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ISOLATION, PARTIAL CHARACTERIZATION, AND BIOLOGICAL PROPERTIES OF POLYSACCHARIDES FROM CRUDE PAPAIN

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ABSTRACT

Two polysaccharides have been isolated from crude papain by precipitation of papain with ammonium sulfate, further precipitation of other proteins with trichloroacetic acid, and chromatography of the supernatant on DEAE-cellulose. The first polysaccharide to be eluted, designated PP-I, contained D-glucuronic acid, D-glucose, D-galactose, L-arabinose, and L-rhamnose, in the approximate molar ratios of 4:1:12:10:4. The other (PP-II), eluted at a higher salt-concentration, contained the same sugars (with about one-third less glucose and more uronic acid) in the approximate molar ratios of 13:1:40:26:12. Reduction of the uronic acid groups of PP-II produced a polysaccharide (PP-II-R) containing the same sugars in the approximate molar ratios of 2:11:37:28:12. Hydrolysis of a mixture of the two polysaccharides yielded an aldobiouronic acid, D-glucosyluronic acid-D-galactose. Neither polysaccharide preparation contained protein. These polysaccharides dramatically affected aggregation and alignment of normal human fibroblasts but had no effect on a mouse embryo fibroblast aneuploid cell-line that does not exhibit contact inhibition of growth or movement. In aggregating cells, these polysaccharides caused the cells to behave as contact-inhibited cells, that is, cell division and nuclear area were decreased.

INTRODUCTION

During an investigation[1] to determine whether qualitative or quantitative changes in glycosaminoglycans of fibroblasts occur as a function of aging, we used crude papain (Sigma Type II) for their isolation. When the isolated glycosaminoglycan preparation was fractionated by the procedure of Schiller *et al.*[2] and then subfractionated by column chromatography on DEAE-Sephadex[3], we found an acidic polysaccharide, different from hyaluronic acid, in fraction I-A, which usually contains only the non-sulfated glycosaminoglycan, hyaluronic acid[3–5]. We identified two polysaccharides as contaminants arising from the crude papain used in the isolation procedure and now report their isolation, preliminary characterization, and interesting biological properties.

