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# FINE STRUCTURE OF (1→3)-β-D-GLUCANS: CURDLAN AND PARAMYLON

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## ABSTRACT

Paramylon, a natural (1→3)-β-D-glucan found in *Euglena gracilis*, and curdlan, a "regenerated" (1→3)-β-D-glucan found in *Alcaligenes faecalis*, have been studied. Differences in chemical and physical properties are compared to each other and it is concluded that this system is a "native–regenerated" pair just as the well known "native cellulose–regenerated cellulose" system. X-Ray diffraction and density measurements indicate for paramylon a very high level of crystallinity, approaching 90%, whereas curdlan powder is only 30% crystalline. The effect of hydrolytic treatment on the crystallinity of the different samples shows the same trends as for cellulosic materials. From the negative birefringence of annealed fibers and positive birefringence of the paramylon granules, a tangential disposition of the chains in the granules may be concluded. Microfibril formation from curdlan precipitated from solution is in line with a proposed triple-helical structure for the crystalline form of this polysaccharide.

## INTRODUCTION

The molecular basis of the fine structure of natural polysaccharides may be explored by comparing polysaccharides in the glucan family. As D-glucose is Nature's most abundant carbohydrate, it is not surprising that a wide range of linear glucans exists, that differ only as regards the nature of the glycosidic linkage. The well known morphological differences between starch and cellulose may be related to the conformational features of the α-D-(1→4) versus the β-D-(1→4) linkage. However, our ability to interpret the chemistry–fine-structure relation in this important field of biopolymers is dependent on extensive exploration of fine structure in glucans other than cellulose and starch. The (1→3)-β-D-glucans would be a logical sequel to the extensive studies on cellulose and starch. They are found in many vegetable and fungal cell-walls, and their chemical compositions are well established[1,2]. (1→3)-β-D-Glucans are also found as the extracellular, bacterial polysaccharide curdlan from the organism *Alcaligenes faecalis*, and as the storage granule paramylon in *Euglena gracilis*. These two forms of (1→3)-β-D-glucan are the object of this study.

Preliminary X-ray studies on cell walls of yeasts and fungi by Kreger *et al.*[3–5]

and by Picciolo[6] provided the first information on the unit-cell parameters and the presence of polymorphs. More recently, X-ray diffraction evidence for a triple helical structure as the basic crystalline organization[2,7,8] has been forthcoming. Stone and Clarke[9] have shown that paramylon granules may constitute as much as 25% of the total weight of the cell, and it is now apparent that the granular form and other properties of paramylon show that it has the same biological functions as starch.

The present effort is aimed at comparing the fine-structural characteristics of "regenerated" solids from a commercial (1→3)-β-D-glucan, curdlan, with those of a native (1→3)-β-D-glucan, paramylon. In this way, information similar to that available in the "native–regenerated" cellulose area will be developed. This type of information is valuable for interpreting relative enzyme-accessibility and comparative mechanical-property data.

## EXPERIMENTAL

*Materials.* — Water-insoluble granules of paramylon extracted from depigmented *Euglena gracilis* were obtained by courtesy of Dr. B. A. Stone (La Trobe University, Australia) and Dr. Miyatake of the Osaka Prefecture University, Japan. The isolation of these granules has been described[9]. Curdlan, a pure polysaccharide powder, was furnished by Takeda Chemical Co., Japan. Proof of the linear (1→3)-β-D-glucan structure, fiber preparation, the different polymorphs, and gelation observations on this bacterial polysaccharide have been reported earlier[7].

*Procedure.* — X-Ray diffraction patterns were recorded on Ilford "b" film, with CuKα radiation from a Philips "fine focus" tube operated at 40 kV and 20 mA. The film-to-sample distance (~50 mm) was calibrated with sodium fluoride powder.

