fact that dynamic membranes can often be destroyed simply by mechanical cleaning of the support surface. Thus, at low protein additive concentrations, it can be considered that surface adsorption leads to pore-entrance blockage, and hence to improved membrane retention properties.

It is generally considered that ion retention by membranes which are dynamically formed from polyelectrolytes can be explained on the
basis of an ion-exclusion mechanism of retention (128 - 132). With such membranes, the retention of electrolytes decreases appreciably with increase in concentration (132). For example, with sodium chloride solution (0.1 M) less than 10% retention was observed with hydrous zirconium oxide membranes, dynamically formed on porous carbon tubes. However, the retention increased to over 90% when the sodium chloride concentration decreased by a factor of 10.

Although the proteinaceous film formed on the cellulose acetate membrane interface will also have ion-exchange properties, it is probable that the 6-APA concentration is too high for retention to be significantly increased simply as a result of ion-exclusion from the pores of the protein layer.

The explanation proposed to explain the results obtained in this work is therefore that the protein is initially adsorbed on the surface of the cellulose acetate membrane. This protein layer develops to form a thin, porous, gel network which overlies the total surface. If large defect pores, similar in size to the protein molecules (3 - 5 nm), are present, the protein will be adsorbed on the membrane surface in the vicinity of the pore entrance, and eventually seal off the pore.

With ionic species such as 6-APA (Na salt), retention will be slightly enhanced because the overall protein gel layer contains a high ionic charge density. Electrolytes will therefore tend to be excluded by virtue of the Donnan ion-distribution equilibrium (184). However, it is not considered that the 6-APA concentration is sufficiently low for this to be the major factor contributing
to the improvements in 6-APA retention observed in this instance.

3. ULTRAFILTRATION STUDIES.

Ultrafiltration studies of 6-APA—enzymic protein solutions were carried out using cellulose acetate type II membranes (185), which retained 97% ovalbumen on filtration of a 0.1% w/v solution (pH 7). The effect of protein concentration on the rate of transport of water, 6-APA, and proteinaceous matter determined. Results are shown in table 42.

It is interesting that protein retention appears to depend on the protein concentration. This phenomenon has been referred to as "self-rejection", and is believed to be caused by protein film formation (158). The protein film which initially forms, acts as a selective barrier to the permeation of smaller proteins, poly-peptides, or peptides which would freely permeate the primary membrane. Assuming that the fraction of the total proteinaceous material which consists of peptides remains constant, it would appear that the peptide retention capacity of the protein membrane which forms on the primary membrane surface, increases with increasing protein concentration.

As the protein concentration in the retained solution increases, the ratio of protein to 6-APA in the membrane permeate, which is determined by their relative transmission factors, does not vary widely. This is because the protein transmission factor decreases much more rapidly than that of 6-APA, due to the self-rejection effect referred to above. The purification efficiency of the ultrafiltration process is not therefore markedly dependent on the
TABLE 42: Effects of Protein Concentration on Mass Transport Across Cellulose Acetate Ultrafiltration Membranes.

<table>
<thead>
<tr>
<th>6-APA Sample No.</th>
<th>Protein Concentrations (mg./l.)</th>
<th>Protein Content of 6-APA (ppm)</th>
<th>Transmission Factors (%)</th>
<th>Water Transport Rate (m./sec.) x 10^5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retentate</td>
<td>Permeate</td>
<td>Retentate</td>
<td>Permeate</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>3.0</td>
<td>1.0</td>
<td>150</td>
<td>60</td>
</tr>
<tr>
<td>20</td>
<td>29</td>
<td>0.2</td>
<td>1450</td>
<td>20</td>
</tr>
<tr>
<td>22</td>
<td>110</td>
<td>0.5</td>
<td>5500</td>
<td>70</td>
</tr>
</tbody>
</table>

6-APA = 6-Aminopenicillin Acid
protein content of the 6-APA. However, the rate at which the purification can be carried out, which depends on the water transport rate, decreases significantly with increasing protein concentration.

Water transport rate and 6-APA transmission both decrease as the protein concentration increases. It is assumed that this is due to a thickening or consolidation of the protein boundary layer, which restricts the transport of water and solutes. The cellulose acetate type II membrane is porous, but since the size of the pores in the membrane is smaller than the molecular dimensions of the proteins, internal pore plugging is not considered to be responsible for the effects observed.

Because of the possible complications of concentration polarization, it is not possible to determine whether 6-APA transport rate is restricted by the protein film to a greater extent than water transport rate. If both rates were equally affected by the protein film, the reduced 6-APA transmission could simply be a consequence of the lower water transport rate and the associated reduction in 6-APA boundary layer concentration (eqns. 56 - 58). An attempt is made to resolve this question in the following section, and a possible mechanism is proposed to account for the effects observed.
II.B. CORRELATION OF MASS TRANSPORT RATES WITH PROTEIN CONCENTRATION.

1. INTRODUCTION.

In the previous section, the effects of protein on the transport rates of water and 6-APA across cellulose acetate ultrafiltration membranes have been briefly described. The transport rates of these species, as measured when pure 6-APA solution was processed, decreased significantly as the protein concentration increased. It was therefore considered that it might be possible to obtain a quantitative correlation between the protein concentration and the observed transport rates for water and 6-APA.

In order to investigate this possibility, it was necessary to determine the transport rates of water and 6-APA when solutions containing variable amounts of protein were filtered. It was not possible to use the same membrane for filtering each protein solution, since the transport properties of the membranes became time dependent when protein boundary layers were formed on the surface. Although it was possible to clean the membrane after each protein solution had been filtered, it proved more convenient simply to use a new membrane sample for each experiment.

It is not possible to closely reproduce membrane transport characteristics from one sample of membrane to another. For the purposes of this work, it was therefore necessary to characterise each membrane sample individually, by measurement of its transport properties when a control solution was filtered. Pure 6-APA would be the ideal control solute, but because 6-APA solutions are unstable (164), an alternative solute, viz. sucrose, of similar molecular size, and with similar transport rates across ultrafiltration membranes, was selected.
While both water and 6-APA transport rates will depend on the protein concentration, only the water transport rate was correlated directly with the latter quantity. This is because measurements of two quantities, viz., the water transport rate and the 6-APA concentration in the permeate, are required in order to determine the 6-APA transport rate. Uncertainties in the measured values of both these quantities will therefore lead to a high uncertainty in its value. A more accurate measure of 6-APA transport rate can be obtained by determining the 6-APA transmission factor (eqn.56), which is effectively a measure of the relative transmission rates of 6-APA and water. This factor is obtained simply from two concentration measurements.

Let $J$ and $T$ refer to water transport rate and solute transmission, and let the subscripts $s,a,p,$ signify that the transport process takes place from aqueous solutions containing the solutes sucrose, 6-APA, and 6-APA-protein mixture, respectively. Since $J_p$ will increase with $J_s$ and $T_p$ with $T_s$, the ratios $(J_p/J_s)$ and $(T_p/T_s)$ will be much less dependent on small variations in $J_s$ and $T_s$ than will $J_p$ and $T_p$. Even if $J_s$ and $T_s$ do not vary significantly from one membrane sample to another, it will therefore be more accurate to correlate the ratios, $(J_p/J_s)$, and $(T_p/T_s)$, with protein concentration, rather than simply to compare mass transport parameters, $J_p$ and $T_p$. The effect of protein on the membrane transport properties was therefore quantified by measurement of these ratios. $(J_p/J_s)$ is called the water transport reduction factor, and $(T_p/T_s)$ is referred to as the 6-APA transport reduction factor.

If the solute concentration and operating conditions remain
identical, the ratio of water transport rates from the sucrose and 6-APA solutions \( \frac{J_w}{J_s} \), and the ratio of sucrose transmission to that of 6-APA, \( \frac{T_s}{T_a} \) will also be constant. Hence the transport reduction factors, as measured in this way, will be a direct measure of the effect of protein on the normal transport properties of the membrane, as measured during ultrafiltration of pure 6-APA solution. The effect of using sucrose instead of 6-APA as control solute, is simply that plots of \( \frac{J_p}{J_s} \) and \( \frac{T_p}{T_a} \) against some function of protein concentration, will extrapolate respectively to \( \frac{J_s}{J_s} \) and \( \frac{T_s}{T_s} \) at zero protein concentration, rather than to unity. Because of the similar dimensions of sucrose and 6-APA, the values of these ratios are in fact very close to 1.

2. SELECTION OF EXPERIMENTAL CONDITIONS.

(a) Membrane Type.

