Goetzl, E. J., & Metzger, H. (1970) *Biochemistry 9*, 1267.
Helm, B., Marsh, P., Vercelli, D., Padlan, E., Gould, H., & Geha, R. (1988) *Nature 331*, 181.
Henson, D. C., Yguerabide, J., & Schumaker, V. N. (1981) *Biochemistry 20*, 6842.
Holowka, D., & Baird, B. (1983) *Biochemistry 22*, 3475.
Holowka, D., Wensel, T., & Baird, B. (1990) *Biochemistry 29*, 4607.
Ishizaka, T., & Ishizaka, K. (1984) *Prog. Allergy* 34, 188.
Jovin, T. M., & Vaz, W. (1989) *Methods Enzymol. 172*, 471.
Kaganov, I. N. (1982) *Sakh. Promst. 8*, 57.
Kinet, J. P. (1989) *Cell 57*, 351.
Kinosita, K., Jr., Kawato, S., & Ikegami, A. (1977) *Biophys. J. 20*, 289.
Kinosita, K., Jr., Kawato, S., & Ikegami, A. (1984) *Adv. Biophys. 17*, 147.
Lipari, G., & Szabo, A. (1980) *Biophys. J. 30*, 489.
Liu, F. T., Bohn, J. W., Ferry, E. L., Yamamoto, H., Molinaro, C. A., Sherman, L. A., Klinman, N. R., Katz, D. H. (1980) *J. Immunol. 124*, 2728.
Matayoshi, E. D., Corin, A. F., Zidovetzki, R., Sawyer, D. H., & Jovin, T. M. (1983) in *Mobility and Recognition in Cell Biology* (Sund, H., & Veeger, C., Eds.) pp 119-134, de Gruyter, Berlin.
McCloskey, M. A., Liu, Z.-Y., & Poo, M.-M (1984) *J. Cell Biol. 99*, 778.
Menon, A. K., Holowka, D., Webb, W. W., & Baird B. (1986a) *J. Cell Biol. 102*, 534.
Menon, A. K., Holowka, D., Webb, W. W., & Baird, B. (1986b) *J. Cell Biol. 102*, 541.
Metzger, H., Alcaraz, G., Hohman, R., Kinet, J. P., Pribluda, K., & Quarto, R. (1986) *Annu. Rev. Immunol. 4*, 419.
Oi, V. T., Vuong, T. M., Hardy, R., Reidler, J., Dangl, J., Herzenberg, L. A., & Stryer, L. (1984) *Nature 307*, 136.
Ortega, E., Schweitzer-Stenner, R., & Pecht, I. (1988) *EMBO J. 7*, 4101.
Ortega, E., Schweitzer-Stenner, R., & Pecht, I. (1990) *Biophys. J. 57*, 284a.
Pecht, I., Schweitzer-Stenner, R., & Ortega, E. (1989) *Prog. Immunol. 7*, 676.
Rivnay, B., Wank, S. A., & Metzger, H. (1982) *Biochemistry 21*, 6922.
Robertson, D., Holowka, D., & Baird, B. (1986) *J. Immunol. 136*, 4565.
Schwarzbaum, S., Nissim, A., Alkalay, I., Ghozi, M. C., Schindler, D. G., Bergman, Y., & Eshhar, Z. (1989) *Eur. J. Immunol. 19*, 1015.
Siraganian, R. P. (1988) in *Inflammation: Basic Principles and Clinical Correlates* (Gallin, J. I., Goldstein, I. M., & Snyderman R., Eds.) pp 513-542, Raven Press, New York.
Small, E. W., & Isenberg, I. (1977) *Biopolymers 16*, 1907.
Szabo, A. (1984) *J. Chem. Phys. 81*, 150.
Yashida, T. M., Jovin, T. M., & Barisas, G. (1989) *Rev. Sci. Instrum. 60*, 29.
Zidovetzki, R., Yarden, Y., Schlessinger, J., & Jovin, T. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6981.
Zidovetzki, R., Yarden, Y., Schlessinger, J., & Jovin, T. M. (1986a) *EMBO J. 5*, 247.
Zidovetzki, R., Bartholdi, M., Arndt-Jovin, D., & Jovin, T. M. (1986b) *Biochemistry 25*, 4397.

Epimerization of D-Glucose to L-Galactose during the Biosynthesis of a Sulfated L-Galactan in the Ascidian Tunic†

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ABSTRACT: The sulfated polysaccharides occurring in the tunic of ascidians are unique among known sulfated polysaccharides in that their major constituent sugar is galactose, which occurs exclusively in the L-enantiomeric form. In vitro incorporation experiments using tunic slices incubated with ^{14}C-labeled sugars revealed that cells from this tissue epimerize D-isomers of hexose into L-galactose during the biosynthesis of their constituent polysaccharides. Compared with other hexoses, the precursor D-[^{14}C]glucose has the highest rate of incorporation and produces the highest proportion of L-galactose units. This metabolic pathway is distinct from the epimerization of D-mannose to L-galactose through its guanosine 5'-diphosphate nucleotide, described previously in an alga and in a snail. Therefore, the epimerization of D-glucose to L-galactose in the ascidian tunic occurs through a novel metabolic route, which involves inversion of the configuration of carbon atoms 2, 3, and 5 of the hexosyl moieties.

Sulfated polysaccharides are widespread in nature, occurring in a great variety of organisms. In marine algae, for example, the carrageenans and fucoidans are composed mainly of sulfated galactose and sulfated fucose, respectively (Painter, 1983). In the animal kingdom, sulfated glycosaminoglycans abound in vertebrate connective tissues (Mathews, 1975) and, in smaller quantities, are also present in invertebrates (Mathews, 1975; Cássaro & Dietrich, 1977).