The scanning electron microscopy was performed at the Pulp and Paper Research Institute of Canada, with a Cambridge Stereoscan microscope, MARK IIA. Transmission electron microscopy was performed at L'Université de Montréal with a Philips EM 300 transmission electron microscope. Samples were negatively stained with 1% phosphotungstic acid solution, or shadowed with tungsten. Small-angle light-scattering on Paramylon powders dispersed in chlorobenzene was performed with an apparatus previously described[10]. Density measurements were performed by flotation in a xylene–carbon tetrachloride density-gradient column calibrated with hollow, glass beads of known density.

Acid hydrolytic studies for determining "weight loss" involved boiling the samples (1% w/w) in 2.5M hydrochloric acid under reflux. The weight loss after 15 min of hydrolysis under these conditions was determined gravimetrically. In the case of fibers, the degree of orientation and crystallinity was evaluated before and after hydrolysis.

Stress–strain curves for curdlan fibers were recorded with an Instron Universal Testing Instrument, Table Model 1130.

TABLE I

HYDROLYTIC WEIGHT-LOSS AND DENSITY OF DIFFERENT SOURCES OF (1→3)-β-D-GLUCAN

| *Sample* | *Density (g/cm³)* | *Hydrolytic weight loss (%)* |
|---|---|---|
| Curdlan powder | 1.44 | 55 |
| Paramylon powder | 1.53 | 9 |
| Curdlan fibers "as spun" | 1.45 | 35 |
| Curdlan fibers, annealed hydrate form | 1.47 | 30 |
| Curdlan fibers, annealed dry form | 1.49 | |
| Hydrolyzed curdlan fiber, annealed dry form | 1.510 | |
| Hydrolyzed curdlan fiber, annealed hydrate form | 1.505 | |

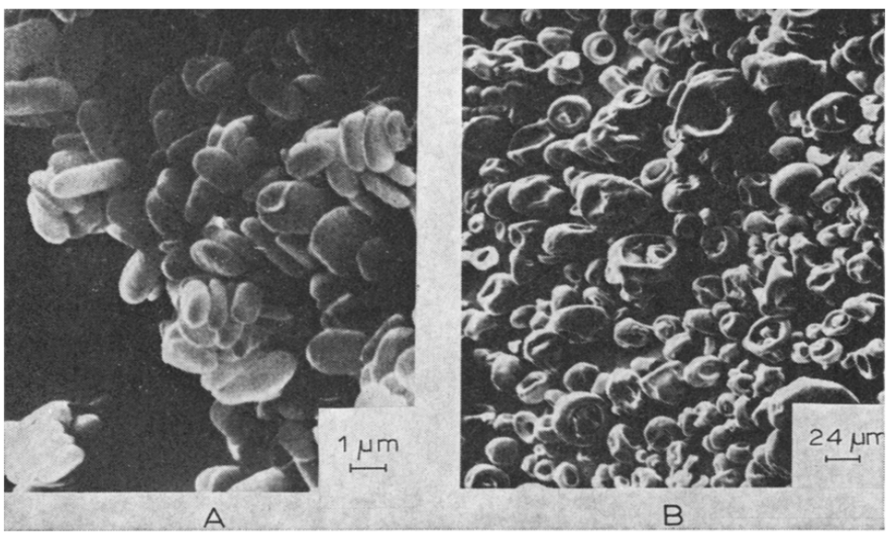


Fig. 1. Scanning electron micrographs of "as received" powders: A, paramylon; B, curdlan.

RESULTS AND DISCUSSION

The results for the two systems studied, paramylon and curdlan, are reported in parallel so that a constant comparison may be made of the two materials. For curdlan, $^{13}$C-n.m.r. analysis has established its chemical homogeneity[11]. Paramylon has not been similarly studied, but the X-ray data suggest that it is identical in chemical purity and structural homogeneity. Molecular-weight information on curdlan indicates a $\overline{DP}_n$ of 540.

The densities of the different samples are listed in Table I, together with the hydrolysis results. The theoretical density of the "dry" crystal form, calculated with the unit cell previously published[7], is 1.548 g/cm$^3$.