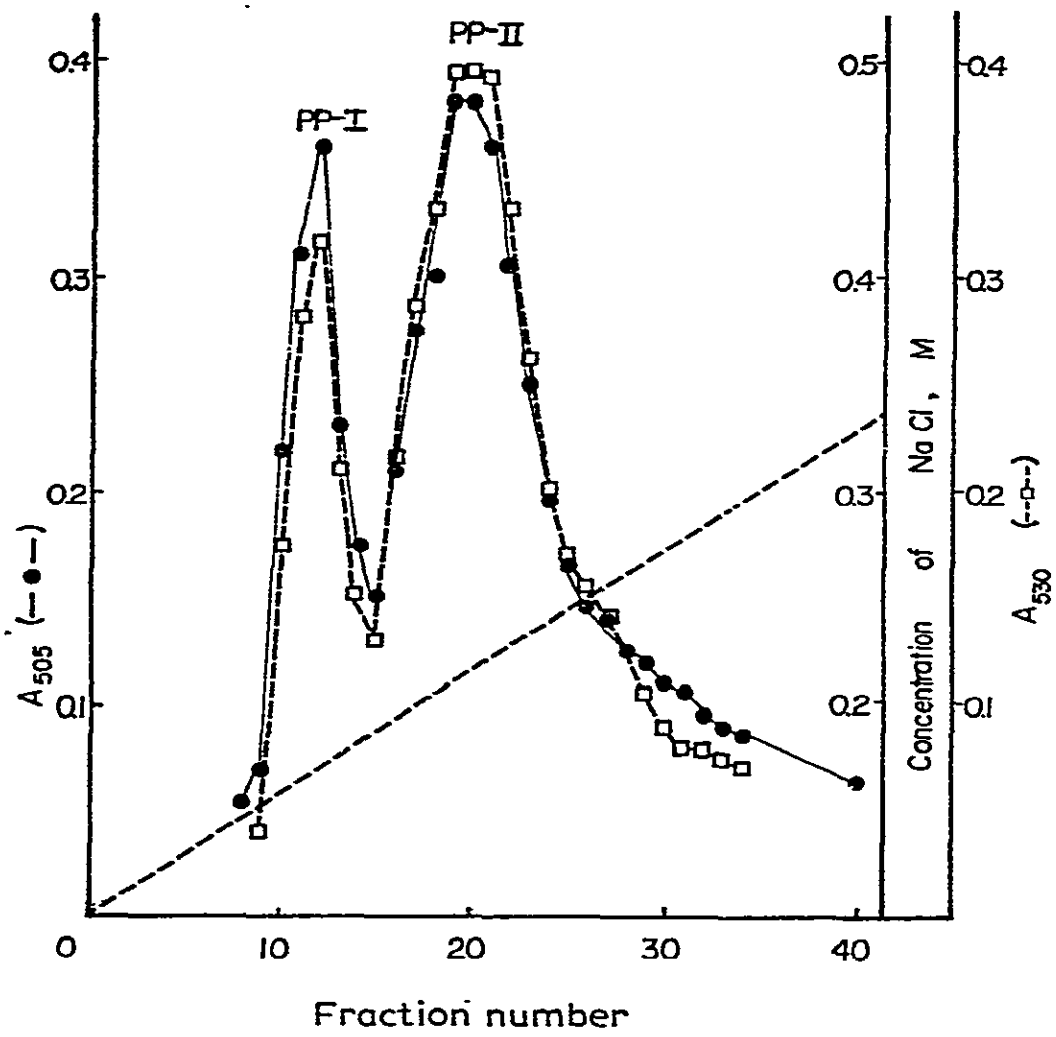

Fig. 1. Fractionation of papain polysaccharides on DEAE-cellulose; --□--, uronic acid by the carbazole reaction[22], —●—, neutral sugar by the orcinol reaction[23].

RESULTS AND DISCUSSION

Isolation and partial characterization of the papaya polysaccharides. — Fractionation of the papaya polysaccharides on DEAE-cellulose produced two fractions (Fig. 1). The material eluted at the higher salt-concentration contained a slightly higher ratio of uronic acid to neutral sugar.

PP-I, the material eluted at the lower salt-concentration, contains (Table I) 45% of hexose (plus 11% as a component of an aldobiouronic acid), 25% of pentose, 11.5% of uronic acid, and 9% of 6-deoxyhexose; PP-II consists of 39% of hexose (plus 15% as a component of an aldobiouronic acid), 23% of pentose, 15% of uronic acid, and 12% of 6-deoxyhexose; whereas PP-II-R (PP-II whose carboxyl group had been reduced) contains 2% of uronic acid, 48% of hexose (plus 2% as a component of an aldobiouronic acid), 24% of pentose, and 11% of 6-deoxyhexose. Estimation of D-galactose with D-galactose oxidase (Galactostat, Worthington Biochemical Corp.) in the hydrolyzates gave values lower than those of the total hexose content of PP-I and PP-II. This result is explained by the fact that not all D-galactose residues were released as free sugar, a considerable proportion remaining in an acidic, oligosaccharide fraction unoxidized by D-galactose oxidase. PP-II-R gave a value for D-galactose higher than that of PP-II, confirming this explanation. When PP-II was converted into PP-II-R, the uronic acid content de-

TABLE I

COMPOSITION (PERCENT) OF THE POLYSACCHARIDES OBTAINED FROM PAPAIN[a]

Polysaccharide	*Total hexose*	D-*Glucose*	D-*Galactose*	*Uronic acid*	*Pentose*	6-*Deoxy-hexose*
PP-I	45.3	2.8	31.9	11.5	25.0	9.3
PP-II	39.2	1.0	27.9	14.7	23.2	11.7
PP-II-R	47.8	11.8	36.0	1.8	24.0	11.3

[a]See the Experimental section for a description of the analytical techniques used in obtaining these data.

creased from 15 to 2%, and the D-glucose content, as determined with D-glucose oxidase (Glucostat, Worthington Biochemical Corp.), increased from 1% to ~12%, indicating that the uronic acid in PP-II is D-glucuronic acid.

When the polysaccharides PP-I, PP-II, and PP-II-R were subjected to electrophoresis on cellulose acetate and stained with Alcian blue, single bands were noticed (Fig. 2) with both PP-I and PP-II, PP-II moving a little faster towards the anode than PP-I, and both PP-I and PP-II moving more slowly than hyaluronic acid. PP-II-R did not show any band when stained with Alcian blue, as it had little anionic charge owing to reduction of the carboxyl groups.