In earlier investigations by the author, the results of which will be published at a later date, a range of ultrafiltration membranes were prepared, with the objective of establishing an effective separation process for the removal of enzymic protein from 6-APA. Whilst in the various membrane types considered, the transport rates of water and 6-APA were reduced by the presence of protein, this effect was greatest in the case of cellulose acetate type II membranes. These membranes were therefore used in this work.

The reproducibility of membrane performance was examined by measuring the water and sucrose transport rates across fifty membrane discs (4.7 cm diam.), prepared from several different batches of casting solution. The results are shown in table 43. At 41.4 bar and 15 °C,
TABLE 43: Water Transport Rate and Sucrose Transmission Characteristics of Cellulose Acetate Ultrafiltration Membranes.

<table>
<thead>
<tr>
<th>Water Transport Rate ( (\text{m.} \text{sec}^{-1}) \times 10^5 )</th>
<th>Sucrose Transmission ( (%) )</th>
<th>Water Transport Rate ( (\text{m.} \text{sec}^{-1}) \times 10^5 )</th>
<th>Sucrose Transmission ( (%) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.90</td>
<td>86.4</td>
<td>2.93</td>
<td>87.0</td>
</tr>
<tr>
<td>2.41</td>
<td>83.7</td>
<td>2.81</td>
<td>90.3</td>
</tr>
<tr>
<td>3.33</td>
<td>88.3</td>
<td>3.08</td>
<td>86.5</td>
</tr>
<tr>
<td>2.61</td>
<td>85.3</td>
<td>2.98</td>
<td>86.7</td>
</tr>
<tr>
<td>3.38</td>
<td>89.9</td>
<td>2.61</td>
<td>84.0</td>
</tr>
<tr>
<td>2.71</td>
<td>84.4</td>
<td>2.62</td>
<td>90.3</td>
</tr>
<tr>
<td>3.18</td>
<td>88.7</td>
<td>2.75</td>
<td>73.0</td>
</tr>
<tr>
<td>2.86</td>
<td>88.7</td>
<td>3.20</td>
<td>86.7</td>
</tr>
<tr>
<td>2.83</td>
<td>88.5</td>
<td>2.64</td>
<td>83.0</td>
</tr>
<tr>
<td>2.72</td>
<td>88.5</td>
<td>3.09</td>
<td>89.0</td>
</tr>
<tr>
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<td>87.0</td>
</tr>
<tr>
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<td>85.9</td>
<td>3.00</td>
<td>84.5</td>
</tr>
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<td>85.2</td>
<td>2.42</td>
<td>83.5</td>
</tr>
<tr>
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<td>3.17</td>
<td>88.7</td>
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<td>78.5</td>
</tr>
<tr>
<td>2.71</td>
<td>84.4</td>
<td>2.65</td>
<td>80.9</td>
</tr>
<tr>
<td>3.38</td>
<td>89.9</td>
<td>2.63</td>
<td>77.1</td>
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<tr>
<td>2.90</td>
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<td>86.5</td>
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<td>88.0</td>
</tr>
<tr>
<td>3.25</td>
<td>86.9</td>
<td>2.83</td>
<td>83.9</td>
</tr>
<tr>
<td>3.03</td>
<td>89.3</td>
<td>3.15</td>
<td>85.9</td>
</tr>
<tr>
<td>3.40</td>
<td>88.5</td>
<td>3.09</td>
<td>84.2</td>
</tr>
</tbody>
</table>
the mean water transport rate was \((2.6 \pm 0.3) \times 10^{-5}\) m./sec., and
the corresponding mean sucrose transmission was \(86 \pm 4\%\). However,
the sucrose control measurements are still desirable for the
reasons given above. In addition, to eliminate any dependence of
\((J_p/J_s)\) and \((T_p/T_s)\) on \(J_s\) and \(T_s\), membranes with transport prop-
erties outside the indicated range were not employed.

(b) **Pressure.**

It is evident from eqn. 58 that the concentration of the protein
boundary layer will increase with increasing water transport rate.
To maximise the effects of protein on the transport rates, condi-
tions were selected to give the highest possible water transport
rate. This was achieved by operating the ultrafiltration process
at a trans-membrane pressure difference of \(41.4 \pm 0.5\) bar. A
pressure of this order is the maximum which the membranes will
tolerate without undergoing rapid changes in transport charact-
eristics due to compaction effects (186).

Solvent and solute flow rates will be proportional to pressure
(eqns. 53, 57) but their ratio, which determines the solute trans-
mission factor, will be pressure independent. Only water transport
reduction factors will therefore be sensitive to pressure setting
errors. Assuming that pressure can be set to \(\pm 1.3\%\), the resultant
uncertainty in the measured water flow ratios (factor A) will be
\(\pm 1.8\%\). Errors in the actual measurement of water flow rates are
negligible compared with pressure setting errors. A small, but
constant pressure drop will occur across each ultrafiltration cell,
but it is not necessary to correct for this effect since the setting
of pressure at one point in the system will automatically define the pressure in all other parts of the assembly.

(c) Temperature.
A temperature of 15 °C. was selected for convenience and ease of control. In order to maintain the solutions at 15 °C. at the membrane surface, the solution reservoir was maintained at 13.5 °C., i.e. a temperature drop of 1.5 °C. was maintained across the water-cooled heat-exchanger which was incorporated in the recirculation loop (fig.41). The effect of temperature on water transport rate and on sucrose transmission over the range 13 °C. - 25 °C. is shown in fig.46.

Water transport rate increases with increasing temperature due to the effects of the latter on rates of viscous pore flow across the membranes (145). The increase in sucrose transmission with increasing temperature is probably due to a preferential increase in solution flow through non-retentive pores, associated with the higher viscosity of the solution relative to solvent.

Assuming that temperature can be controlled to ± 0.25 °C., it is evident from fig.46 that the probable error in the ratio of water transport rates is ± 1% (± 0.7% error in each flow measurement). In the ratio of solute transmissions, a ± 0.25 °C. variation in temperature introduces an error of 0.2%.

(d) Velocity Across Membrane Surface.
The rate of flow of pressurised solution across the membrane surface is an important parameter which determines the rate of mass
FIG. 46: EFFECTS OF TEMPERATURE ON WATER TRANSPORT AND ON SUCROSE TRANSMISSION DURING ULTRAFILTRATION OF SUCROSE SOLUTION (2% w/v, 41.4 bar)
transport across the membrane (eqn.56). When protein is present, the concentration of protein in the boundary layer, and hence the reduction in the transport rate of small molecules, is markedly dependent on flow velocity. If the flow velocity is too high, the water and 6-APA transport rates will be insufficiently sensitive to the presence of traces of protein. On the other hand, if the flow is too low, the membrane transport rates may become strongly time dependent, due to time dependent changes in the boundary layer resistance (158). This degree of time dependence may in turn be dependent on protein concentration, which would complicate any correlation of the latter with transport rates. An intermediate flow rate was therefore selected, which corresponds to a Reynolds Number of about 3000 in the flow channels of the spiral baffle (assumed semi-circular). The flow is thus not fully turbulent (187). The degree of turbulence at the membrane surface is only sufficient to be able to exercise some control over protein boundary layer formation, while ensuring that the transport parameters remain sufficiently sensitive to variations in protein concentration.

Using a metering pump, it is possible to exercise a sufficient degree of control over flow rate to ensure that no errors in the transport reduction parameters should arise from this source.

(e) Processing Time.
Although effects of pressure on membrane transport properties occur principally within the first five minutes of operation, it was considered advisable to employ a longer period of 30 minutes to allow transport rates to stabilise before measurement.
significant time-dependent changes in membrane transport properties would then occur over the short period while transport rates were being measured.

(f) Concentration of Solutions.

(i) Sucrose.
An arbitrary sucrose concentration of 2.0% w/v was selected. Although deviations from this concentration will affect the transport reduction factors to some extent, it is possible to prepare solutions to a sufficient degree of accuracy for this to be eliminated as a variable which might affect transport rate measurements.

(ii) 6-APA.
The 6-APA concentration selected for the purposes of the test was also 2.0% w/v. At this 6-APA concentration, the corresponding protein concentrations in solutions of the 6-APA samples were sufficiently high to cause measurable differences in the 6-APA and water transport rates over those for pure 6-APA. The 6-APA concentration was set at 2.0% w/v by polarimetry.