In previous studies we have reported the isolation of novel sulfated polysaccharides from invertebrate tissues: the tunic of ascidians (Albano & Mourão, 1983, 1986; Mourão &

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Perlin, 1987; Pavão et al., 1989a) and the body wall of the sea cucumber (Albano & Mourão, 1983; Mourão & Bastos, 1987; Vieira & Mourão, 1988). We speculated that the occurrence of high amounts of sulfated polysaccharides in these tissues indicates that they are playing important structural roles, analogous to those of the glycosaminoglycans in vertebrate connective tissue.

In the tunic of *Styela plicata* (Chordata, Tunicata), these sulfated polysaccharides occur as three main fractions, which are markedly distinct in their molecular weight and chemical composition (Albano & Mourão, 1986; Mourão & Perlin, 1987). The high molecular weight fraction, denominated F-1, contains a high proportion of galactose and minor amounts of glucose, whereas the other two fractions of low molecular weight, denominated F-2-A and F-2-B, contain, besides galactose, a higher proportion of glucose, amino sugars, and small amounts of mannose.

The galactose from the sulfated polysaccharides of the ascidian occurs entirely in the L-enantiomeric form (Mourão & Perlin, 1987; Pavão et al., 1989b). Although L-galactose is a constituent of several polysaccharides (Bell & Baldwin, 1941; Anderson & Lowe, 1947; Correa et al., 1967; Painter, 1983), this was the first report of sulfated polysaccharides that contain high amounts of L-galactose and that lack the D-enantiomorph of this sugar. In the high molecular weight fraction (F-1), galactose undoubtedly exists mainly as α-L-galactopyranosides, linked glycosidically through positions 1 $\rightarrow$ 4 and sulfated at position 3. The glucose units present in these polysaccharides are the D-isomer, most of them as β-D-glucopyranosyl units (Mourão & Perlin, 1987; Pavão et al., 1989a).

Although the role of several sugar nucleotides in the assembly of complex carbohydrates has been established for a long time, the pathway involved in the incorporation of L-galactose into polysaccharides is not known. In the present work, we investigate the incorporation of ^{14}C-labeled sugars into the sulfated polysaccharides found in the ascidian tunic. Our results demonstrate that D-glucose epimerizes to L-galactose during the biosynthesis of these molecules.

Material and Methods

Materials. D-[1-^{14}C]Glucose, D-[U-^{14}C]mannose, D-[1-^{14}C]galactose, and D-[2-^{3}H]mannose were purchased from Du Pont (Wilmington, DE), L-[1-^{14}C]fucose was from Amersham International (Buckinghamshire, England), *Dactylium dendroides* D-galactose oxidase, *Aspergillus niger* D-glucose oxidase, D-glucose 6-phosphate, uridine 5′-diphosphate D-glucose, L-galactose, proteinase K (protease type XI), and papain (type IV) were from Sigma Chemical Co. (St. Louis, MO), and agarose (standard low M_r) was from Bio-Rad (Richmond, CA).

Labeling of Sulfated Polysaccharides in the Ascidian Tunic. The ascidian *S. plicata* was collected in Guanabara Bay, Rio de Janeiro, and transported to the laboratory immersed in sea water. Thereafter, the ascidian was cut longitudinally, the viscera were removed, and the tunic was cut transversely into slices approximately 1 mm thick. These slices were immersed immediately in the incubation medium containing 423 mM NaCl, 9 mM KCl, 9 mM Na_2SO_4, 23 mM $MgCl_2$, 9 mM $CaCl_2$, and 2 mM $NaHCO_3$ and washed three times with 5 mL of this solution. Approximately 10 mg (wet weight) of the slices was incubated for different times at 20 °C either with 0.7 μCi (1.05 nmol) of the ^{14}C-labeled sugars or with 20 μCi (0.66 nmol) of the ^{3}H-labeled sugar, in 100 μL of the incubation medium. At the end of the labeling period, the medium was decanted and the tunic slices were washed five times with 5 mL of the incubation medium. The slices were then immersed in 3 mL of acetone, where they were kept for 24 h at 4 °C and then dried at 80 °C for 60 min.

Isolation and Quantitation of the ^{14}C- and ^{3}H-Labeled Polysaccharides from Ascidian Tunic. ^{14}C- and ^{3}H-labeled polysaccharides were isolated from the ascidian tunic following the method previously described for fresh tissue (Albano & Mourão, 1986). Briefly, the dried slices (originally 10 mg wet weight) were suspended in 0.5 mL of 0.1 M sodium acetate buffer (pH 5.0) containing 30 μg of papain (type IV), 5 mM EDTA, and 5 mM cysteine and incubated at 60 °C for 24 h. The incubation mixture was then centrifuged (2000*g* for 10 min at room temperature), and incubation of the pellet with papain was repeated. The clear supernatants were combined and mixed with 2 volumes of absolute ethanol. The precipitate formed after standing at −10 °C for 24 h was collected by centrifugation (2000*g* for 15 min at room temperature), dried at 80 °C for 1 h, and dissolved in 100 μL of distilled water.

To estimate the amounts of ^{14}C- or ^{3}H-labeled sugars incorporated into polysaccharides, the solution of glycans described above was applied to Whatman 3MM chromatographic paper and developed in 1-butanol/pyridine/water (3:2:1 v/v) for 48 h. The origins (containing the polysaccharides, now free of low molecular weight contaminants) were cut out, added to 10 mL of 0.5% PPO/toluene solution, and counted in a liquid scintillation counter.

Identification of the ^{14}C- and ^{3}H-Labeled Sugar Residues in the Ascidian Polysaccharides. The origins of the chromatograms described above were removed from the PPO/toluene solution, washed 3 times with 10 mL of acetone, and dried. Thereafter, the sulfated polysaccharides were eluted from the paper with distilled water and concentrated on a rotary evaporator. After acid hydrolysis (6.0 M trifluoroacetic acid at 100 °C for 4 h), the mixtures were applied to Whatman No. 1 paper and separated by descending chromatography in 1-butanol/pyridine/water (3:2:1 v/v) for 48 h. The chromatogram was cut transversely into 3-mm-wide strips, which were added to 5 mL of 0.5% PPO/toluene solution, and counted in a scintillation counter. A longitudinal strip showing migration of a standard solution containing 10 μg each of D-galactose, D-glucose, and D-mannose was developed with silver nitrate.