*Microscopy and X-ray diffraction.* — Pictures (Fig. 1) by scanning electron microscopy show the oblate elliptical form of the paramylon granules and the col-

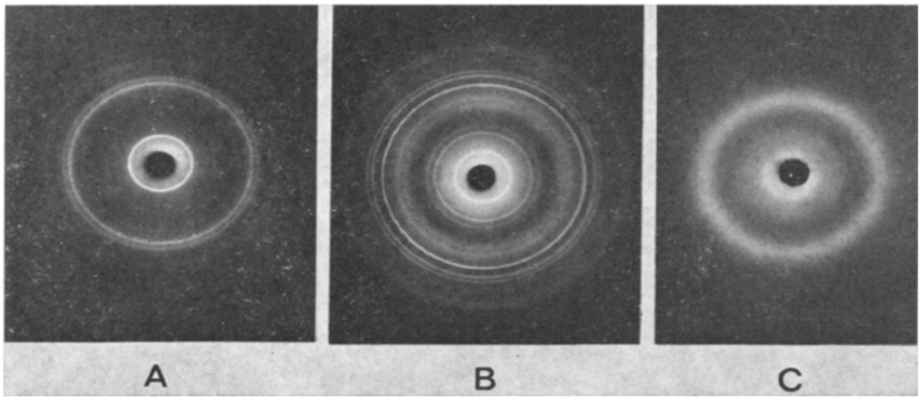


Fig. 2. X-Ray powder diagrams of samples: A, paramylon powder "as received"; B, paramylon powder after the annealing treatment; C, curdlan powder "as received".

lapsed or invaginated form of the curdlan grains. The former are native granules found inside the cells[13,14]. The latter, much larger, are regenerated granules obtained by spray-drying and aqueous solution of curdlan derived from a culture of *Alcaligenes faecalis*[12].

As reported[7], (1→3)-β-D-glucan shows two crystalline polymorphs, according to the relative humidity (r.h.). Below 20% r.h., a different X-ray pattern is observed compared to the one obtained at a higher percent r.h. The difference in unit-cell dimensions arises from the presence of water molecules in the lattice of the hydrate form. According to the density measurements, the hydrated form would contain 2 water molecules per glucose residue, whereas the "dry" form would not have any. For the curdlan fibers, the crystalline transformation from "dry" to "hydrate" occurs under the influence of relative humidity, and the transformation is always complete if X-ray observations are made above 80% r.h.

X-Ray powder diagrams of both systems are shown in Fig. 2. The "d" spacings of the "as received" paramylon granules (pattern A) correspond to the "dry" form of the (1→3)-β-D-glucan. This result was surprising, as the sample had had a long period of exposure to high, relative humidity. In fact, even if paramylon is immersed in water for several weeks at room temperature, the hydrated form is not obtained. The granules must be annealed in water, in a sealed bomb at 140°, to be transformed into the hydrated form (pattern B). The shape of the granules is the same after the annealing treatment, although some swelling of the granules was observed.

The higher crystallinity of the paramylon granules, as compared with curdlan (pattern C), is the reason for this difference in ease of hydratability. The high crystallinity does not permit accessibility of water without a heating treatment. The latter allows the water molecules to penetrate into the crystallite, a phenomenon similar to the resistance[15] of native cellulose to transformation from cellulose I to cellulose II. Under no circumstances could curdlan powder be transformed to a level of crystallinity equivalent to that of paramylon.

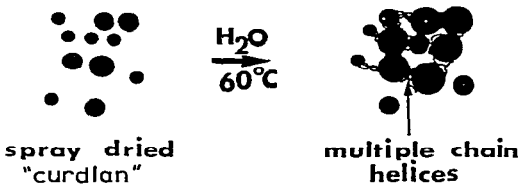


Fig. 3. Gelation mechanism of curdlan powder in water; free chains at the surface of curdlan particles interact at 60° to form "pseudo-crosslinks".