Preliminary identification of the sugars present in PP-I by paper chromatography revealed components (Table II) corresponding to galactose, glucose, and arabinose in two solvent systems. When hydrolyzed in two different ways and then subjected to paper chromatography in two solvent systems, PP-II gave spots corresponding to galactose and arabinose; PP-II-R gave three spots corresponding to

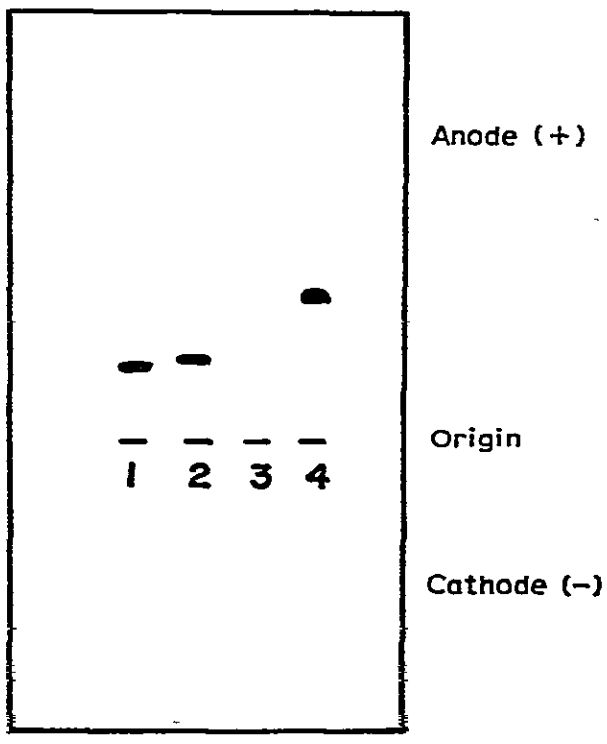

Fig. 2. Electrophoresis on cellulose acetate at 200 V for 45 min with the Gelman Sepratek System and high-resolution buffer: 1, PP-I; 2, PP-II; 3, PP-II-R; 4, hyaluronic acid.

TABLE II

PAPER CHROMATOGRAPHY OF SUGARS FROM THE POLYSACCHARIDES

Standard sugars and samples	*Chromatography of neutral sugars*[a]		*Chromatography of formic acid hydrolyzates*[b]	
	Solvent A R_{Glc}	*Solvent* B R_{Glc}	*Solvent* A R_{Glc}	*Solvent* B R_{Glc}
Gal	0.94	0.89	0.89	0.83
Ara	1.19	1.26	1.16	1.24
Xyl	1.42	1.56	1.36	1.49
Fuc	1.46	1.56	1.38	1.52
Rha	1.82		1.77	
Disaccharide not hydrolyzed			0.33	0.05
PP-I components[c]				
	0.87	0.87		
	0.97	1.01		
	1.15	1.28		
PP-II components[c]				
	0.87	0.92	0.86	0.83
	1.15	1.32	1.12	1.20
			0.34	0.05
PP-II-R components[c]				
	0.89	0.93	0.85	0.87
	1.01	1.07	0.94	1.00
	1.19	1.33	1.11	1.23

[a]Hydrolyzed with M hydrochloric acid for 3 h at 100°. [b]Hydrolyzed with concentrated formic acid for 4 h at 100°. [c]When the chromatography was done for shorter periods by loading with larger amounts of sample, a spot corresponding in R_F value to rhamnose was also detected.

galactose, glucose, and arabinose. PP-II gave a third component of very low mobility in the two solvent systems. Paper chromatography of the formic acid hydrolyzates of PP-I and PP-II revealed one additional component, which had the same mobility as the third spot from PP-II. This slowly moving component (aldobiouronic acid) was not given by PP-II-R. When paper chromatography of neutral sugars was conducted for shorter periods (24 h instead of 40 h) by applying large amounts of sample, PP-I, PP-II, and PP-II-R each gave an identifiable spot that had the mobility of L-rhamnose.