Isolation of ^{14}C-Labeled Metabolic Intermediates from the Ascidian Tunic. The slices of ascidian tunic were labeled with D-[^{14}C]glucose, as described above. At the end of the labeling period, the medium was decanted and the tunic slices were washed with 20 mL of the incubation medium. The slices were suspended in 0.4 mL of 0.01 M Tris-HCl buffer (pH 8.0) containing 300 μg of proteinase K and 5 mM EDTA and incubated at 37 °C for 4 h. The incubation mixture was then centrifuged (2000*g* for 10 min at room temperature), and the clear supernatant was applied to Whatman 3MM chromatographic paper and developed in isobutyric acid/1 N NH_4OH (5:3 v/v) for 12 h. The chromatogram was cut transversely into 3-mm-wide strips, which were added to 5 mL of 0.5% PPO/toluene solution, and counted in a scintillation counter. A longitudinal strip showing migration of a standard solution containing 20 μg each of D-glucose, D-glucose 6-phosphate, and uridine 5′-diphosphate D-glucose was visualized by a short-wave ultraviolet lamp and by silver nitrate staining.

Enzymatic Degradation

Oxidation with D-Galactose Oxidase. The mixture of ^{14}C-labeled sugars obtained by acid hydrolysis of the ascidian polysaccharides (2000 cpm) and a mixture of standard sugars containing 10 μg each of D-galactose, D-glucose, and D-mannose were incubated with 1 unit of *D. dendroides* D-

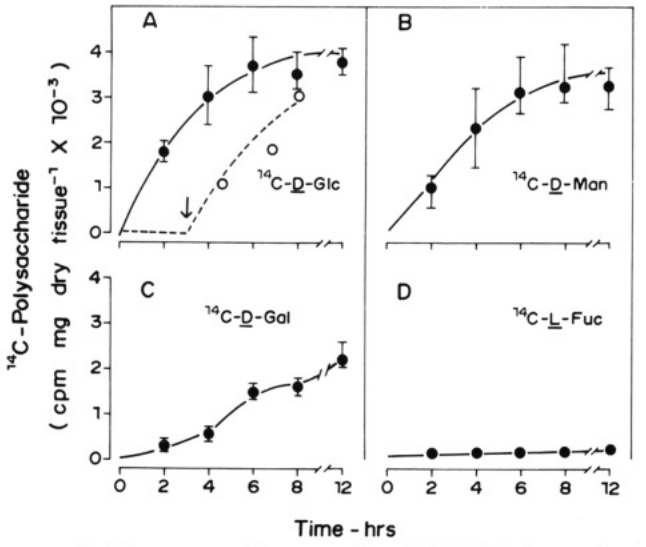

FIGURE 1: Time course of incorporation of ^{14}C-labeled sugars into the ascidian polysaccharides. Slices of ascidian tunic (about 10 mg wet weight) were incubated at 20 °C with 0.7 μCi (1.05 nmol) of D-[1-^{14}C]glucose (A), D-[U-^{14}C]mannose (B), D-[1-^{14}C]galactose (C), or L-[1-^{14}C]fucose (D) in 100 μL of the incubation medium (see Materials and Methods). At different times, the medium was decanted and the slices were washed five times with the incubation medium, then immersed in 3 mL of acetone, kept for 24 h at 4 °C, and dried. The ^{14}C-labeled polysaccharides were extracted and quantitated as described under Materials and Methods. In the experiment shown by a broken line (panel A), the tissue was incubated in the absence of sugar. At the time indicated by the arrow, 0.7 μCi (1.05 nmol) of D-[1-^{14}C]glucose was added to the medium and the medium was incubated for an additional 2, 3, or 5 h.

galactose oxidase (Avigad et al., 1962) in 50 μL of 0.05 M sodium acetate buffer (pH 7.0). After 4 h at 37 °C, the reaction mixtures were developed by descending chromatography on Whatman No. 1 paper in 1-butanol/pyridine/water (3:2:1 v/v) for 48 h, and transverse strips 3 mm wide were counted as before. In addition, a longitudinal strip from the chromatogram was developed with silver nitrate for the identification of nonlabeled standard sugars, before and after incubation with D-galactose oxidase.

Oxidation with D-Glucose Oxidase. The mixture of ^{14}C-labeled sugars obtained by acid hydrolysis of the ascidian polysaccharides (2000 cpm) was incubated with 1 unit of *A. niger* D-glucose oxidase in 50 μL of 0.05 M sodium acetate buffer (pH 7.0). After 4 h at 37 °C, the reaction mixtures were analyzed by descending chromatography, as described above.

Identification of the ^{14}C-Labeled Polysaccharides. Twenty microliters of the intact ^{14}C-labeled polysaccharides eluted from the origin after the paper chromatography, described for quantitation of radiolabel incorporation, was applied to 0.5% agarose gels in 0.05 M 1,3-diaminopropane/acetate buffer (pH 9.0) and run for 1 h at 120 V. The polysaccharides in the gel were fixed with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide in water and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5 v/v). After staining, the gel was washed for about 15 min in acetic acid/ethanol/water (0.1:5:5 v/v). The ^{14}C-labeled polysaccharides were visualized by autoradiography of the stained gels. The radioactive bands having identical electrophoretic migration with standard polysaccharides were scraped into 5 mL of 0.5% PPO/toluene solution and counted in a scintillation counter.