The effect of variations in the 6-APA concentration (sample 14, 1.5 - 2.5% w/v) on water and 6-APA transport reduction factors, as measured under standard conditions, is shown in fig. 47. Assuming that the concentration can be controlled to ± 0.1% w/v, the variations in the measured factors are ± 1.8% in the 6-APA transmission reduction factor, and ± 2.9% in the water transport reduction factor. Control of the concentration to better than
FIG. 47: EFFECT OF 6-APA CONCENTRATION ON WATER AND 6-APA TRANSPORT REDUCTION FACTORS
± 0.1% w/v is only possible if relatively large volumes of solution (1 litre) are tested. The volume removed by membrane permeation during sampling is then sufficiently small compared to the retentate volume that the concentration of the latter does not alter significantly.

When the 6-APA concentration is increased, the protein concentration also increases. Both factors contribute towards a lowering in water transport rate, resulting in a lower water transport reduction factor. However, increased protein concentration causes a decrease in 6-APA transmission, whereas increased 6-APA concentration causes an increase in this parameter. Since it is evident from fig. 47 that the overall effect of an increase in 6-APA protein concentration is to decrease the 6-APA transport reduction factor, the influence of the higher 6-APA concentration must outweigh that of the higher protein level. For this reason, the 6-APA transport reduction is much less sensitive to changes in concentration than is the water transport reduction factor.

(g) 6-APA Solution Holding Time.
During preliminary work, it was found that if 6-APA solutions were tested several hours after being prepared, the measured transport reduction factors were higher than those determined for freshly prepared 6-APA solutions. This effect, which must be associated with changes occurring in the 6-APA solution, necessitates that the transport parameters be measured at some fixed time after preparation. A holding time of one hour was selected for convenience. This allows time for the sucrose solution to be processed, and also ensures that
in the case of 6-APA samples with high protein contents, the protein is completely dissolved.

The increase in water and 6-APA transport reduction factors with increase in holding time of 6-APA solution (sample 14, 2.0% w/v) is indicated in fig. 48. The greatest part of the increase occurs over the first hour (pH 7.8, 20 °C.). 6-APA solutions are unstable, and undergo transformation by two principal pathways, (i) by β-lactam ring opening (164), to form penicilloic (penicic) acid (VI) and (ii) by polymerisation (173). The replacement of 6-APA molecules by those of penicilloic acid is unlikely to cause any changes in the effect of protein on membrane transport properties, since the molecules formed are similar to 6-APA, both in size and in chemical nature. Likewise, 6-APA polymerisation does not appear to afford an explanation for the results, since the extent of the reaction will be virtually negligible at low 6-APA concentration (165).

The transport reduction factors were determined for a 6-APA solution (sample 14, 1.9% w/v), in which penicilloic acid was incorporated at a concentration of 0.1% w/v. No significant difference in the measured transport reduction factors relative to those for pure 6-APA solution, was observed. This finding is consistent with the fact that penicilloic acid transmission is comparable to that of 6-APA. The presence of an average 4% penicilloic acid in the 6-APA samples (165) would thus appear to be unimportant in so far as the effects on the measured transport reduction factors are concerned.
FIG. 48: EFFECT OF 6-APA SOLUTION HOLDING TIME ON WATER AND 6-APA TRANSPORT REDUCTION FACTORS
An investigation was also carried out to determine whether 6-APA polymers (173) were responsible for the time dependent changes in the transport reduction factors. A concentrated 6-APA solution (sample 14, 12% w/v) was held for 27 hours at 20 °C. to promote polymerisation. The solution was subsequently diluted to 2% w/v 6-APA, (3.2 mg./l. protein), and the mass transport parameters were determined under standard conditions. The water and 6-APA transport reduction factors are shown in table 44, together with those determined for a 2% w/v 6-APA solution of the same sample, after holding times of 1 and 25 hours.

**TABLE 44: Time Dependent Changes in 6-APA and Water Transport Reduction Factors.**

<table>
<thead>
<tr>
<th>6-APA Solution Holding Conditions</th>
<th>6-APA Transport Reduction Factor (T_p/T_s)</th>
<th>Water Transport Reduction Factor (J_p/J_s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (hrs.)</td>
<td>Concentration (% w/v)</td>
<td>0.82</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>0.90</td>
</tr>
<tr>
<td>25</td>
<td>2</td>
<td>0.81</td>
</tr>
<tr>
<td>27</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

Assuming that partial polymerisation has taken place in the 12% w/v 6-APA solution, the effect of this reaction is to decrease the transport reduction factors, relative to those observed for a 2% w/v solution with the same holding time. On the basis of these results, 6-APA polymerisation would not appear to be responsible for the time-dependent increase in transport reduction factors.
6-APA polymer, at a concentration of 150 mg./litre, was incorporated in a pure 6-APA solution (sample 14), and the transport reduction factors determined. No detectable difference in the measured factors over those for pure 6-APA solution was observed. This confirms directly that the presence of 6-APA polymer in 6-APA solutions would not affect the results of protein determinations based on transport rate measurements.

The most likely explanation for the observed effects would appear to lie with the proteinaceous contaminants. It is possible that time dependent conformational changes take place in the protein molecules, which renders them less effective at inhibiting the passage of water and 6-APA molecules. This may be due to a decreased affinity for the membrane (lower tendency to adsorb), or to a reduction in gel porosity at the membrane surface, i.e. weaker interactions between protein molecules. Both effects would account for the time-dependent increase in the permeability of the protein boundary layer. It is also possible that time-dependent aggregation effects may occur, which would result in the protein being distributed over a much smaller number of sites on the membrane surface. The sensitivity of antibiotic polypeptide conformations to changes in solvent, pH, temperature etc. has been investigated by Craig (188). In this work conformational changes in the polypeptides were detected from observations of their diffusion rates through dialysis membranes. It is not therefore surprising that mass transport through thin films composed of such materials would likewise be affected by changes in the conformation of their component molecules.
If any departure from the 6-APA solution holding time of one hour occurs, it is evident from fig.48 that errors on both of the measured transport reduction factors will be considerable. This is especially true for holding periods of less than one hour. For the purposes of estimating the probable combined errors in the transport reduction factors, the errors resulting from holding time and holding condition variations were considered to be ± 2% for the 6-APA transport reduction factor, and ± 1% for the water transport reduction factor, assuming the holding time was not less than 1 hour. Since it is very probable that temperature will affect the rate of changes occurring in the 6-APA solution, the temperature conditions while dissolving the 6-APA and while holding the solution were carefully standardised (20 °C.).

3. **ESTABLISHMENT OF CORRELATION CURVES.**

The 6-APA and water transport reduction factors were determined for some twenty 6-APA samples of different protein content. The operating conditions defined in the previous section were employed during measurement of the transport rates. In each case, 6-APA and water transport reduction factors were plotted as a function of the logarithm of the protein concentration, and the results are shown in fig.49 and fig.50 respectively. The plots are apparently linear. While on the basis of eqn.58, there is some justification for pre-supposing that the plot of water transport reduction factor against log (protein concentration) should be linear, the linearity of the analogous 6-APA transport reduction factor plot does not follow. A further discussion of the form of the correlation curve is given in the following section. However, for the purpose of
FIG. 49: CORRELATION OF SOLUTE TRANSPORT REDUCTION FACTOR WITH PROTEIN CONCENTRATION
Correlation of Water Transport Reduction Factor with Protein Concentration

**FIG. 50**

**CELLULOSE ACETATE ULTRAFILTRATION MEMBRANES**

- O - 6-APA-protein co-precipitate
- x - 6-APA-isolated protein
- ● - sucrose-ovalbumin

PROTEIN CONCENTRATION (mg/l.)

PROTEIN CONCENTRATION (mg/l.)
establishing a rapid method of determining protein levels in 6-APA solutions, the correlation curves can be considered on an empirical basis. It is evident from figs. 49 and 50 that the protein concentration of a 6-APA solution can be determined from trans-membrane 6-APA and water transport rates, as measured under the conditions described.

