The use of N-acetylglucosamine: agarose as an acceptor in the galactosyltransferase reaction

	Galactose incorporated (cpm/3 h)
Complete system [a]	1154
Minus enzyme	42
Minus Mn^{2+}	111
Minus adsorbent + CNBr activated ethanolamine inactivated agarose	62
Minus adsorbent + *p*-aminophenyl galactose-agarose	87

[a] As described in Figure 2.

stirring overnight. The beads were subjected to hydrolysis with 2 *N* HCl at 100 °C for 4 h. The supernatant was deionized by passing over a mixed ion exchange resin (Biorad No. AG501-X8), the eluate lyophilized, redissolved in H_2O and chromatographed on Whatman No. 1 paper in 2 solvent systems: 1. butanol-pyridine-H_2O, 6:4:3, for 33 h, and 2. pyridine-ethylacetate-H_2O-acetic acid, 5:5:3:1, for 24 h. The paper strip containing the radioactive sample was cut into 2.5 cm wide pieces and put into scintillation vials for counting. The sugar standards, including N-acetylglucosamine, D-galactose, D-glucose and lactose were detected on the chromatograms by the alkaline-$AgNO_3$ reaction[7]. The label co-chromatographed in both systems with D-galactose. Protein was determined according to LOWRY et al.[8].

Result and discussion. Figure 1 shows the purification of serum galactosyltransferase from 0.2 ml of normal human serum by 1 ml of packed adsorbent. The elution buffers A and B (for composition cf. legend to Figure 1) are made according to BARKER. Fractions of 1 ml were collected and dialyzed against 0.1 *M* Na-cacodylate buffer, pH 7.4, before assaying for activity and protein concentration. The increase in specific activity varied between 50- and 150-fold by this single purification step. The recovery of activity was close to 100% when measured immediately after dialysis, in agreement with the almost complete depletion of galactosyltransferase activity from serum. The affinity column proved suitable for the purification of soluble galactosyltransferases from different sources such as fetal calf serum, calf serum and human amniotic fluid.

Labeling of the adsorbent. These experiments were carried out with partially purified galactosyltransferase from fetal calf serum (150-fold purified) suspended in bovine serum albumin (1 mg/ml). As demonstrated by TRAYER and HILL[9], the purified galactosyltransferase required bovine serum albumin (1 mg/ml) for stabilization of activity. As shown in Figure 2, the reaction with the beads as acceptor was linear up to 3 h. The Table demonstrates that the reaction, as with other acceptors, required Mn^{2+} and that agarose without the N-acetylglucosamine arm could not act as acceptor.

These data demonstrate that soluble galactosyltransferase can be purified by affinity chromatography with a system similar to that described by BARKER et al.[3] but using beads prepared by a simple and rapid method. BLOCH and BURGER[4] originally made these agarose beads for the purification of various lectins. The main advantage of this procedure lies in the rapidity of coupling the adsorbent to the activated agarose, taking advantage of the *p*-nitrophenyl group as a spacer group already attached to different sugars which are commercially obtainable and inexpensive.

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Abnormally Soluble Collagen Produced in Fibroblasts Cultures[1]

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Summary. Abnormally soluble collagen is synthesized in vitro not only by skin fibroblasts of Marfan patients but also by those of patients with Ehlers-Danlos type V and cutis laxa. The excessive solubility of collagen is corrected by the addition to the culture medium of a synthetic flavonoid, (+)-catechin.

In 1973 PRIEST, MOINUDDIN and PRIEST[2] demonstrated that cultured skin fibroblasts derived from patients affected by Marfan syndrome produced a collagen which was more soluble than normal. Although the molecular defect responsible for this excessive solubility was not identified, the authors concluded that the findings were important because they might lead to the clarification of the basic defect of the disease and might be useful for early and possibly prenatal diagnosis.

We report here that the synthesis of excessively soluble collagen is not a peculiarity of Marfan cultured fibroblasts because it occurs also in cultures of fibroblasts from patients affected respectively by the sex-linked form of Ehlers-Danlos (Type V)[3] and by the autosomal recessive form of cutis laxa. Moreover, the addition of a synthetic flavonoid capable of stabilizing collagen[4] to the culture medium of the various types of mutant fibroblasts studied decreased the abnormal solubility of their collagen, while it did not affect the solubility of the collagen synthesized by normal, control fibroblasts.

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Materials and methods. Punch skin biopsies were performed under 1% procaine local anesthesia. The fibroblasts used had undergone from 3 to 10 doublings from the primary culture and none of them had been stored in liquid nitrogen. The cells, carried in stationary cultures with MEM medium, Earle's base, supplemented with 10% fetal calf serum, nonessential aminoacids and antibiotics[5], were cultured at 37 °C in 5% CO_2-95% air, humid atmosphere. The medium was changed every 3 day and the cells were subcultured once a week.