Identification of the ^{3}H-Labeled Polysaccharides. Fifty microliters of the ^{3}H-labeled ascidian polysaccharides (2000 cpm) was applied to a 0.5% agarose gel and electrophoresed as described above. After the run, the gel was cut into 2-mm-wide strips, which were frozen. Upon thawing, the agarose lost its gel structure and was removed by centrifugation (5000*g* for 15 min). Four hundred microliters of the clear supernatants was added to 5 mL of 0.4% PPO, 0.0004% POPOP solution in toluene/Triton X-100 (2:1 v/v) and counted in a scintillation counter.

Results and Discussion

Incorporation of ^{14}C-Labeled Sugars into Polysaccharides of the Tunic. The time course of labeling the sulfated polysaccharides from *S. plicata* with ^{14}C-labeled sugars (Figure 1) indicates that incorporation is higher when the tunic is incubated in vitro with D-[^{14}C]glucose and D-[^{14}C]mannose (Figure 1A,B). Incubation with D-[^{14}C]galactose leads to about half of the incorporation observed with the other two sugars (Figure 1C), whereas no incorporation occurs when the tissue is incubated with L-[^{14}C]fucose (Figure 1D). Incorporation begins immediately and increases steadily for D-[^{14}C]glucose and D-[^{14}C]mannose, whereas with D-[^{14}C]-galactose incorporation is slow in the first 4 h and then increases rapidly to a plateau value. Slices of the tunic incubated for 3 h in the absence of sugar retain their capacity to incorporate D-[^{14}C]glucose into the polysaccharides at approximately the same rate as when the labeled compound is present from the beginning (see broken line, Figure 1A). This observation ensures that the tissue retains its biosynthetic capacity during in vitro incubation.

In order to show whether the ^{14}C-labeled sugars were incorporated into sulfated polysaccharides, the solution of glycans obtained in the experiment of Figure 1 was subjected to electrophoresis on agarose gels (Figure 2). On autoradiograms of the stained gels, the radioactive bands coincide with the two

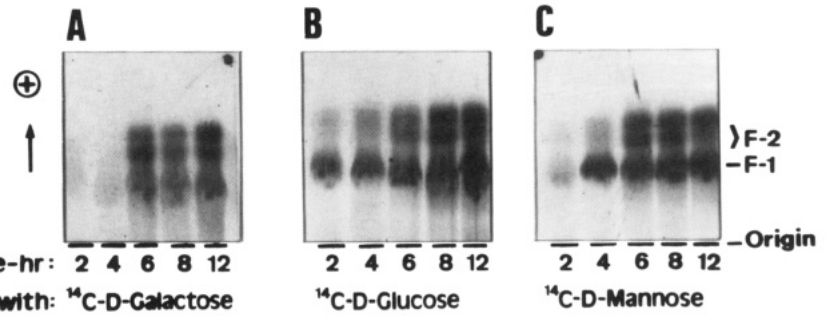

FIGURE 2: Agarose gel electrophoresis of the ^{14}C-labeled ascidian polysaccharides. Twenty microliters of the ascidian polysaccharides labeled with D-[^{14}C]glucose (A), D-[^{14}C]mannose (B), or D-[^{14}C]galactose (C) was applied to a 0.5% agarose gel, and electrophoresis was carried out in 0.05 M 1,3-diaminopropane/acetate buffer (pH 9.0) for 1 h at 120 V. The sulfated polysaccharides from the gel were fixed with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide in water for 12 h and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5 v/v). The radioactive bands corresponding to the ^{14}C-labeled polysaccharides were detected by autoradiography of the fixed and stained gel. The electrophoretic migrations of standard F-1 and F-2 fractions are indicated at the right.

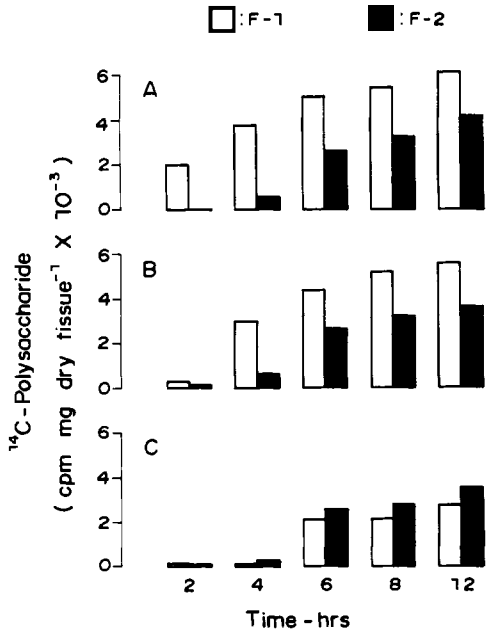

FIGURE 3: Time course of incorporation of D-[^{14}C]glucose (A), D-[^{14}C]mannose (B), and D-[^{14}C]galactose (C) into the F-1 and F-2 fractions of the ascidian polysaccharides. The radioactive bands having identical electrophoretic migration with standard F-1 and F-2 fractions (see Figure 2) were scraped into 5 mL of 0.5% PPO/toluene solution and counted in a liquid scintillation counter.