In each case, the values plotted for the 6-APA and water transport reduction factors represent the mean of four determinations. The sources of error contributing to the uncertainty in a single measured transport reduction factor are summarised in table 45. For any given 6-APA sample, the spread in the four measured values of the 6-APA and water transport reduction factors about the mean value, is consistent with the magnitude of the uncertainties deduced from table 45, viz. $\pm 5\%$ and $\pm 7\%$, respectively.
<table>
<thead>
<tr>
<th>Source and Magnitude of Uncertainty</th>
<th>Contribution to Uncertainty in 6-APA Transport Reduction Factor (± %)</th>
<th>Contribution to Uncertainty in Water Transport Reduction Factor (± %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polarimeter Readings (0.01°)</td>
<td>0.7</td>
<td>Negligible</td>
</tr>
<tr>
<td>Pressure Setting (± 0.5 bar)</td>
<td>Negligible</td>
<td>1.8</td>
</tr>
<tr>
<td>Temperature Setting (+ 0.25°C.)</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>6-APA Concentration (+ 0.1% w/v)</td>
<td>1.8</td>
<td>2.9</td>
</tr>
<tr>
<td>6-APA Holding Time (+ 15 mins.)</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Total Probable Uncertainty</td>
<td>5</td>
<td>7</td>
</tr>
</tbody>
</table>
The correlation curves show that the transport reduction factors are sensitive to protein concentrations in the range 2 - 100 mg./litre. At the higher protein concentrations, the scatter in the experimental points about the best straight line is considerable. However, when account is taken of the estimated ± 20% uncertainty in the protein concentration (165), and the uncertainties in the values of the transport reduction factors (table 45), the best straight line lies within the errors associated with the position of most of the points. Based on the established correlation, the probable error in a determination of protein concentration would be of the order of ± 40%. This degree of accuracy is adequate for distinguishing between 6-APA samples which are sufficiently low in protein content for conversion to semi-synthetic penicillins, and those which are not. In view of the rapidity of the test (30 mins.), its value for purposes of quantity control during manufacture of penicillins is therefore considerable.

4. GENERAL INVESTIGATION OF CORRELATIONS.

Although the correlations between trans-membrane water and 6-APA transport rates and protein concentration, which were established in the previous section, can be put to practical use, no theoretical basis for the form of the correlations has been proposed. In addition, it is not known whether the derived relationships are true generally, or whether they are specific for the 6-APA-enzymic protein system. It is also unknown whether the scatter in the results is due to inhomogeneity in the enzymic proteinaceous material, or to errors in determining its concentration by the amino-acid analytical method. The objective of the work in this section was to investigate these questions, and to propose an explanation
The effects of retained proteinaceous materials on the transport rates of smaller molecules across ultrafiltration membranes, was further investigated in the following aqueous solution systems: -

(i) sucrose solutions (pH 7.5) containing up to 100 mg./litre of ovalbumen, a protein of molecular weight 45,000.

(ii) 6-APA solutions (pH 7.5) containing up to 100 mg./litre of enzymic protein (protein-6-APA conjugate) which had been isolated from fermentation liquors containing 6-APA. These synthetic solutions are to be distinguished from the 6-APA-protein solutions previously investigated, in which the enzymic protein had not been isolated from the 6-APA after biosynthesis, and was co-precipitated during isolation of the 6-APA. Both proteins are in fact protein-6-APA conjugates (166). The solute and water transport reduction factors determined for these two systems are shown in fig. 49 and fig. 50 respectively.

(a) Comparison of Membrane-Forming Capacities of Different Protein Systems.

The isolated enzymic protein and ovalbumen have similar capacities for reducing the transport rates of water and of small solutes. Under the particular experimental conditions employed, it does not therefore appear that a proteinaceous film has the capacity to preferentially reduce the transport rate of the charged amino-acid over that of the neutral sucrose molecule.

In the presence of protein which has been co-precipitated with 6-APA, water transport rate is more markedly affected than when isolated.
enzymic protein is present at the same concentration. However, the gradient of the transport rate-protein concentration plot is the same in each case. Water transport rate thus extrapolates to zero at a lower protein concentration when the co-precipitated protein rather than the isolated enzymic protein is present. This suggests that in the case of the co-precipitated protein, the actual protein concentrations are higher than is indicated by the amino-acid analytical method. This is in fact partly true, since the 6-APA content of the 6-APA-protein conjugate is not included in the summation of the amounts of amino-acid derived from the macromolecular residue after dialysis (experimental section 1 (c) ). However, the amounts of bound 6-APA would normally be insufficient to explain the results (165).

A more likely explanation is that the protein which has been co-precipitated with 6-APA is in a more effective conformation for forming a transport-limiting film on the primary membrane surface. This view is supported by the fact that with the synthetic 6-APA-protein solution, the transport reduction factors did not increase with solution holding time. In addition, the factors were virtually identical to those determined for the sucrose-ovalbumen system. However, with solutions of the 6-APA-protein co-precipitates, the transport reduction factors decreased with solution holding time (fig. 48), tending towards the values for the synthetic solution. This suggests that history-dependent changes either in the conformation, or in the degree of association of the protein-6-APA conjugate, are responsible for the apparently superior membrane forming characteristics of the redissolved 6-APA-protein co-precipitates.
In the case of interfacial protein films formed from the 6-APA-enzymic protein co-precipitates, 6-APA transmission is also affected to a greater degree than with films formed from the isolated enzymic protein. This is again most likely due to the differences in the conformation or degree of association of the enzymic protein in the former instance.

It is evident from figs. 49 and 50 that with the synthetic protein solutions, there is much less scatter in the experimental points, about the best straight line. This suggests that in the un-purified 6-APA samples in which the enzymic protein content had been determined by amino-acid analysis, the variability of the results may be due to variations in the content of low molecular weight species. Such species would be retained by the cellulose acetate ultrafiltration membrane, but would be transmitted by the dialysis membrane during isolation of the macromolecular residue for amino-acid analysis.

(b) Theoretical Basis of Correlations.

(i) Water Transport Rate-Protein Concentration Correlation.

The fact that water transport rate appears to decrease inversely with the logarithm of the protein concentration suggests that a relationship of the form given in eqn. 58 might apply. However, the protein concentration at the interface, $c_s^w$, might be expected to increase with concentration in the bulk solution, whereas the form of the observed relationship would suggest that the boundary layer concentration is constant. Extrapolation of the transport rate to zero water transport rate indicates an interfacial concentration of the order of 7500 mg./litre. At this low
proteins concentration coherent gel formation is unlikely. It is possible that the apparently constant interface concentration is due to adsorption effects, and that above a critical bulk solution concentration (10 - 20 mg./litre), increasing concentration acts to reduce water transport by building up a thicker boundary layer, rather than by increasing the wall concentration.

(ii) Solute Transmission-Protein Concentration Correlation.

The water transport reduction factors and solute transmission reduction factors appear to depend more or less equally on protein concentration. Hence, at any protein concentration,\[ \frac{J_p}{T_p} = \frac{J_s}{T_s} = \text{constant} \quad (59) \]

Since from eqn.57, \( T_p \) is equal to \( (1 - \alpha) \), the fraction of total solvent carried by non-retentive pores, then,\[ \frac{J_p}{(1 - \alpha)} = \text{constant} \quad (60) \]

Since \( J_p \) decreases with increasing protein concentration, this implies that \( (1 - \alpha) \) must also decrease in proportion. The decrease in \( (1 - \alpha) \) could be due either to a reduced boundary-layer 6-APA concentration, resulting from the lower water transport rate, or to an increase in the number of solute retentive pores, due to the introduction of the proteinaceous membrane. The former effect would appear to be unlikely, since pure 6-APA transmission from a 2% w/v solution was found to be independent of flow velocity above a Reynolds Number of 2000. This suggests that concentration polarisation would not enhance solute transport under these conditions. In this work, since the Reynolds number which characterised flow was 3000, and since the water transport rates were lower, it is improbable that concentration polarisation effects were responsible for the observed decrease in 6-APA transmission with
decreasing water transport rate, i.e. with increasing protein concentration. It is therefore concluded that the effect of the protein is to decrease the number of solute permeable pores. This may be due to a mechanism which is similar to that proposed to explain the reduced 6-APA transport rate during reverse osmosis concentration. Initially, protein is adsorbed onto the cellulose acetate membrane surface, to form a uniform interfacial layer. Since access to the pore entrances will be restricted by the protein molecule network, solute transport will be decreased. The fact that water transport is also reduced significantly in the case of ultrafiltration suggests that viscous pore flow of water is also being decreased. In order to keep the ratio \( J_p/(1 - \alpha) \) constant, when the protein concentration increases, water flow in solute-permeable pores must decrease faster than the total water transport rate. This is reasonable, since a proteinaceous interfacial layer would be expected to influence water transport through the large solute-permeable pores to a greater extent than water transport through smaller pores which retain 6-APA.
CONCLUSIONS

Traces of heterogeneous, enzymic protein contaminants (4 mg./litre) improve 6-APA retention during reverse osmosis concentration of 6-APA solutions (20 g./litre), using cellulose acetate membranes. Since water transport rate is not significantly affected under the same conditions, the efficiency of the reverse osmosis separation is improved.

During studies of ultrafiltration of 6-APA - enzymic protein solutions, the following conclusions emerged: -

(i) the low molecular weight fraction of the heterogeneous protein material, which presumably consists of peptides, permeates the membrane.