*Gelation phenomenon.* — The gelation properties of paramylon and curdlan are quite different. When curdlan powder is placed in water (2–4%) and heated, a resilient gel is formed[12] at about 55°. Paramylon, on the other hand, does not form a gel, even at higher temperature. The strong crystallinity, which prevents solubilization, explains the lack of gelation properties of the granules under conditions comparable to those successful with curdlan powder.

The gelation phenomenon for curdlan is one of its important physical properties. However the non-homogeneity of the gel in question should be noted, as well as the irreversible character of the gelation reported previously[12]. When the resilient gel is observed in a polarizing microscope, a weak birefringence is apparent, as is also the case for the "as received" powder particles. It appears that the pseudo-crosslinks between these particles must be in the form of molecular junctions arising from the formation of multiple-chain helices between surface-solubilized chains of adjacent particles.

A model to explain the irreversible gelation phenomenon is shown in Fig. 3. It should be stated that the gelation observed for curdlan is reminiscent of that for starch; starch is a native granule, but of rather poor crystalline perfection. The lack of gelling behavior in water, observed with paramylon, places this native granule in a class apart from the starches. The latter form rather weak gels compared to those from curdlan, which are properly described as resilient[12].

A uniform gel of curdlan may readily be formed by dialysis against water of curdlan in 0.3M sodium hydroxide. The stiffness and turbidity of the gel is a function of the original concentration of the curdlan solution. Concentrations of curdlan from 0.05–3% were used. The former gave such a weak gel that it could readily be dispersed mechanically, and the structural elements examined by transmission electron microscopy after negative staining with uranyl acetate (Fig. 4). The appearance of the structural elements is distinctly microfibrillar. The average diameter of ~200 Å and "fringed" appearance suggests that a number of triple-helical systems are in parallel alignment along the fibril direction. Under negative-staining conditions, the fibrils display a twisted or helical ribbon-texture, which may indicate a propensity for formation of a superhelix.

*Light scattering.* — Small-angle light-scattering studies on solid materials have been particularly useful for examining the anisotropic characteristics of starch granules[16]. For this reason, the scattering properties of paramylon granules and

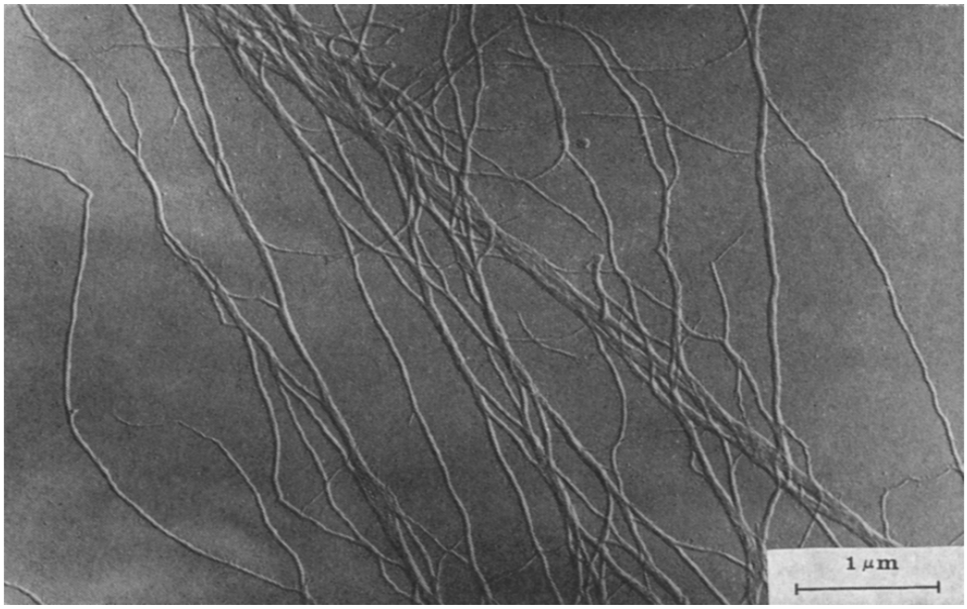


Fig. 4. Transmission electron micrograph of shadowed microfibrils from a dialyzed solution of curdlan in 0.3M sodium hydroxide.