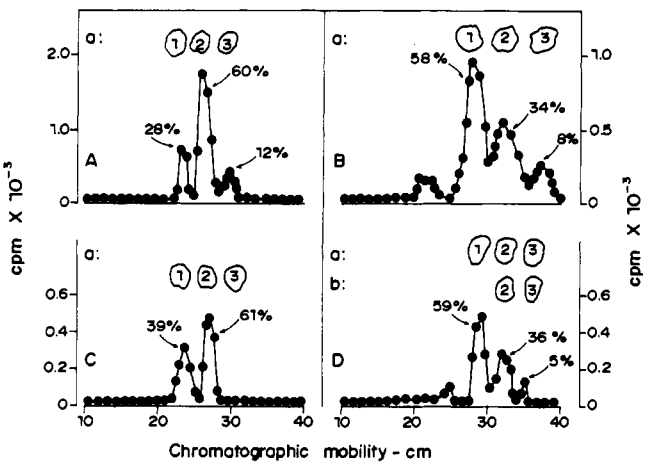

FIGURE 4: Identification of the ^{14}C-labeled sugars in the acid hydrolysates of the ascidian polysaccharides after an 8-h incubation with D-[^{14}C]mannose (A), D-[^{14}C]glucose (B, D), or D-[^{14}C]galactose (C). Panel D shows that D-galactose oxidase has no effect on the hydrolysate from the D-[^{14}C]glucose incubation of panel B. The hydrolysates were applied to Whatman No. 1 paper and chromatographed in 1-butanol/pyridine/water (3:2:1 v/v) for 48 h. The chromatogram was cut into 3-mm-wide strips and counted in a liquid scintillation counter. The relative amounts (%) of label appearing in each of the sugars, on the basis of the area of each peak compared with total area, are shown beside each peak. The upper portion of each panel (a) shows a strip from the chromatogram where a standard solution was applied, containing 10 μg each of D-galactose (1), D-glucose (2), and D-mannose (3), and then the strip was developed with silver nitrate. The upper portion of panel D shows the migration of a standard solution containing 10 μg each of D-galactose (1), D-glucose (2), and D-mannose (3) before (a) and after a 4-h incubation with D-galactose oxidase (b). Incubation conditions for standards and the polysaccharides hydrolysates are described under Materials and Methods.

fractions of sulfated polysaccharides that have been characterized in previous studies (Albano & Mourão, 1986). Both fractions (F-1 and F-2) are labeled, regardless of whether the slices of tunic are incubated with D-[^{14}C]glucose, D-[^{14}C]-mannose, or D-[^{14}C]galactose.

Inspection of the autoradiograms in Figure 2 suggests that label may be incorporated more rapidly into the F-1 fraction. This is documented quantitatively in Figure 3, which also shows that the proportion of label incorporated into F-1 and F-2 fractions is the same whether the tunic is incubated with D-[^{14}C]glucose or D-[^{14}C]mannose, despite the fact that F-2 has a higher D-glucose content than F-1 (Albano & Mourão, 1986; Mourão & Perlin, 1987). With D-[^{14}C]galactose, the F-1 and F-2 fractions are labeled at about equal rates.

After incubation of the tunic with D-[^{14}C]glucose, a higher proportion of label appears in the galactose residues (Figure 4B), whereas after incubation with D-[^{14}C]mannose or D-[^{14}C]galactose most of the ^{14}C label is in the glucose residues (Figure 4A,C). The [^{14}C]galactose obtained from incubation with D-[^{14}C]glucose (Figure 4D) or D-[^{14}C]mannose (not shown) is totally resistant to oxidation by D-galactose oxidase, indicating that this sugar is entirely in the L-enantiomeric form, as already observed for the nonlabeled polysaccharide (Mourão & Perlin, 1987; Pavão et al., 1989b). The measurement of the oxidation by D-galactose oxidase included a control, which shows the total oxidation of the D-galactose standard (b in Figure 4D). The ^{14}C-labeled glucose present in the hydrolysate is the D-isomer, since it was oxidized completely by D-glucose oxidase (not shown).

Overall, these results demonstrate that cells in slices of the ascidian tunic synthesize the L-galactose units of its sulfated polysaccharides in vitro. The best precursor appears to be D-glucose, since this sugar shows the highest rate of incorporation among the sugars tested (Figure 1) and forms a higher proportion of L-galactose units (Figure 4).

Epimerization of D-*Mannose to* L-*Galactose Involves the Action of* D-*Mannose Phosphate Isomerase.* Epimerization of D-mannose to L-galactose through its guanosine 5′-diphosphate nucleotide has been shown to occur in extracts from a green alga (Barber, 1975, 1979; Feingold, 1982) and a land snail (Goudsmit & Neufeld, 1967; Feingold, 1982), although the transfer of sugar from guanosine 5′-diphosphate L-galactose to other molecules has never been reported. However, since D-glucose and D-mannose belong to the same hexose monophosphate pool and are readily interconverted in most cells, it is possible to speculate from our results (Figures 1–4) that epimerization of D-glucose to L-galactose in the ascidian tunic could occur through the same guanosine 5′-diphosphate D-mannose pathway described for algae and the snail. On the other hand, the conversion of D-mannose to L-galactose in ascidian tunic could involve the formation of a metabolic intermediate through the action of D-mannose phosphate isomerase.

In order to distinguish between these two possibilities, slices of tunic were incubated with D-[2-^{3}H]mannose. If the epimerization of D-mannose to L-galactose involves the action of D-mannose phosphate isomerase, the [^{3}H]hydrogen atom at carbon 2 of D-mannose should be exchanged with the protons of water. If not, and, if epimerization occurs through the guanosine nucleotide pathway, the ^{3}H marker should remain in the L-galactose.