(ii) transmission of such peptides decreases rapidly with increasing protein concentration.

(iii) transmission of 6-APA decreases with increasing protein concentration, but to a much lesser extent than peptide transmission.

(iv) Water transport rate decreases with increasing protein concentration.

Thus, while the purification efficiency of the process, i.e. the ratio of peptides to 6-APA in the permeate, is not markedly affected as the protein concentration increases, the rate of purification, which depends on water transport rate, decreases considerably.

If experimental conditions are closely controlled during ultrafiltration, correlations can be established between both water and 6-APA transport rates, and protein concentration. Such correlations
can be used for the rapid determination of protein in 6-APA, to an accuracy of about ± 40%.

The ability of the enzymic protein to reduce water and 6-APA transport rates depends on its history. In the case of enzymic protein which has been co-precipitated with 6-APA, this ability decreases with solution storage time. After 24 hours storage, the effect of this protein on the mass transport rate of smaller species approaches that observed with the same protein, but which had been isolated separately from 6-APA. The effect is then the same as that observed in the case of the well-characterised protein, ovalbumen. It is possible that conformational changes in the co-precipitated enzyme protein may be responsible for these effects.

The observed scatter in the experimental points about the line defining the correlation between mass transport rates and protein concentration, may be due to (i) uncertainties in the determination of protein in 6-APA-protein co-precipitates, (ii) time-dependent changes in the nature of the proteinaceous material (iii) variations in the molecular weight distribution of the protein. Much more accurate correlations are obtained with synthetic 6-APA-enzymic protein solutions, in which the protein has been separated from the 6-APA, and can be used to prepare solutions of known protein concentration.
1. ANALYTICAL PROCEDURES.

(a) Penicillin Intermediates and Derivatives.

The samples of 6-APA, penicilloic acid, 6-APA polymer, and 6-APA enzymic protein conjugate were supplied by Beecham Research Laboratories.

The 6-APA solutions (2% w/v) used in the reverse osmosis studies were filtrates of the fermentation liquor, in which benzylpenicillin had been enzymatically degraded to give 6-APA and phenylacetic acid. These solutions contained approximately 4 mg./litre residual enzymic or cell-wall protein.

(b) Determination of Specific Rotation of 6-APA.

6-APA (2.0 g.) was dried over phosphorus pentoxide in a vacuum desiccator for 2 days. The sample was then accurately weighed, suspended in water (50 ml.), and sodium hydroxide solution (10% w/v) added to bring the pH to 7.8. The solution was then made up to 100 ml. in a volumetric flask, and the optical rotation was determined using a Hilger polarimeter (2 dm.cell). The calculated values for the specific rotation, \([\alpha]_D^{20} \), (see eqn.49) are given in table 46. The measured specific rotation of pure 6-APA (samples 1 and 2) is in good agreement with the value of +337 ° reported in the literature (189). With the other 6-APA samples, the specific rotation is lowered to an average value +325 °. This is consistent with the presence of approximately 4% penicilloic acid in these samples (3 - 22).
TABLE 46: Enzymic Protein Content and Specific Rotation of 6-APA Samples.

<table>
<thead>
<tr>
<th>6-APA Sample No.</th>
<th>Protein Content (ppm)</th>
<th>Specific Rotation $\pm [\alpha]_{D}^{20^\circ}$ pH 7.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>335</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>337</td>
</tr>
<tr>
<td>3</td>
<td>70</td>
<td>325</td>
</tr>
<tr>
<td>4</td>
<td>110</td>
<td>325</td>
</tr>
<tr>
<td>5</td>
<td>120</td>
<td>---</td>
</tr>
<tr>
<td>6</td>
<td>135</td>
<td>326</td>
</tr>
<tr>
<td>7</td>
<td>150</td>
<td>326</td>
</tr>
<tr>
<td>8</td>
<td>165</td>
<td>327</td>
</tr>
<tr>
<td>9</td>
<td>165</td>
<td>325</td>
</tr>
<tr>
<td>10</td>
<td>175</td>
<td>327</td>
</tr>
<tr>
<td>11</td>
<td>175</td>
<td>324</td>
</tr>
<tr>
<td>12</td>
<td>190</td>
<td>328</td>
</tr>
<tr>
<td>13</td>
<td>215</td>
<td>328</td>
</tr>
<tr>
<td>14</td>
<td>215</td>
<td>324</td>
</tr>
<tr>
<td>15</td>
<td>380</td>
<td>323</td>
</tr>
<tr>
<td>16</td>
<td>500</td>
<td>320</td>
</tr>
<tr>
<td>17</td>
<td>550</td>
<td>322</td>
</tr>
<tr>
<td>18</td>
<td>700</td>
<td>325</td>
</tr>
<tr>
<td>19</td>
<td>900</td>
<td>318</td>
</tr>
<tr>
<td>20</td>
<td>1450</td>
<td>326</td>
</tr>
<tr>
<td>21</td>
<td>3500</td>
<td>321</td>
</tr>
<tr>
<td>22</td>
<td>5500</td>
<td>317</td>
</tr>
</tbody>
</table>
A mixed 6-APA-penicilloic acid solution (1.92% w/v 6-APA, 0.08% w/v penicilloic acid) was prepared at pH 7.8, and the optical rotation determined; a value of 6.49 (1 dm. cell,) was obtained. If the penicilloic acid were optically inactive, the polarimeter reading would be 6.44 (1 dm. cell, $[\alpha]^2^0_D = +335, 1.92\%$ w/v 6-APA). The higher observed value shows that the penicilloic acid contributes slightly to the rotation of the 6-APA-penicilloic acid mixture. This is consistent with the value of +30°, determined for the specific rotation of penicilloic acid in aqueous solution at pH 7.8. Thus, the incorporation of 4% penicilloic acid in 6-APA lowers the specific rotation to +325°, calculated on the basis that the sample is 100% 6-APA. This explains the apparent low specific rotations determined above. With these samples, the polarimeter reading was set to 6.49 (1 dm.cell) in order to adjust the combined 6-APA/penicilloic acid concentration to 2.0% w/v.

(c) Determination of Protein in 6-APA.
Analyses were carried out by members of staff at Beecham Research Labs. A sample of 6-APA (10 g.) was suspended in distilled water (100 ml.), and the pH adjusted to 7.8 with sodium hydroxide solution (10% w/v) to dissolve the amino-acid. The solution was placed in a Visking dialysis bag, and dialysed against tap water for 5 days. The macromolecular retentate was evaporated to dryness, and hydrolysed with hydrochloric acid (10 M) for 12 hours at 50 °C. The amino-acid mixture was resolved by ion-exchange chromatography, and the individual amino-acids determined colorimetrically on an auto-analyser. An amino-acid index was determined from the results, based on a summation of the number of the number of μmoles of each.
amino-acid present. 6-APA and other amino-acids such as glycine, methionine, penicillamine, penicillamine disulphide, etc., which could originate from 6-APA or 6-APA derivatives are not included in the summation. The protein content of a 6-APA sample (ppm) is obtained by multiplying the amino-acid index by 13.6, where 136 is the average molecular weight of the amino-acids present. Results of protein determination in the 6-APA samples used in this work are given in table 46.

2. MEMBRANE PREPARATION.

(a) Type I Membranes (Reverse Osmosis) (177).

Cellulose acetate (Eastman Kodak, E398 - 3, 300 g.) was dried over phosphorus pentoxide at 60 °C. for 24 hours. The polymer was dissolved in acetone (B.D.H., Analar Reagent, 1140 ml.) by placing the polymer-solvent mixture in a stoppered bottle, and continuously rolling the bottle for 24 hours at 50 °C. An aqueous zinc chloride solution (50% w/w) was prepared by dissolving zinc chloride (B.D.H., Technical Grade Reagent, 150 g.) in distilled water (300 g.) containing concentrated hydrochloric acid (B.D.H., Analar Reagent, 7.5 ml.). The solution was filtered through an acid-hardened (Whatman) filter paper before use. Zinc chloride solution (270 ml.), in aliquots 10 ml.), was added to the cellulose acetate solution, with vigorous shaking. The air bubbles were allowed to disperse from the casting solution before use.

A film of freshly prepared casting solution (0.30 mm. thickness) was spread over the surface of a clean glass plate using a thin-layer chromatography spreader. After a period of 45 seconds, the plate,
containing the cast film, was immersed in distilled water. The cellulose acetate solution gelled, and the membrane separated from the plate within 3 minutes. The active side of the membrane (uppermost on casting) was marked for later identification. The casting and immersion operations were carried out with the casting solution, glass plate, and immersion liquid at ambient temperature (20 °C). The membrane was cut into discs (47 mm diameter), which were heated between metal plates in a water bath for 15 minutes, at temperatures in the range 70 - 85 °C. The membranes were stored in dilute copper sulphate solution (1000 ppm).