When the cells became confluent in a 75 cm^2 Falcon plastic flask, they were harvested by trypsinization and inoculated into a roller flask containing the same culture medium supplemented with 50 µg of sodium ascorbate per ml. The flasks were placed in a New Brunswick roller drum and rotated at 1/4 rpm for a period of 4–5 days. When the cells became confluent the medium was replaced, and paired cultures of the same line were either replenished with the same medium (supplemented with ascorbate) or with the same medium containing in addition 200 µg of (+)-catechin per ml. After additional 3 days in culture the cells were harvested by trypsinization, collected by centrifugation, washed once with ice-cold 0.9% NaCl and stored frozen until analyzed for collagen content and solubility.

Each batch of harvested fibroblasts was sequentially extracted for 48 h at 4 °C, with stirring, with 7 ml of the following solvents: a) 1.0 *M* NaCl buffered to pH 7.5 with 0.05 *M* *Tris*-HCl; b) 0.5 *M* acetic acid; and c) 4 *M* guanidinium chloride. After each extraction, the residue was obtained by centrifugation at 12,000 *g* for 90 min. Each extract was dialyzed against distilled water and lyophilized.

The final residue and the various lyophilized extracts were hydrolyzed for 24 h at 100 °C with 6 *N* HCl; the hydrolysates were brought to dryness under vacuo, dissolved in a minimum volume of distilled water and duplicate aliquots were used to measure hydroxyproline according to the method of SWITZER and SUMNER[6].

Results and discussion. The table shows the results of the analyses performed on lines of fibroblasts derived from 2 normal control individuals, 2 Marfan patients, 1 patient with Ehlers-Danlos Type V and 1 with the autosomal recessive type of cutis laxa.

The amounts of hydroxyproline found in each extract and in the final residue are expressed as percent of the total hydroxyproline in a given cell line. The open figures represent the distribution of hydroxyproline in the various solvents when the cells were cultured in the medium supplemented with ascorbate only; those enclosed in parentheses represent the distribution of hydroxyproline in the various solvents when the cells were grown in presence of (+)-catechin and ascorbate.

The results obtained indicate that excessively soluble collagen is synthesized by the cultured fibroblasts of the various patients studied, despite the fact that, unquestionably, they represent phenotypes of different genetic defects. Thus, the finding of an excessively soluble collagen in cultures of fibroblasts, while not pathognomonic for a single molecular defect, might be useful for the study and the prenatal diagnosis of various genetic defects involving collagen synthesis or its post-ribosomal modifications. However, the results obtained with the cultured fibroblasts of the Marfan patients suggest that the findings be interpreted with due caution. When those fibroblasts were cultured for 5 days instead of 3, the collagen produced by the cells of case 2 underwent a spontaneous process of maturation, leading to the formation of considerable amounts of insoluble collagen. A similar phenomenon has been described previously by MACEK et al.[7]. The same period of culture, however, produced only a very modest change in the solubility profile of the collagen synthesized by the cells of case 1. While these findings may be related to the recognized variability of Marfan patients[8], they suggest that a rigid standardization of culture conditions be observed if a diagnostic value has to be ascribed to them.

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Hydroxyproline extracted from cultured fibroblasts of 2 normal controls, 2 Marfan, 1 Ehlers-Danlos type V and 1 cutis laxa patients

Phenotype	Total hydroxyproline (µg)	Percentage extracted in			
		Neutral salt	Acetic acid	Guanidinium Cl	Insoluble residue
Normal	1.85	18.4	11.1	19.4	51.0
	(2.06)	(13.3)	(21.3)	(24.0)	(41.3)
Normal	1.71	10.4	19.3	15.7	54.4
	(1.40)	(10.7)	(13.2)	(36.4)	(40.0)
Marfan 1	2.0	21.3	11.8	9.2	57.6
	(1.9)	(6.2)	(7.0)	(11.6)	(75.2)
Marfan 2	2.6	24.5	14.6	17.9	43.0
	(2.5)	(11.4)	(11.1)	(7.2)	(70.2)
Ehlers-Danlos V	2.0	31.5	6.9	30.0	31.5
	(1.5)	(10.9)	(27.3)	(9.0)	(52.8)
Cutis laxa	6.2	30.9	3.2	33.9	31.8
	(1.5)	(17.2)	(17.7)	(17.3)	(47.8)
Marfan 1 5 days	4.4	15.8	16.6	18.6	50.8
	(2.0)	(5.8)	(7.8)	(13.4)	(73.3)
Marfan 2 5 days	3.2	11.1	10.6	8.4	69.8
	(3.1)	(7.8)	(8.9)	(5.6)	(77.6)

The addition of 200 µg of (+)-catechin to the culture media of the various type of mutant fibroblasts produced invariably a considerable decrease in the amount of neutral salt-soluble collagen and an increase in the amount of the insoluble one.