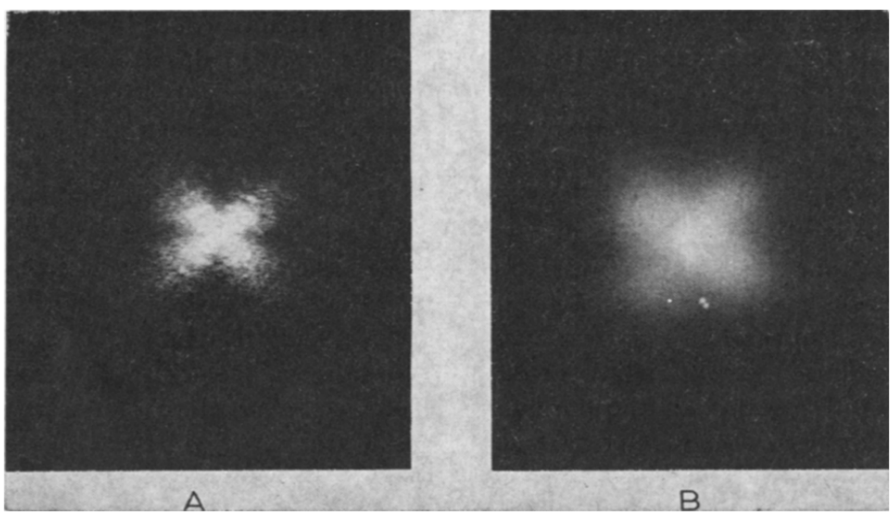


Fig. 5. Small-angle, laser, light-scattering pattern, $H_v$ conditions, from (1→3)-β-D-glucan powders: A, curdlan (film-to-sample distance, $R$ = 27.5 cm); B, paramylon ($R$ = 7 cm).

"as received" curdlan powder were observed by this method. The study involved $H_v$ scattering conditions, that is, scattered, monochromatic radiation from vertically polarized, incident light is filtered through a crossed analyzer before recording of the scattering envelope on a flat Polaroid film. This condition of observation is sensitive to fluctuations in anisotropy and to spherulitic structure, which gives rise

to a "four-leaf clover" scattering-envelope[10]. From the radial position of the scattering maximum, the average particle-diameter (the incident beam covers 50–100 granules) may be calculated. The scattering observations were conducted with particles suspended in chlorobenzene ($n = 1.523$) as suspending fluid.

The two pertinent $H_v$ scattering-patterns are shown in Fig. 5. The one for curdlan shows considerable "speckling", as is often the case for such systems[17]. Nevertheless, from the observable maxima at 45° azimuthal angle, it was possible to calculate that the average particle-diameter was 46 $\mu$m. For paramylon, the scattering maximum was much less distinct, and only an approximate value for the average diameter could be obtained, namely 3 $\mu$m.

The important observations relate to the general appearance of the scattering envelopes under $H_v$ scattering conditions. Whereas the curdlan powder seems to show the typical "four-leaf clover" appearance corresponding to a collection of rather irregular spherulites, this is not so for paramylon. The scattering envelopes for the latter never showed distinct maxima; rather, the impression of superposed "rod-like" and "spherulite-like" scattering was conveyed from a qualitative examination of the scattering pattern[18]. This observation would be in keeping with scattering from a collection of anisotropic disks seen either "edge on" or "face on". This hypothesis was confirmed by observation of the granules in suspension in a non-swelling liquid of matching refractive-index. Between crossed polarizers, two different, regular extinction-patterns were found: a classical Maltese cross, which would correspond to the "face on" view, and two hyperbolae symmetrically at each end of the disk. The latter correspond to the "edge on" view, as the hyperbolae would be positions of pseudo-isotropy in transmission for the edge-viewed object[28]. The unanswered question is the chain orientation inside the disks.