Our results indicate that, after incubation of slices of tunic with D-[2-^{3}H]mannose, some ^{3}H marker is incorporated into the polysaccharide (Figure 5A), but mostly in the F-2 fraction (Figure 5B), which has a higher mannose content (about 10% of all sugar residues) (Albano & Mourão, 1986; Mourão & Perlin, 1987). Analysis of the ^{3}H-labeled sugars obtained by acid hydrolysis of the total ascidian polysaccharide shows that most of the label is in the mannose residues, with only small amounts in L-galactose and D-glucose (Figure 5C).[1]

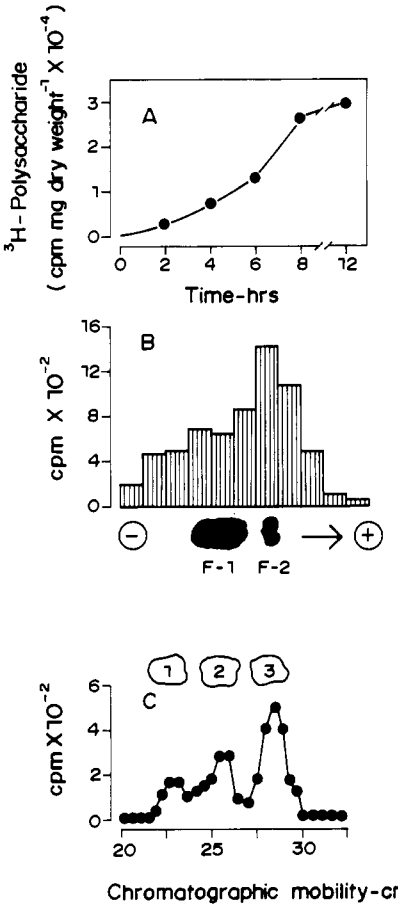

FIGURE 5: Labeling of the ascidian polysaccharides with D-[2-^{3}H]-mannose. (A) Time course of incorporation of the ^{3}H-labeled D-mannose. About 10 mg (wet weight) of the isolated slices of ascidian tunic was incubated for different times with 20 μCi (0.66 nmol) of D-[2-^{3}H]mannose. At the end of the incubation period, the ^{3}H-labeled polysaccharides were isolated and quantitated as described under Materials and Methods. (B) Identification of the ^{3}H-labeled ascidian polysaccharides. About 2000 cpm of the ^{3}H-labeled polysaccharides was applied to a 0.5% agarose gel and electrophoresed in 0.05 M 1,3-diaminopropane/acetate buffer (pH 9.0) for 1 h at 120 V. The ^{3}H-labeled polysaccharides in the gel were quantitated as described under Materials and Methods. In the lower part of panel B, the polysaccharides were stained with toluidine blue, which shows the electrophoretic migration of standard F-1 and F-2 fractions. (C) Identification of the ^{3}H-labeled sugars in the acid hydrolysates of ascidian polysaccharides. The acid hydrolysates of the ^{3}H-labeled polysaccharides from both fractions combined were applied to Whatman No. 1 paper and chromatographed in 1-butanol/pyridine/water (3:2:1 v/v) for 48 h. The chromatogram was cut into 3-mm-wide strips and counted in a liquid scintillation counter. The upper part of the figure shows the chromatographic migration of D-galactose (1), D-glucose (2), and D-mannose (3) standards.

These results suggest that epimerization of D-mannose to L-galactose involves the D-mannose phosphate isomerase step and not the conversion to guanosine 5′-diphosphate D-mannose. Therefore, the conversion of D-mannose (and D-glucose) to L-galactose in the ascidian tunic occurs through a different metabolic pathway, distinct from that described previously in other organisms (Goudsmit & Neufeld, 1967; Barber, 1975, 1979; Feingold, 1982).

[1] Small amounts of D-[^{3}H]galactose and D-[^{3}H]glucose in acid hydrolysates of ascidian polysaccharides labeled with D-[2-^{3}H]mannose might be attributed to contamination with mannose containing [^{3}H]hydrogen atoms in positions other than carbon 2. Even though such contaminants, if present, occur only in small amounts, they would carry the ^{3}H marker to L-galactose and D-glucose more rapidly than into D-mannose units (compare Figures 4A and 5C).

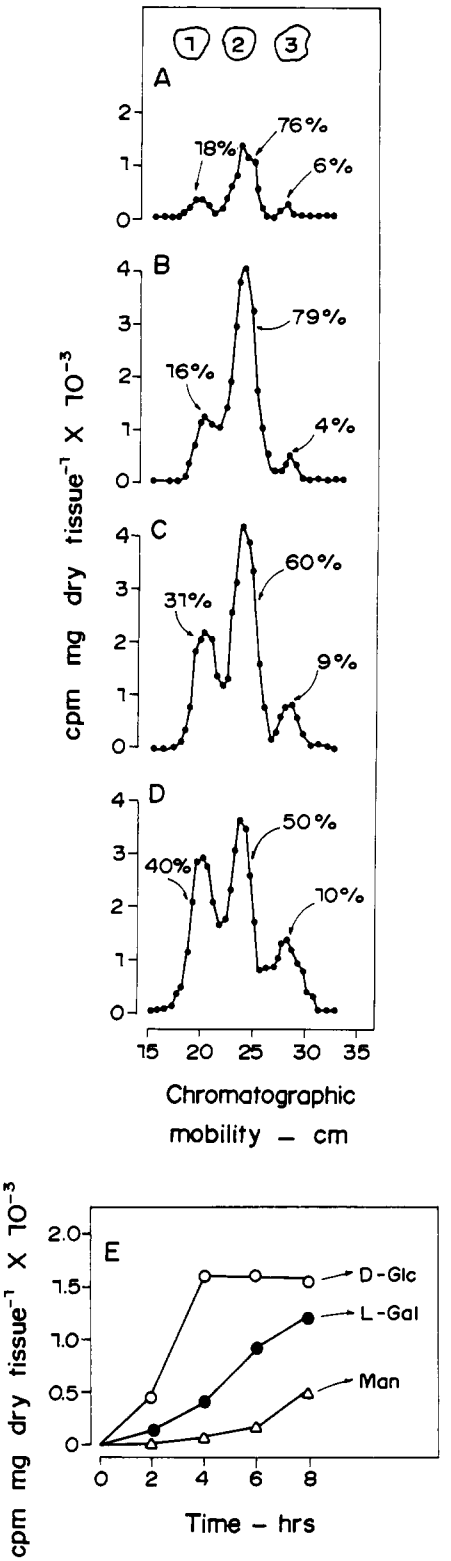

FIGURE 6: Identification of the ^{14}C-labeled sugars in the acid hydrolysates of ascidian polysaccharides labeled by incubation with D-[^{14}C]glucose for 2 (A), 4 (B), 6 (C), and 8 h (D). The sugars were separated and quantitated as described in the legend of Figure 4. The relative amounts of each of the ^{14}C-labeled sugars (in %) are based on the area of each peak compared with the total area. Panel E shows the absolute amount of label incorporated into each sugar; L-galactose (●), D-glucose (○), and D-mannose (△).