The oligosaccharide isolated from the polysaccharide preparation containing both PP-I and PP-II contained almost equimolar amounts of uronic acid and neutral hexose (Table III). Most of the neutral hexose was galactose. The reducing-sugar value indicated that this oligosaccharide was a disaccharide, specifically, an aldobiouronic acid. As the uronic acid group was not reduced by sodium borohydride but the galactose moiety was, this disaccharide must therefore be a glycuronosylgalactose. As reduction of the intact polysaccharide (PP-II) had indicated that the uronic acid component was D-glucuronic acid, the aldobiouronic acid must thus be a D-glucosyluronic acid-D-galactose. The isolation of this disaccharide in good yield

TABLE III

ANALYTICAL DATA FOR THE OLIGOSACCHARIDE CONTAINING URONIC ACID[a]

Constituents	*Molar ratio*
Neutral sugar	1.00
Uronic acid	1.25
Uronic acid after reduction with $NaBH_4$	1.22
Neutral sugar after reduction with $NaBH_4$	0.12
Reducing sugar	0.95
Galactose[b]	0.89
Pentose	0
6-Deoxyhexose	0

[a]See the Experimental section for a description of the analytical techniques used in obtaining these data. [b]Estimated after hydrolysis in M hydrochloric acid by using Galactostat reagent.

(30 mg obtained from 200 mg of polysaccharide), when considering the amount of uronic acid in the polysaccharide (less than 15%), suggests that the uronic acid and galactose are present in the same polysaccharide, that is, the polysaccharide preparation is not a mixture of a galactan and a glycuronan.

The fact that the aldobiourunic acid is not oxidized by D-galactose oxidase is an indication that the D-galactosyl residue is substituted either at O-6 (ref. 6) or at O-4 (ref. 7).

The presence of these polysaccharides, which behave similarly to hyaluronic acid upon electrophoresis and ion-exchange chromatography, eluting with hyaluronic acid, might explain why L-arabinose has been reported to be a component of some hyaluronic acid preparations in variable proportions (see, for example, ref. 9). However, if this is the source of L-arabinose, it is surprising that D-galactose has not been detected, even by g.l.c.[8,9].

Effects of polysaccharides isolated from papain on fibroblasts in culture. — Fibroblasts were grown in normal growth-medium and in normal growth-media containing added PP-I, PP-II, and PP-II-R. After decanting the medium, the fibroblasts were fixed and photographed at X200 and X500 magnifications.

The normal, human diploid fibroblasts grown in media containing the polysaccharides exhibited marked aggregation and orientation as compared to the fibroblasts grown in control medium (Fig. 3). (No differences in the effectiveness of PP-I, PP-II, and PP-II-R in effecting this aggregation could be detected by this method.) However, when compared to mouse embryo 3T12 fibroblasts grown in control medium, the 3T12 fibroblasts grown in medium containing the various polysaccharides exhibited no apparent aggregation or orientation.

This fact, namely, that these polysaccharides bring about aggregation and alignment of normal human fibroblasts but have little effect on mouse embryo 3T12 fibroblasts (which, unlike normal cells are aneuploid and do not exhibit contact inhibition of growth or movement)[10] is most interesting.