In the case of the fibroblasts from the patient with cutis laxa, the solubility profile of the collagen was shifted toward a normal pattern and the excessive amount of collagen produced, as indicated by the level of intracellular hydroxyproline, was decreased to normal levels, to suggest that an adequate level of insoluble collagen might exercise some feed-back control on the process of collagen synthesis.

When the flavonoid was added to the medium of normal, control fibroblasts, changes in collagen solubility were minimal and, indeed, the amount of insoluble collagen decreased.

Although the exact mechanism of action of (+)-catechin in decreasing collagen solubility is not clearly defined, two hypotheses have been proposed: 1. that in complex with copper ions, it might increase the oxidative deamination of lysine ε-amino groups, especially in absence of lysyl oxidase[9]; 2. that it might establish hydrogen bonds between adjacent collagen chains[4] and, possibly, covalent bonds through formation of free radicals.

The effectiveness of the flavonoid in decreasing the solubility of collagen produced by skin fibroblasts derived from different genotypes would favor a non-specific mechanism of action rather than a specific one.

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Heavy Metal Hydrolysis of Polyisoprenoid-Phosphate Mono- and Oligosaccarides[1]

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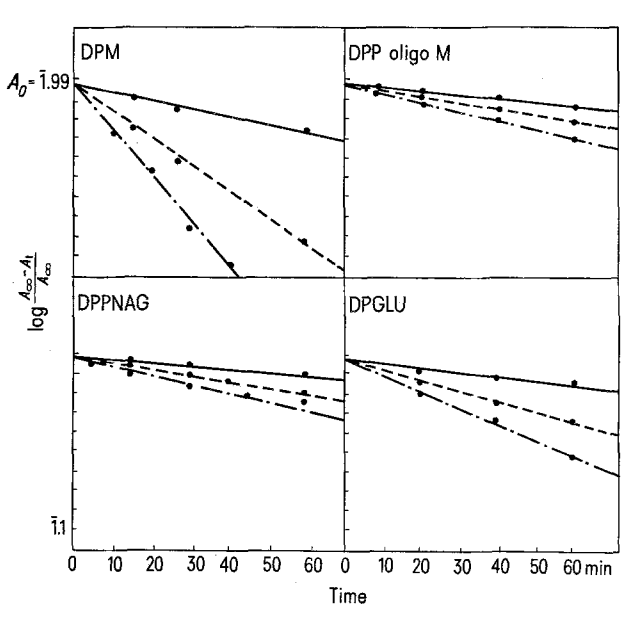
Summary. Mono- and oligosaccharide derivatives of dolichol phosphate can be hydrolyzed by heavy metal, preferably Zn^{++} at 100 °C or 65 °C. The reactions follow first order kinetics. The reaction proceeds through hydrolysis of the sugar phosphate bond.

Long-chain polyisoprenoid compounds function as glycosyl carriers in biosynthesis of complex polysaccharides of bacteria[2,3], yeasts[2], plants[4,5] and animals[6,7]. The first reaction in these pathways is the transfer of single sugar residue from sugar nucleotide to the phosphoryl derivative of dolichol[2,8], a C_{90}–C_{100} polyisoprenoid alcohol[9]. These sugar residues are believed to be transferred via a series of lipid-bound oligosaccharide intermediates[2]. Characterization of these pathways requires structural analysis of the oligosaccharide chains bound to dolichol. It would be facilitated by a technique for splitting of the intact oligosaccharide portion from the lipid-portion of the intermediate. A mild procedure for hydrolyzing polyisoprenoid-phosphate-sugars has been developed and is presented in this paper.



—, Kinetics of Zn^{++} hydrolysis at 100 °C; ——, Zn^{++} 0 M; – – –, Zn^{++} 10^{-3} *M*; —·—·, Zn^{++} 10^{-2} *M*; A_o = 1.99.

Materials and methods. Dolichol-phosphate-mannose-[^{14}C][10] (DPM), a dolichol-phosphate-glucose[^{14}C][7] (DPGlu) were prepared according to the literature in the following way. 50 µl GDP-mannose[^{14}C] (20 µCi/ml) or 50 µl UDP-glucose[^{14}C] (20 µCi/ml, both purchased from New England Nuclear, was incubated with 10 µl $MgCl_2$ 0.1 *M*. 15 µl AMP 20 m*M*, 60 µl *tris*-buffer pH 7.5 μ = 0.25, 100 µl microsomes (35 mg/ml of protein). After 15 min at 37 °C the reactions were stopped with 1 ml of chloroform, methanol, water (60:40:8). The organic phases were collected and washed several times with a solution of chloroform, methanol, water (1:16:16). Dolichol-P-P-N-acetyl-glucosamine [^{14}C] (DPPNAG) was prepared according to Ghalambor et al.[11] and separated from Dolichol-P-P-dichitobiose by thin layer chromato-

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