Epimerization of sugars in carbohydrate metabolism has been studied extensively. It may lead to inversion of the configuration of a single atom of the hexosyl moieties, as in the interconversion of UDP-D-glucose and UDP-D-galactose,

or it may involve a double epimerization, as in the synthesis of UDP-L-rhamnose from UDP-D-glucose, where the intermediate UDP-4-keto-6-deoxy-D-glucose is formed (Feingold, 1982). However, the conversion of D-glucose to L-galactose requires a triple epimerization, which brings about inversion of the configuration of carbon atoms 2, 3, and 5 of the hexosyl moieties.

L-Galactose Is Incorporated into the Ascidian Polysaccharides at a Lower Rate than D-Glucose. When the ascidian tunic is incubated in vitro with D-[^{14}C]glucose, the content of D-[^{14}C]glucose units in the ascidian polysaccharides increases rapidly up to 4 h and thereafter remains constant until the end of the labeling period. In contrast, the content of L-[^{14}C]galactose units increases throughout the experiment (Figure 6). This difference may be attributed to the delay in epimerization of D-glucose to L-galactose, whereas the D-glucose residues are incorporated more rapidly, possibly through its uridine 5′-diphosphate nucleotide.

Epimerization of D-glucose to L-galactose could occur after incorporation of D-glucose into the polymer, as previously described for the conversion of polysaccharide-bound β-D-glucuronic acid to α-L-iduronic acid in the course of heparin biosynthesis (Malmstrom et al., 1980). According to this line of reasoning, the experiment of Figure 6 could be interpreted as showing that the conversion of polysaccharide-bound D-[^{14}C]glucose to L-galactose occurs at a rate identical with that of the incorporation of new D-[^{14}C]glucose. However, the experiment of Figure 7 excludes such possibility, since when the D-[^{14}C]glucose is removed from the incubation medium after 2 h (Figure 7), the content of D-[^{14}C]glucose units in the polysaccharide remains constant until the end of the incubation period, and the amount of L-[^{14}C]galactose is negligible throughout the experiment (Figure 7). This experiment totally excludes the possibility that polysaccharide-bound D-glucose is converted to L-galactose.

Finally, the delay in the labeling of the D-mannose units compared with L-galactose (Figure 6E) reinforces our proposal that epimerization of D-glucose to L-galactose does not occur through the D-mannose pathway.

Attempts To Isolate Intermediates of the Metabolic Route. Attempts to isolate intermediates of this metabolic route after incubating homogenates with ^{14}C-labeled precursors were unsuccessful, since they were unable to incorporate ^{14}C-labeled sugars into polysaccharides or to form any detectable ^{14}C-labeled intermediate when incubated with D-[^{14}C]glucose, D-[^{14}C]mannose, or D-[^{14}C]glucose 6-phosphate, even after the addition of ATP, UTP, GTP, TTP, or CTP. This result may reflect the low content of cells in the tunic, which contains an extensive extracellular matrix, composed of large amounts of fibers and amorphous substance (Barnes, 1980).

An alternative attempt to isolate metabolic intermediates involved protease digestion of the tissue slices, after labeling with D-[^{14}C]glucose for 4 h. Besides the ^{14}C-labeled polysaccharide and [^{14}C]glucose, three other products, denominated I, II, and III, were detected. Compounds I and II have the same mobility on paper chromatography and on paper electrophoresis as standard uridine 5′-diphosphate D-glucose and D-glucose 6-phosphate, respectively; after acid hydrolysis both compounds form [^{14}C]glucose, which was oxidized completely by D-glucose oxidase.[2] Compound III does not migrate on paper electrophoresis (at pH 5.0), and after acid hydrolysis it forms only D-[^{14}C]glucose. This compound does not appear

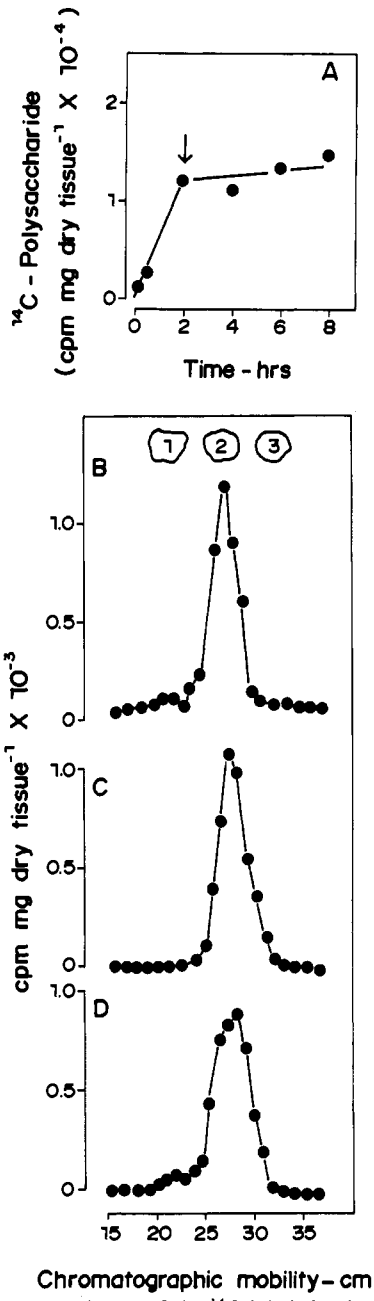

FIGURE 7: Chase experiment of the ^{14}C-labeled polysaccharides of the tunic labeled by incubation with D-[^{14}C]glucose. (A) Slices of ascidian tunic were incubated with D-[^{14}C]glucose as described in the legend of Figure 1. At the time indicated by the arrow (3 h), the incubation medium was removed, and a new medium without D-[^{14}C]glucose was added to the tissue slices and incubated for different times. The ^{14}C-labeled polysaccharides were extracted and quantitated as described under Materials and Methods. (B–D) Identification of the ^{14}C-labeled sugar in the acid hydrolysates of the ascidian polysaccharides incubated in the experiment shown in panel A for 2 (B), 4 (C), and 8 h (D). The sugars were separated and quantitated as described in the legend of Figure 4.