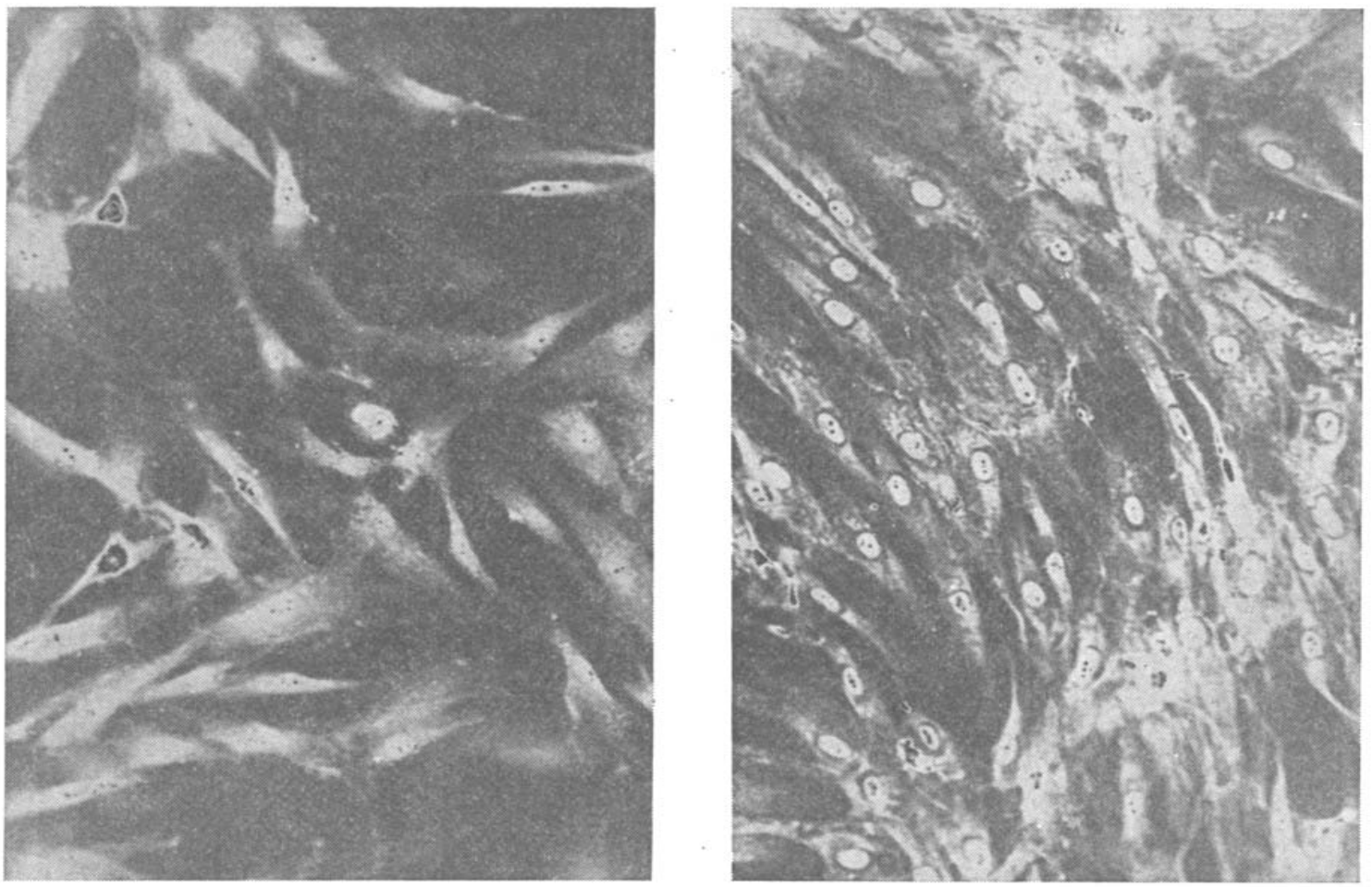

Fig. 3. Treatment of normal, human, skin-fibroblast cells from an 84-year-old, male donor. Left-hand photograph: cells in Eagle's medium supplemented 10% with calf serum. Right-hand photograph: cells in Eagle's medium supplemented 10% with calf serum and with 0.1% PP-II. Cells were photographed by using a Leitz interference microscope fitted with a Leitz automatic camera and Kodak Kodachrome film.

Little is known about the interaction of polysaccharides with cell surfaces. Cell surfaces do contain glycoproteins and glycolipids, and there is indirect evidence that glycoproteins play an important role in several cellular functions, including cell–cell adhesion. Less is known about the function of the glycolipids, but there is also indirect evidence that it is the carbohydrate portions of glycoproteins or glycolipids that are involved in adhesion.

Much of the speculation concerning the role of cell-surface carbohydrates in cell–cell adhesion has centered around Roseman's model[11] that cell recognition and aggregation result from enzyme–substrate interaction between glycosyltransferases on the surface of one cell and an oligosaccharide acceptor present on another cell. Roth *et al.*[12] found that treatment of dissociated, embryonic, neural retinal cells with β-D-galactosidase alters the adhesive properties of these cells, suggesting that terminal β-D-galactopyranosyl groups on the surface may be partly responsible for the adhesive selectivity of these cells. Chipowsky *et al.*[13] found that SV40-transformed, mouse embryo 3T3 fibroblast cells can distinguish β-D-galactopyranosyl end-groups from β-D-glucopyranosyl and 2-acetamido-2-deoxy-β-D-glucopyranosyl end-groups, that they adhere to Sephadex beads having covalently attached β-D-galactopyranosyl groups, and that cells which adhere to the beads act as a nucleus for cell–cell adhesion, leading to the formation of large, mixed aggregates containing cells and