(b) Type II Membranes (Ultrafiltration) (185).
Cellulose acetate (400 g.) was dissolved in acetone (2000 ml.), and zinc chloride solution (50% w/w, 660 ml.) added, as before. The casting procedure was identical to that for type I membranes, except that the cast film was allowed to stand in an undisturbed atmosphere for 250 seconds before being immersed in water. No heat treatment of the membranes is necessary.

3. FILTRATION STUDIES.
(a) Equipment.
The ultrafiltration studies were carried out using the equipment shown in figs. 41 and 42. In the reverse osmosis studies, the equipment employed was basically similar, except that it was necessary to substitute a high pressure pump (Milton-Roy Co., Duplex Diaphragm Pump, rated max. capacity 3.0 l/min., against a max. working pressure of 110 bar), and pressure controller (Fisher Controls Ltd., air-actuated pressure governor, max. working pressure 140 bar). In each case, temperature was controlled by adjusting the rate of flow of cooling water through the heat exchanger.
(b) **Preparation of Solutions.**

(i) 6-APA.

6-APA (20 g.), containing variable amounts of enzymic protein (10 - 4000 ppm) as contaminant, was suspended in water (500 ml.) at 20 °C., and sodium hydroxide solution (10% w/v) was added to bring the pH to 7.8. The volume was then made up to approx. 1000 ml. The 6-APA concentration was accurately adjusted to 2.00% w/v, by polarimetry, using the values for the specific rotation given in table 46.

(ii) **6-APA-Enzymic Protein Mixtures.**

Pure 6-APA (20 g.) together with various quantities of enzymic protein (2, 4, 10, 20, 40 mg.) were dissolved as described above, to give a 2.00% w/v 6-APA-protein solution at pH 7.8.

(iii) **Sucrose-Ovalbumen Mixtures.**

Sucrose (B.D.H., Analar Reagent, 20 g.) together with various quantities of ovalbumen (B.D.H., 5, 10, 20, 40, 60, 80, 100 mg.) were dissolved in water (500 ml.). Each solution was adjusted to pH 7.8 with sodium hydroxide solution (10% w/v), and the volume made up to 1000 ml. with water. The sucrose concentration was accurately adjusted to 2.00% w/v by polarimetry, using a value + 66.5° for the specific rotation.

(iv) **6-APA-Penicilloic Acid Mixtures.**

Pure 6-APA (sample 1, 19.2 g.) together with penicilloic acid (0.8 g.), were suspended in water (500 ml.) and the pH adjusted to 7.8 with sodium hydroxide solution (10% w/v). The volume was made up to 1000 ml. with water in a volumetric flask.
Pure 6-APA (sample 1, 19.99 g.) together with 6-APA polymer (0.01 g.) were suspended in water, and the pH adjusted to 7.8 with sodium hydroxide solution. After making the value up to 1000 ml. with water, the 6-APA concentration was adjusted to 2.00% w/v by polarimetry.

(c) Operating Procedures.

(i) Reverse Osmosis Studies.
The filtration system was thoroughly flushed with distilled water, and new membranes (type I cellulose acetate) inserted into the cells, ensuring that the active surface was in contact with the pressurised solution. Sodium chloride solution (0.4% w/v, 1000 ml.) was introduced with the fluid reservoir, and the solution filtered for 30 minutes, (temperature 20 °C., mean pressure 100 bar, solution flow rate 1500 ml./mn.). During this period, the permeate was recycled to the reservoir. After 30 minutes, the solution flow times were recorded by timing the passage of a given volume (5 ml.) in the inverted pipettes (fig.41). Samples of permeate and retentate were taken, and the sodium chloride concentration determined from conductivity measurements. 6-APA solution (2% w/v) was then subjected to reverse osmosis under the same operating conditions.

(ii) Ultrafiltration Studies.
In the preliminary studies, filtration equipment was thoroughly cleaned and new membranes (type II cellulose acetate) introduced into the cells. 6-APA solutions (2% w/v, 1000 ml.) were placed in the fluid reservoir, and ultrafiltered for 30 minutes (temperature 10 °C., pressure 20 bar, flow rate 1.5 litres/mn.). During
this period, the permeate was recycled to the reservoir. After ultrafiltration for 30 minutes, the solution flow times were measured and samples of ultrafiltrate and retentate were taken. The 6-APA content of the samples was determined by polarimetry, and the protein content was determined by amino-acid analysis.

In the studies of effects of protein concentration on mass transport rates, the filtration system was thoroughly flushed out with distilled water, and new membranes (type II cellulose acetate) inserted into the cells. Sucrose solution (2.0% w/v, 500 ml.) was introduced into the fluid reservoir, and recirculated under pressure (41.1 bar) for 5 minutes, in order to flush the water from the system. A further volume of sucrose solution (2.0% w/v, 500 ml.) was placed in the reservoir, and ultrafiltered for 30 minutes (temperature 15 °C., mean pressure 41.1 bar, solution flow rate 400 ml./min.). During this period the permeate was recycled to the reservoir. After filtration for 30 minutes, the solution flow rates were recorded, samples of permeate and retentate were taken, and the sucrose content was determined by polarimetry. Using the same membranes, the whole procedure was then repeated with 6-APA solutions (2.0% w/v, 500 ml.), containing variable amounts of protein, or with sucrose solutions containing various quantities of ovalbumen.
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Untersuchungen zur Charakterisierung von klinischen Dextranen

N. C. BEATON / K. P. STEINHOFF

Verfahren zur Produktion und Charakterisierung von Dextran

Dextran ist ein Polysaccharid, welches Glukose-Einheiten enthält, die vornehmlich in der α-1,6-Stellung miteinander verbunden sind (Abbildung 1). Natürliches Dextran wird aus Saccharose synthetisiert durch Anlegen von Kulturen bestimmter Bakterien, von denen insbesondere Leuconostoc mesenteroides kommerziell nutzbare Erträge ergibt. Der B.512-Stamm dieses Bakteriums ergibt ein Produkt mit einem hohen Prozentsatz (95%) α-1,6-Bindungen und wird allgemein für die weltweite Herstellung klinischer Dextrane gebraucht. Das Verhältnis von α-1,6-Bindungen und nicht α-1,6-Bindungen wird oft als "branching factor" (Verzweigungs faktor) bezeichnet. Nach Gabe von Dextranen mit weniger α-1,6-Bindungen (80 bis 90%) wurden ernste Nebenwirkungen bekannt. Das Polymer aus dieser Fermentation, das ein Molekulargewicht bis zu 50 Millionen erreichen kann, läßt sich durch Äthanol-Ausfällung leicht aus der Lösung isolieren. Für die klinische Anwendung ist ein viel niedrigeres Molekulargewicht wesentlich – Dextran mit hohem Molekulargewicht beeinträchtigt die normalen Blutgerinnungsmechanismen und kann eine Erythrozyten-Aggregation herbeiführen. Andererseits wird Dextran mit sehr niedrigem Molekulargewicht zu rasch aus den Nieren ausgeschieden, um wirksam werden zu können. Das natürliche Polymer

Abbildung 1: Strukturformel von Dextran-α-1,6-Glykosid-Bindung [6]
wird deshalb durch Säure-Hydrolyse teilweise depolymerisiert, so daß ein Produkt mit einem durchschnittlichen Molekulargewicht im Bereich von 60 000 bis 150 000 entsteht. Dieses Material besitzt jedoch immer noch einen weiten Streubereich von Molekulargewichten und ist in diesem Stadium noch kein zufriedenstellendes pharmazeutisches Produkt.

Es ist notwendig, das hydrolysierte Dextran durch nichtlösende Ausfällung einer Fraktionierung zu unterwerfen, und die in diesem Stadium angewandten Bedingungen entscheiden über die endgültige molekulare Beschaffenheit des klinischen Materials.

Eine ständige genaue Kontrolle über die Hydrolyse- und Fraktionierungsverfahren ist notwendig bei der Herstellung einer Dextran-Fraktion von einheitlicher Zusammensetzung, die sich für die pharmazeutische Anwendung eignet. Ferner muß jede hergestellte Dextran- Charge strengen Qualitätskontrollprüfungen unterzogen werden, um sicherzustellen, daß das Endprodukt den diesbezüglichen Spezifikationen entspricht. Es wurden verschiedene Verfahren für die angemessene Charakterisierung von polymeren Substanzen im allgemeinen entwickelt, und diese, für klinische Dextrane am besten anwendbaren Methoden, sind nachfolgend aufgeführt.