*Sign of birefringence and fibrillar structure.* — The sign of the birefringence was measured for paramylon granules as well as "as spun" and annealed fibers. A polarizing microscope equipped with a quarter-wave plate and the "Michel–Levy" color chart was used. Under these conditions, paramylon granules displayed the same color distribution as starch granules, which are known to have a positive birefringence[19]. This observation means that the radial refractive-index is greater than the tangential index.

"As spun" fibers also display positive birefringence, whereas annealed fibers display negative birefringence, indicating that the principal refractive-index for the latter is perpendicular to the fiber axis. This birefringence has the same sign as that observed for the microfibrils of (1→3)-$\beta$-D-xylan in the cell walls of siphoneous green algae[25].

According to these observations and in view of the similarity of the X-ray diffraction data for paramylon and "annealed" curdlan fibers, it may be concluded that the chains are arranged tangentially in the granules. It should be noted that Holt and Stern[14], and also Guttman[13], reported the presence of concentric layers ~200 Å thick as a substructure in granules of paramylon.

Transmission electron microscopy performed on (1→3)-$\beta$-D-glucan, synthesized

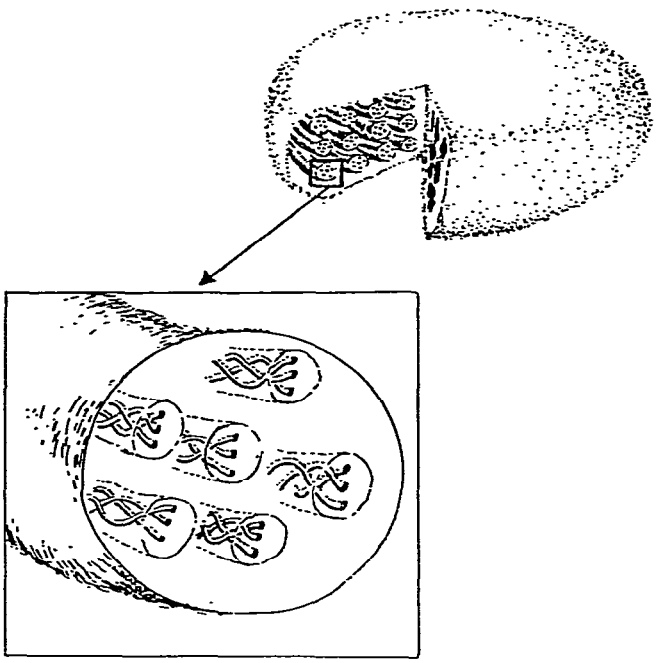

Fig. 6. Schematic representation of a paramylon granule, showing layering and the tangential chain-orientation.

*in vitro* by cell-free extracts of various plants and fungi[20], shows microfibrils ~300–400 Å thick. Similar microfibrils were observed after hydrolytic treatment of fungal cells-walls[4] and, although those microfibrils are not very crystalline, orientation of chains along the axis of the microfibrils has been established by electron diffraction[21]. For these reasons, a "microfibrillar ultrastructure" in the layers in paramylon granules appears the most probable hypothesis.

Our final model for the paramylon granule is illustrated in Fig. 6, and consists of layers of tangentially disposed cylinders. The cylinders may be individual chains or agglomerates of chains that form a microfibril.

Finally it should be noted that callose, a (1→3)-β-D-glucan found as a deposit in wood, has been reported to be isotropic[26]. In view of the change in sign of birefringence in passing, via annealing, from curdlan of low crystallinity to a form of higher crystallinity, this property is not in cont adiction with our observations. It was not possible to establish the sign of the birefringence of "as received" or annealed curdlan powder because of the irregular shape.

*Acid hydrolysis.* — Acid hydrolysis was performed for powders and fibers under the conditions previously described. Table I gives comparative weight losses and densities of different samples. The weight loss is related to the density of the samples. The lower the density, the lower the crystallinity and the more effective the hydrolytic treatment for degrading a quantity of the polysaccharide.