TABLE IV

AREA OF NUCLEI IN CONTROL AND POLYSACCHARIDE-TREATED FIRBROBLASTS[a]

Cell line		*Nuclear area (10^{-7} cm^2)*		
		Average	*S.d.*	*Median*
Al Pos[b]	Control	14.19	4.22	13.76
	PP-I	11.85	3.77	11.36
	PP-II	13.14	3.03	12.89
Ar Mor[c]	Control	15.56	5.34	14.92
	PP-I	11.03	3.64	10.27
	PP-II	14.58	5.18	13.34
Ar Mor[c]	Control	18.19	6.58	16.92
	PP-I	11.45	2.92	11.20
	PP-II	14.55	3.52	14.10
	PP-II-R	10.83	2.74	10.54

[a]See the Experimental section for the experimental techniques used to obtain these data. [b]Normal, human, diploid fibroblasts from a 84-year-old male donor; passage 15. [c]Normal, human, diploid fibroblasts from a 40-year-old male donor; passages 16 and 17, respectively.

TABLE V

EFFECT OF POLYSACCHARIDES ON CELL MULTIPLICATION[a]

Polysaccharides	*Number of cells in the culture plate × 10^{-5}*		
	Initial	*Final*	*Difference*
Control	5.00	12.10	7.10
PP-II	5.00	6.94	1.94
Galactan[b]	5.00	10.47	5.47
Larch arabinogalactan[c]	5.00	8.52	3.52
Partially hydrolyzed larch arabinogalactan[d]	5.00	7.59	2.59

[a]Normal, human, diploid fibroblasts from a 40-year-old male donor (Ar Mor); passages 14–15; four-day growth period. [b]A lupine galactan isolated from *Lupinus albus*[18]. [c]Reprecipitated, commercial larch arabinogalactan from St. Regis Paper Co., Tacoma, Washington. [d]Larch arabinogalactan partially hydrolyzed to cleave arabinofuranosyl groups[16,17]. By this procedure, the arabinose content was decreased from 9.3 to 6.8%.

β-D-galactopyranosyl beads. Oppenheimer[14] found that mouse teratoma cells contained surface sites that recognized and bound terminal D-galactose groups of an aggregation factor.

Burger[15] has used sponge reaggregation as a biochemical model for cell–cell adhesion. He purified an aggregation factor from *Microciona prolifera* and observed that the D-glucuronic acid residues in this factor were mainly responsible for the specificity of the interaction between the cell surface and the factor.

That the adhesion effected by the papaya polysaccharides is similar to normal cell–cell adhesion was demonstrated by two pieces of evidence. One was that the

nuclear area of cells treated with the galactans was smaller than that of normal cells in the log phase of growth (Table IV), a phenomenon we also have found with contact inhibition of growth in the same cell strains (S.-C. D. Lee, A. J. Pappelis, P. M. Be-Miller, and J. N. BeMiller, unpublished data). The other was that addition of the polysaccharides to the growth medium decreased cell division (Table V), another characteristic of contact inhibition of growth. (The data in Table V are characteristic of those obtained in several investigations. In another, using the Ar Mor cell strain at other passages, it was determined that PP-I was more effective in decreasing cell multiplication than was PP-II). There was no inhibition of growth, in the presence of the papaya polysaccharides, of the 3T12 fibroblast cells, which exhibited no apparent aggregation or orientation, indicating that the observed decrease in cell division of normal fibroblast cells is not due to a toxic effect of the polysaccharides.

Because PP-I and PP-II-R, both of which contain less uronic acid than does PP-II, were more effective than PP-II in decreasing cell division and nuclear area, it may be concluded that the uronic acid residues of these polysaccharides do not have a role in cell–cell adhesion.

Perhaps, the papaya polysaccharides (very rich in D-galactose) aggregate normal cells by mimicking cell-surface oligosaccharide moieties of glycoproteins or glycolipids; they may interact with receptor sites on the cell surface and cement them together, providing a spacer between cells. Perhaps, they mimic a naturally occurring, glycoprotein aggregation-factor.