Klinische Dextrane enthalten ein Gemisch von Dextran-Molekülen mit verschiedenen Molekulargewichten, oder – mit anderen Worten – sie sind charakterisiert durch eine Molekulargewichtsstreuung. Die physikalisch-chemischen Parameter und Techniken, die für die Charakterisierung klinischer Dextrane am häufigsten Verwendung finden, sind daher folgendermaßen:

a) Gewichtsmäßig durchschnittliches Molekulargewicht (Mw): Der Mw-Wert von Dextran in der Lösung läßt sich entweder durch Untersuchungen der Sedimentation und Diffusion in der Ultrazentrifuge oder durch Lichtstreumessungen bestimmen; diese Methoden geben das Gewicht der vorhandenen Moleküle an. Die letztgenannte Methode gilt als besonders nützlich für Zwecke der Qualitätskontrolle der endgültigen pharmazeutischen Form klinischer Dextrane. Ein anderes häufig angewendetes Verfahren – die Viskositätsmessungen – ist nicht so exakt insofern, als man die

b) Zahlenmäßig durchschnittliches Molekulargewicht (Mn): Der Mn-Wert für Dextran-Fraktionen wird bestimmt durch Techniken wie die Osmometrie oder Endgruppen-Analyse, die die Zahl der vorhandenen Moleküle messen.

c) Molekulargewichtsverteilung: Das Verhältnis zwischen Mw und Mn hängt von der Form der Molekulargewichtsverteilung ab, wobei die beiden Durchschnittswerte nur für ein homogenes Polymer gleich, sonst aber Mw>Mn sind. Das Verhältnis Mw/Mn ist ein Maß für die Breite der Streuung, doch ist die aus diesem Verhältnis zu entnehmende Information begrenzt. Eine exakte Charakterisierung von klinischem Dextran auf der Basis seiner Molekulargewichtsverteilung ist nur möglich durch den Prozeß der Fraktionierung. Das Dextran wird in mehrere Fraktionen eingeteilt, von denen jede im wesentlichen ein einheitliches Molekulargewicht hat. Dann ist es möglich, eine Distributionskurve für die Probe anzufertigen (in Abbildung 2 mit Bezug auf den Gewichtsanteil jeder Spezies und ihrem Molekulargewicht). Zur Anfertigung von Distributionskurven werden häufig zwei Fraktionierungsverfahren angewendet, nämlich (a) die fraktionierte Ausfällung und (b) die Gel-Permeationschromatographie. Bei der erstgenannten Methode macht man sich die Tatsache zunutze, daß die Polymer-Löslichkeit mit steigendem Molekulargewicht abnimmt; die Zufügung eines nichtlösenden Mittels, z. B. Äthanol, zu einer wäbrigen Dextranlösung fällt zuerst das Material mit hohem Molekulargewicht aus, und bei weiterer Zufügung des Mittels werden Fraktionen mit forschreitend niedrigeren Molekulargewichten isoliert.

Das letztenannte chromatographische Verfahren ist schneller und einfacher für Routinezwecke und bedient sich der Trennung von Dextranmolekülen auf Größen-
Abbildung 2: Verteilungskurve für Dextran-Molekulargewichte
oben: Fraktion mit enger Verteilung; unten: Fraktion mit weiter Verteilung bei gleichem Mw

basis. Die Dextranlösung wird in eine Säule gebracht, welche das fraktionierende Medium (häufig Sephadex) enthält; dieses Medium besteht aus aufgetriebenen Gelpartikeln. Kleine Moleküle können rasch in das Gel diffundieren, so daß ihr weiteres Absinken in der Säule gehemmt wird. Größere Moleküle dagegen können nur in unterschiedlichem Ausmaß, abhängig von ihrer Molekulgröße, in das Gel eindringen. Die Moleküle werden also in der Reihenfolge der Molekulargewichtsverminderung ausgewaschen, und die aus der Säule gesammelten Fraktionen lassen sich zur Anfertigung der Molekulargewichtsverteilungskurve für die Probe verwenden. Der Wert des Verhältnisses Mw/Mn läßt sich ebenfalls aus den Daten errechnen.
Für praktische Zwecke der Qualitätskontrolle genügt es, die Molekulargewichtsverteilung mittels eines vereinfachten Fraktionierungsverfahrens zu bestimmen.

Bestimmung des Mw durch Lichtstreuung


licht eliminieren und die austretende Lichtintensität messen. Die Lichtintensität bei einem beliebigen Winkel $\alpha$, multipliziert mit $\sin \alpha$ zur Korrektur von Veränderungen in dem beobachteten Volumen, sollte dann konstant sein, zumindest im Bereich von 30° bis 150°, falls die optische Ausrichtung des Instrumentes korrekt ist.


### Tabelle 1 Rayleigh-Quotienten für Wasser

<table>
<thead>
<tr>
<th>Wellenlänge des Lichtes</th>
<th>Rayleigh-Quotienten, $R_{90°} \times 10^6$ (cm⁻¹)</th>
<th>Literatur</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blau, 436 nm</td>
<td>2,72</td>
<td>2,89</td>
</tr>
<tr>
<td>Grün, 546 nm</td>
<td>1,03</td>
<td>1,05</td>
</tr>
</tbody>
</table>

Bei Lichtstreuungsmessungen muß man das Auftreten von Effekten wie etwa Absorption, Fluoreszenz, optischer Depolarisierung und Asymmetrie der Streuung berücksichtigen und bei Bedarf entsprechende Korrektur-

Für Lichtstreuungsmessungen an Dextranen ist die Methode der Probenzubereitung von entscheidender Bedeutung. Da die Proben, insbesondere solche, die durch Äthanol-Ausfällung zubereitet wurden, Stoffpartikel enthalten können, ist es wesentlich, diese Lösungen vor der Messung ihrer Turbidität sorgfältig zu reinigen.

Autoklav-Erhitzung der Lösungen mit anschließender Filtration durch ein Millipore-Filter mit einem mittleren Porendurchmessen von 0,1 bis 0,2 µ hat sich als zufriedenstellendes Verfahren herausgestellt. Die Asymmetrie (45°/135°) von so zubereiteten wässrigen Dextranlösungen liegt unter 1,05.

Die exakte Messung von dn/dc, der Änderung des Brechungskoeffizienten mit der Konzentration, ist unerläßlich bei jeder Molekulargewichtsbestimmung durch Lichtstreuung, da sie in der Lichtstreuungsgleichung als ein Quadratwert im konstanten H vorhanden ist:

\[
\frac{H_c}{\tau} = \frac{1}{M} + 2 B \cdot c.
\]

Tabelle 2 Vergleich der dn/dc-Werte für Dextran in Wasser

<table>
<thead>
<tr>
<th>Wellenlänge des Lichtes</th>
<th>Gemessen ( \text{sen} ) Wert</th>
<th>Literaturwerte</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[1]</td>
<td>[2]</td>
</tr>
<tr>
<td>Blau, 436 nm</td>
<td>0,151</td>
<td>0,151</td>
</tr>
<tr>
<td>Grün, 546 nm</td>
<td>0,149</td>
<td>0,154</td>
</tr>
</tbody>
</table>

Bei den Messungen des Mw unter Verwendung verschiedener Lichtwellenlängen wurde ein Unterschied von 5% beobachtet; die aufgeführten Werte sind Mittelwerte von Messungen bei \( \lambda = 436 \text{ nm} \) (blau) und \( \lambda = 546 \text{ nm} \) (grün). Andere Untersucher stellten ähnliche Differenzen fest, doch der genaue Grund für die Diskrepanz ist nicht bekannt [10, 11]. Zur Errechnung des Mw für jede Wellenlänge wurden Turbiditätsmessungen von mindestens sechs verschiedenen Konzentrationen für erforderlich gehalten für eine genaue Extrapolation auf die Nullkonzentration. Eine geradlinige Extrapolation war bei allen Dextranen möglich, sofern die Konzentrationen unter 0,01 g/ml lagen. Die Reproduzierbarkeit von Messungen, die an demselben Lichtstreuungsphotometer unter Verwendung einer einzigen Eichtechnik durchgeführt werden, liegt im Bereich von ± 2 bis 3%. Allerdings liegt in Anbetracht der vielen möglichen, oben beschriebenen Fehlerquellen, von denen die meisten abhängig sind vom Instrument und von der Eichung, die absolute Unsicherheit mehr im Bereich von ± 7 bis 8%.