Removal of the amorphous region in the fiber breaks "pseudo-crosslinks"

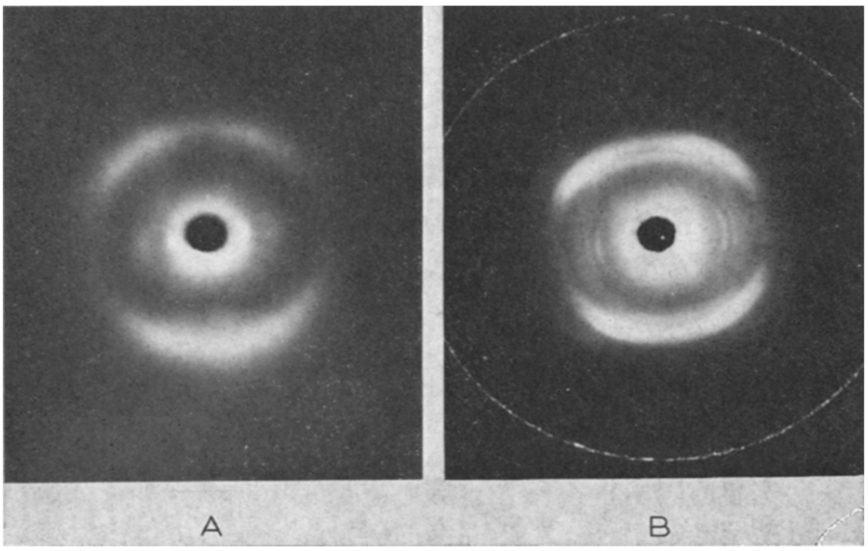


Fig. 7. X-Ray fiber diagrams, showing the effect of acid hydrolysis on "as spun" fibers: A, before hydrolysis; B, after hydrolysis. The crystalline forms correspond to the "A" and "B" polymorphs, respectively[7], of curdlan.

TABLE II

MECHANICAL PROPERTIES OF CURDLAN FIBERS

| *Sample* | *Breaking strength (g/den)* | *Breaking elongation (%)* | *Stiffness (g/den)* |
|---|---|---|---|
| "As spun" | 0.6→0.8 | 40→50 | 12 |
| "Annealed" | 1→1.5 | 15→18 | 25 |

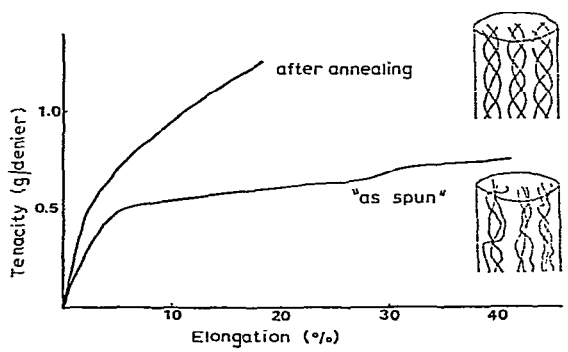


Fig. 8. Stress–strain curves of annealed and "as spun" curdlan fibers.

between the chains inside the fibers and allows the free chains to crystallize[22,23]. At 105°, hydrolysis has the same effect as the annealing treatment performed at 145°. Fig. 7 illustrates the effect of the acid hydrolysis on "as spun" curdlan fibers. The same treatment performed on annealed fibers does not change the crystallinity.

Taking 1.548 as the density of the completely crystalline material and 1.40 as that of completely amorphous, paramylon having a density of 1.53 would be 88% crystalline. This value is found by solving the equation

$$1.53 = 1.548(\alpha) + 1.40\,(1 - \alpha),$$

where $\alpha$ is the crystalline fraction of the compound. Curdlan powder, on the other hand, is 30% crystalline.

*Mechanical properties of fibers.* — Table II shows the mechanical properties of curdlan fibers, measured at room temperature. The stress–strain curves appearing in Fig. 8 show two different sets of behavior. The curve for "as spun" fibers resembles the stress–strain behavior of wool, whereas that of the "annealed" fiber is more silk-like.