PP-II, which was less effective than PP-I in decreasing cell division, was much more effective than were other galactans, although all were effective (Table V). Of the galactans tested, the next most effective was a larch arabinogalactan partially hydrolyzed to cleave arabinofuranosyl bonds[16,17]; then larch arabinogalactan itself, and finally a galactan isolated from *Lupinus albus*[18], which was about one-third as effective. Each contains β-D-galactopyranosyl residues. Guaran, a polysaccharide having α-D-galactopyranosyl, nonreducing end–groups, was ineffective. It can be speculated that polysaccharides with multiple, terminal, nonreducing, β-D-galactopyranosyl groups are capable of effecting cell–cell adhesion and that the effectiveness of the various polysaccharides is related to the degree to which they exhibit this structure; however, this hypothesis needs to be tested.

EXPERIMENTAL

Materials. — Papain (Sigma Type II), the standard sugars, DEAE-cellulose, and 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide were purchased from Sigma Chemical Co.; sodium borohydride from Fisher Scientific Company; Glucostat and Galactostat reagents from Worthington Biochemical Corp. All other chemicals were reagent grade or the purest preparations available.

Fibroblast cultures. — Human-skin, fibroblast strains from 40- (Ar Mor) and 84- (Al Pos) year-old, normal, male donors were obtained from American Type Culture Collection, Rockville, Maryland. The mouse embryo 3T12 fibroblast cell-

line was obtained from Dr. J. K. Dorsey of this department. All cells were grown in Falcon plastic Petri dishes in an atmosphere of 5% CO_2–95% air by feeding on alternate days with Eagle's medium supplemented 10% with calf serum. Cultures were determined to be free from mycoplasm by the method of Hayflick[19].

Isolation of polysaccharides from papain. — The polysaccharides were isolated by following the procedure of Kimmel and Smith[20] for the purification of papain. Crude papain (40 g) was dissolved in 0.04M cysteine, pH 9.0 (1 g papain/10 ml 0.04M cysteine). The pH of the solution was further adjusted to 9.0 with M sodium hydroxide, and the gray precipitate that appeared was removed by centrifugation at 4°. The supernatant was adjusted to 0.4 saturation with solid ammonium sulfate and kept for 1 h at 4°. The white precipitate, which contained the enzyme activity, was collected by centrifugation at 4° and washed once with 0.4 saturated ammonium sulfate solution. The ammonium sulfate supernatant fluid was dialyzed against running tap-water overnight and then against distilled water for 24 h. The dialyzate was adjusted to 10% trichloroacetic acid concentration by adding 100% trichloroacetic acid and then centrifuged at 4°. The supernatant fluid was again dialyzed against running tap-water and then against distilled water. The dialyzate was concentrated to low volume and subjected to column chromatography on DEAE-cellulose (Fig. 1). A DEAE-cellulose column of 150-ml bed volume (3 × 21 cm) equilibrated with 0.05M sodium chloride was used. The uronic acid-containing material isolated from 40 g of papain (20 ml) was applied to the column. Linear gradient-elution was achieved by placing 500 ml of 0.5M sodium chloride in the reservoir and 500 ml of 0.05M sodium chloride in the mixing vessel.

Fractions of 14 ml were collected. Neutral sugar and uronic acid were determined by assaying 0.01 ml of each fraction. Fractions 10–15 and 17–40 were pooled separately, dialyzed exhaustively against distilled water to remove salt, concentrated under diminished pressure to low volume, and then freeze dried. These materials were designated as PP-I and PP-II, respectively.

Reduction of uronic acid residues of PP-II[21]. — PP-II (100 mg) and 300 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were dissolved in 2 ml of water. The pH of this solution was maintained at 4.75 for 1 h by adjusting it with 0.1M hydrochloric acid. Then 500 mg of sodium borohydride was added slowly with stirring, and the mixture was heated in a water bath for 2 h at 50°. Then, excess borohydride was decomposed with 4M hydrochloric acid, added dropwise until effervescence ceased. The whole mixture was dialyzed against several changes of distilled water for 24 h, concentrated to a small volume, and freeze dried; yield of PP-II-R, 75 mg.