Ergebnisse der Dextran-Charakterisierung

Nach Feststellung der Vorsichtsmaßregeln, die für einen Erfolg bei der Anwendung von Lichtstreuungstechniken zur Molekulargewichtsbestimmung von Dextranlösungen notwendig ist, werden mehrere Chargen des klinischen Materials nach den beschriebenen Methoden charakterisiert. Die Resultate dieser Charakterisierungsuntersuchungen werden nachfolgend wiedergegeben, um den Wert der Lichtstreuungsmethode bei beiden detaillierten Produktcharakterisierungen zu veranschaulichen.
Die eingehenden Untersuchungen werden mit Dextran-40 (Schiwadex 40) durchgeführt, doch werden die Befunde der Lichtstreuungsmessungen bei Dextran-60-Produkten (Schiwadex 60) ebenfalls dargelegt.

Es wurde eine Dextran-40-Charge für die Untersuchung ausgewählt, ihr gewichtsmäßig durchschnittliches Molekulargewicht, ermittelt nach der Lichtstreuungsmethode, betrug 37 000. Das zahlenmäßig durchschnittliche Molekulargewicht wurde nach einer Endgruppenanalysenmethode bestimmt, bei der die Dextran-Endgruppen mit Borhydrid reduziert wurden, gefolgt von einer Oxydation mit Perjodat, wobei das Formaldehyd kolorimetrisch mit Chromotropsäure bestimmt wurde. Der nach diesem Verfahren ermittelte Mn-Wert war 28 000, was bedeutet, daß der Mw/Mn-Quotient gleich 1,3 ist. Zwar kann man schon von dem niedrigen Wert dieses Quotienten auf eine Dextranfraktion mit einem schmalen Spektrum der Molekulargewichtsverteilung schließen, doch ist eine genaue Charakterisierung des Produktes notwendig. Dies erfordert die Erstellung der Molekulargewichtsverteilungskurve für die Probe. Dem entsprechend wurde die Dextran-40-Charge eingeteilt in dreizehn Unterfraktionen, wobei Äthanol als nichtlösendes Mittel diente, um die einzelnen Fraktionen mit fortschreitend geringer werdendem Molekulargewicht auszufallen. Das Molekulargewicht jeder Fraktion wurde nach den oben beschriebenen Lichtstreuungsverfahren bestimmt und die Integral- und Differentialkurven der Verteilung angelegt (Abbildung 3).
Die Kurven demonstrieren das Fehlen von unerwünschtem Material mit hohem und niedrigem Molekulargewicht. Die Befunde deuten darauf hin, daß die oberen 10% der Fraktion ein mittleres Mw von 60 bis 70 000 und die unteren 10% ein Mw im Bereich von etwa 15 000 haben. Die aus den Fraktionierungsdaten errechneten Werte für Mw und Mn betragen 38 000 bzw. 29 000. Diese Zahlen und die Größe des Mw/Mn-Quotienten stimmen weitgehend mit den experimentell ermittelten Werten überein.


Die integralen Verteilungskurven für jede dieser Chargen sind im wesentlichen deckungsgleich; der Verlauf der Kurve ist in Abbildung 4 dargestellt. Die nach diesem Verfahren ermittelte Form der Molekulargewichtsverteilung stimmt weitgehend mit jener überein, welche bei der durch Äthanolausfällung fraktionierten Charge aufgezeichnet worden war.

Abbildung 4: Überlagerte Integral-Molekulargewichtsverteilungskurve für Schiwadex-40-Dextran-Chargen: Fraktioniert durch Gel-Permeations Chromatographie (Sphadex)

Weitere Chargen von Schiwadex-40 wurden ebenfalls durch Lichtstreuungsbestimmungen des gewichtsmäßig durchschnittlichen Molekulargewichtes charakterisiert. Zusätzlich wurden die oberen und unteren 10% jeder Probe durch Ausfällungsverfahren mit Äthanol heraustrennten und die Mw-Werte dieser Unterfraktionen
gemessen. Die Mittel- und Streuwerte des Mw für die Gesamtprobe sowie die oberen und unteren 10-%-Fraktionen sind:

<table>
<thead>
<tr>
<th>Dextran-Fraktion</th>
<th>Mw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gesamtprobe</td>
<td>40 000 ± 4 000</td>
</tr>
<tr>
<td>Obere 10%</td>
<td>67 000 ± 6 000</td>
</tr>
<tr>
<td>Untere 10%</td>
<td>16 000 ± 2 000</td>
</tr>
</tbody>
</table>

Aus diesen Ergebnissen wird gefolgt: Schiwadex-40-Dextrane sind gut fraktionierte Produkte und Abweichungen der molekularen Zusammensetzung von Charge zu Charge sind gering.


Tabelle 3 Dextran 60 Charakteristika

<table>
<thead>
<tr>
<th>Dextran-Fraktion</th>
<th>Gesamtprobe</th>
<th>Obere 10%</th>
<th>Untere 10%</th>
<th>Osmometrie</th>
<th>End-Gruppe Analyse</th>
<th>Mw/Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charge 1</td>
<td>62 000</td>
<td>119 000</td>
<td>23 000</td>
<td>46 500</td>
<td>—</td>
<td>1.33</td>
</tr>
<tr>
<td>Charge 2</td>
<td>56 000</td>
<td>114 000</td>
<td>20 000</td>
<td>43 000</td>
<td>—</td>
<td>1.30</td>
</tr>
<tr>
<td>Charge 3</td>
<td>55 000</td>
<td>92 000</td>
<td>26 000</td>
<td>43 400</td>
<td>46 000</td>
<td>1.27</td>
</tr>
<tr>
<td>Charge 4</td>
<td>64 000</td>
<td>119 000</td>
<td>24 000</td>
<td>49 100</td>
<td>—</td>
<td>1.30</td>
</tr>
<tr>
<td>Charge 5</td>
<td>63 000</td>
<td>111 000</td>
<td>24 000</td>
<td>45 800</td>
<td>—</td>
<td>1.38</td>
</tr>
</tbody>
</table>

* Obere 10% und untere 10% beziehen sich auf die höchsten bzw. niedrigsten Molekulargewichtsfraktionen, die zwischen 5% und 10% der gesamten Dextran-Charge enthalten.

Auch hier wurde das Fehlen großer Mengen von Stoffen mit hohem und mit niedrigem Molekulargewicht, welche ungünstige klinische Wirkungen haben könnten, demonstriert. Das Verhältnis von Mw/Mn lag in dem Rahmen 1.27 bis 1.38, was eine weitere Bestätigung
dessen ist, daß die Molekulargewichtsverteilung genügend eng ist für eine klinische Anwendung des Produktes. Schiwadex-Dextrane werden unter Verwendung des B.512-Stammes von Leuconostoc mesenteroides hergestellt. Dextran-Proben werden regelmäßig von der Routineherstellung genommen und durch periodische Titration auf ihren Prozentsatz an α-1,6-Bindungen untersucht. Die veröffentlichte Abbildung über 95% α-1,6-Bindung ist bestätigt worden [7].

Bis andere Informationsmöglichkeiten verfügbar werden und internationale Spezifikationen für Dextran-Produkte festgesetzt werden, muß der Dextran-Hersteller auf diesem Wege detaillierte Charakteristika der Marktprodukte liefern, damit der Arzt voll informiert ist über die molekulare Beschaffenheit des betreffenden Dextrans. Deshalb befaßte sich dieser Artikel hauptsächlich mit einer Dextran-Charakterisierung durch exakte Messungen des Molekulargewichts in Dextran-Fraktionen durch Lichtstreuungsmethoden. Nur durch molekulare Charakterisierungsverfahren dieser Art lassen sich optimale Spezifikationen für ein klinisches Dextran systematisch auswerten.

Zusammenfassung

Die molekulare Beschaffenheit von Dextranfraktionen bedarf einer Charakterisierung, bevor das Produkt zur klinischen Anwendung auf den Markt gebracht wird. Als Verfahren zur Bestimmung des durchschnittlichen Molekulargewichtes (Mw) erweist sich die Technik der Lichtstreuung für diesen Zweck als besonders nützlich; die verschiedenen Vorsichtsmaßregeln, die zur Gewährleistung der Genauigkeit bei dieser Methode notwendig sind, werden erörtert. Die bei Untersuchungen von klinischen Dextranen (Schiwadex 40 und 60) ermittelten Daten über das Mw und die Molekulargewichtsverteilung werden dargelegt, um den Wert des Lichtstreuungsverfahren bei der detaillierten Produktcharakterisierung zu veranschaulichen.

Literatur


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