The difference between the two samples arises from differences in the proportion of crystalline material and degree of orientation, as confirmed by the X-ray patterns and by the relative weight-loss after acid hydrolysis. As reported in a previous paper[7], the unit cell of the "as spun" fiber is larger than that of the "annealed" fiber, which may also contribute to the observed differences.

A notable feature of the fiber studies presented here was the high level of orientation of the "as spun" fiber. As the spinning system was of "low shear", this result was rather unexpected. It would appear that some anisotropy or liquid-crystalline organization may be operative in the 10% dimethyl sulfoxide solution used for spinning. In fact, a syringe extrusion as used in preparing fibers for this study[7], corresponds to a system giving rise to extensional flow[24]. No optical anisotropy was noted, however, in the quiescent solution in dimethyl sulfoxide.

## CONCLUSIONS

The high level of crystallinity of paramylon is comparable to that of *Valonia* cellulose, which is the most crystalline, native cellulosic material yet known. On the other hand, curdlan, which crystallizes during drying of its solution, may be compared to regenerated cellulose (rayon), where a lower degree of crystallinity is usually found as compared with the native state. Thus, the paramylon–curdlan system may be compared with the *Valonia*-regenerated cellulose system, with the exception that both crystal structures for the former are the same, whereas the latter display two different crystal forms: cellulose I and II. Furthermore, hydrates are not found with cellulose, whereas curdlan crystallizes as "hydrated" and "dry" polymorphs. Likewise, acid hydrolysis promotes recrystallization in the two systems, the effect being most pronounced for a regenerated sample of low crystallinity[23]. Concerning this effect, the presence of crystalline (1→3)-β-D-glucan in the cell walls of yeasts and

fungi[3-5] may be affected by the purification process, which involves a 3-h extraction with boiling, 2% hydrochloric acid. Thus, the native form could be less crystalline than that observed after hydrolysis, or it may be a different polymorph.

The microfibrillar texture of native cellulose is literally its signature. In the case of (1→3)-β-D-glucan, only the regenerated form, namely curdlan gels, have demonstrated the tendency of this polysaccharide to form microfibrils[12]. Published reports[4,20] indicate, however, that fibrils are a constituent of nascent (1→3)-β-D-glucan.

For paramylon, allusions to a fibrillar organization are frequently made[2]. In general, paramylon is similar to starches in its overall texture, except for the exceedingly well developed crystallinity, wherein it resembles native celluloses, especially that from *Valonia*.

Curdlan powder is reminiscent of erythrocytes (Fig. 1), because of the invaginated appearance of the particles. As these were spray-dried from a dilute solution[27], it is assumed that a hollow sphere is formed around the droplets initially and some of these collapse on drying. The shape of curdlan powder and its fine structure is therefore expected to vary with the method of drying. On the other hand, paramylon particles have a shape similar to that of certain starches[19], namely, disk-like. However the tangential chain-orientation is opposite to that established for starches.

The heterogeneity of the irreversible curdlan gels should be noted. In this respect, they resemble those from starch, but are much stronger: "resilient". The reason for this must be related to the strength of the intermolecular interactions, that is, multiple-chain helices. The effect of heat treatment on these gels is to bring about syneresis and the development of crystallinity. This result emphasizes that a high degree of crystallinity is incompatible with a gel structure based on crystallites as pseudo-crosslinks.

There is a tendency to seek out structure–function relations in all biopolymers. However, (1→3)-β-D-glucan frustrates this objective as it is found to play both a structural and a storage role. This polysaccharide is rarely found outside species of primitive organisms. Its properties make it an excellent polyfunctional compound for non-specialized organisms. As the cells became more and more sophisticated, Nature replaced it by more-specialized polysaccharides, such as cellulose or starch, which can perform their function more efficiently.

Extensive studies of the paramylon–curdlan system, and of other polysaccharides, are needed to correlate chemical and morphological properties with biological function. Certainly the present data for paramylon and curdlan offer convincing evidence of a native–regenerated granular pair to be compared with the fibrous celluloses.

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