Isolation of an aldobiouronic acid consisting of D-*glucuronic acid and* D-*galactose.* — Polysaccharide material (200 mg) that had not been fractionated into PP-I and PP-II by DEAE-cellulose chromatography was dissolved in 40 ml of formic acid; the reaction flask was flushed with nitrogen for 15 min, glass stoppered, and heated in an air oven for 4h at 100°. The hydrolyzate was evaporated under diminished pressure to dryness, dissolved in water, and passed through a 25-ml column of Dowex-1

(formate). After washing with 100 ml of water, the column was eluted with 100 ml of M formic acid. The formic acid eluate was evaporated to dryness, the residue dissolved in a small volume of water, and the solution freeze dried; yield 30 mg.

Effect of polysaccharides on cells in culture. — By trypsinization, approximately the same number of cells (human fibroblasts, 5×10^5; mouse embryo 3T12 fibroblasts, 3×10^5) were transferred from 150-mm culture dishes to 60-mm culture dishes to which four cover slips had been added. The next day, the medium in these plates was changed as follows: by using a disposable, sterile syringe fitted with disposable sterile Millipore filter, 10 ml of fresh culture medium containing 10 mg of polysaccharide was added to each plate; the control plate received 10 ml of fresh culture medium containing no polysaccharide. The human fibroblasts were allowed to grow for 4 days, feeding on the second day with polysaccharide-containing culture medium, and the mouse embryo fibroblasts for 2 days. After this growth period, the medium was decanted and the plates (containing the cover slips at the bottom) were rinsed with balanced phosphate-buffered saline, fixed for 15 min in 3:1 (v/v) ethanol–acetic acid, and allowed to air-dry. Each cover slip was photographed by using a Leitz interference microscope fitted with a Leitz automatic camera and Kodak Tri-X black and white film. The films were developed in Diafine A and B solutions, 3.5 min in each solution, and fixed for 2 min in Kodak Rapid Fixer. The photographic image of each nucleus was projected on a screen; the circumference of each was traced on a sheet of paper, and the area within the tracing was determined with a planimeter (Keuffel and Esser Company, type R E A 620015, giving direct reading in cm^2 with an accuracy of 0.1 cm^2). A photomicrograph of a stage micrometer was also projected onto the screen in order to determine the magnification. The planimeter-measured area was divided by the square of the linear magnification to yield the actual area in cm^2; 150 randomly selected interphase nuclei were measured.

For counting the number of cells in the culture plates, the cells were grown in the appropriate medium, as already described (1 mg polysaccharide/ml medium; without cover slips at the bottom). When the cells in the control plate reached confluence, the medium was decanted from each of the plates, and the cells were counted after detaching by mild trypsinization.

Analytical techniques. — Uronic acid was determined by the carbazole method of Bitter and Muir[22], neutral sugar by Winzler's modification of the orcinol procedure[23] or Roe's anthrone method[24], pentose by the orcinol reaction of Brown[25], and 6-deoxyhexose by using thioglycolic acid[26]. Appropriate corrections were made for the color contributed by other sugars. D-Glucose was determined with Glucostat reagent and D-galactose with Galactostat reagent (Worthington Biochemical Corp.).

For identification of neutral sugars, the samples were hydrolyzed in M hydrochloric acid for 3 h at 100° in sealed ampules and then evaporated to dryness in a vacuum desiccator in the presence of sodium hydroxide pellets. The residue was dissolved in water, passed first through a column of Dowex-1(HCO_3^-), and then through Dowex-50 (H^+). The breakthrough and water washings were pooled, con-

centrated, and then subjected to paper chromatography. Descending paper chromatography was conducted on Whatman No. 1 paper with two different solvent-systems: (*A*) 4:1:2 (v/v) 2,2-dimethyl-1-propanol–2-propanol–water[27], (*B*) 10:10:5:1:5 (v/v) ethyl acetate–pyridine–1-butanol–butanoic acid–water[28] for about 40 h. The sugars were detected by staining with alkaline silver nitrate[29].

Electrophoresis. — Electrophoresis on cellulose acetate[30] was effected with a Gelman Sepratek System, using Gelman's high-resolution buffer, at 200 V for 45 min, and the polysaccharides were stained with 0.5% Alcian blue in 3% acetic acid (Fig. 2).

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