to be a metabolic precursor of the ascidian polysaccharides, since its concentration remains constant in a cold chase experiment whereas those of compounds I and II and D-[^{14}C]-glucose decrease markedly. It is conceivable that compound III is a small polymer of D-[^{14}C]glucose.

The absence of detectable amounts of L-galactose among the metabolic precursors isolated from tunic slices, together with the unequivocal evidence that this sugar appears in the ascidian polysaccharides, suggests that the L-galactose intermediates may be formed in minimal amounts, perhaps as a limiting step in the biosynthetic pathway, or that they are very

[2] It is possible to speculate that L-galactose could arise from L-glucose through a 4-epimerase. However, the absence of L-glucose in these metabolic precursors does not support this hypothesis.

unstable and could not be detected under the experimental conditions used.

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Registry No. D-Glucose, 50-99-7; L-galactose, 15572-79-9; D-mannose, 3458-28-4; mannose phosphate isomerase, 9023-88-5.

REFERENCES

Albano, R. M., & Mourão, P. A. S. (1983) *Biochim. Biophys. Acta 760*, 192–196.
Albano, R. M., & Mourão, P. A. S. (1986) *J. Biol. Chem. 261*, 758–765.
Anderson, E., & Lowe, H. J. (1947) *J. Biol. Chem. 168*, 284–297.
Avigad, G., Amaral, D., Ascencio, C., & Horecker, B. L. (1962) *J. Biol. Chem. 237*, 2736–2743.
Barber, G. A. (1975) *Arch. Biochem. Biophys. 167*, 718–722.
Barber, G. A. (1979) *J. Biol. Chem. 254*, 7600–7603.
Barnes, R. D. (1980) *Invertebrate Zoology*, pp 1030–1032, W. B. Saunders Co., Philadelphia, PA.
Bell, D. J., & Baldwin, E. (1941) *J. Chem. Soc.*, 125–131.
Bretting, H., Jacobs, G., Benecke, I., Konig, W. A., & Thiem, J. (1985) *Carbohydr. Res. 139*, 225–236.
Cássaro, C. M. F., & Dietrich, C. P. (1977) *J. Biol. Chem. 252*, 2254–2261.
Correa, J. B. C., Dmytraczenko, A., & Duarte, J. H. (1967) *Carbohydr. Res. 3*, 445–452.
Feingold, D. S. (1982) *Encycl. Plant Physiol., New Ser. 13A*, 37–39.
Goudsmit, E. M., & Neufeld, E. F. (1967) *Biochem. Biophys. Res. Commun. 26*, 730–735.
Malmstrom, A., Rodén, L., Feingold, D. S., Jacobsson, I., Backstrom, G., & Lindahl, U. (1980) *J. Biol. Chem. 255*, 3878–3883.
Mathews, M. B. (1975) *Connective Tissue. Macromolecular Structure and Evolution*, pp 93–206, Springer-Verlag, Berlin.
Mourão, P. A. S., & Bastos, I. G. (1987) *Eur. J. Biochem. 166*, 639–645.
Mourão, P. A. S., & Perlin, A. S. (1987) *Eur. J. Biochem. 166*, 431–436.
Painter, T. J. (1983) in *The Polysaccharides* (Aspinall, G. O., Ed.) Vol. 2, pp 195–285, Academic Press, New York.
Pavão, M. S. G., Albano, R. M., Lawson, A. M., & Mourão, P. A. S. (1989a) *J. Biol. Chem. 264*, 9972–9979.
Pavão, M. S. G., Albano, R. M., & Mourão, P. A. S. (1989b) *Carbohydr. Res. 189*, 374–379.
Vieira, R. P., & Mourão, P. A. S. (1988) *J. Biol. Chem. 263*, 18176–18183.

Purification and Biochemical Characterization of Recombinant α_1-Antitrypsin Variants Expressed in *Escherichia coli*

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ABSTRACT: Site-directed variants of α_1-antitrypsin (α_1AT) expressed in a recombinant strain of *Escherichia coli* have been isolated with an overall process yield of 50% following tangential flow ultrafiltration, anion-exchange, immobilized metal affinity, and hydrophobic interaction chromatography. The primary structure of the purified variants including the integrity of the N- and C-termini has been verified by electrospray mass spectrometry of the intact molecules (44 kDa) for two of the variants (α_1AT Leu-358 and α_1AT Ala-357, Arg-358). Complementary classical peptide mapping and automated amino acid sequencing have verified 75% of the primary sequence of α_1AT Ala-357, Arg-358. Isoelectric focusing in an immobilized pH gradient revealed some microheterogeneity which proved to be reproducible from one purification batch to another. The isolated variants of α_1AT did not show any signs of proteolytic degradation during the purification process and proved to be fully active against their target proteases. The described process also allowed the complete removal of endotoxins from the preparations, opening the possibility to evaluate these novel protease inhibitors for their in vivo efficacy in different animal models of human disease.

α_1-Antitrypsin (α_1AT),[1] one of the major protease inhibitors in human plasma, is present at concentrations of 1.5–3.5 mg/mL (Travis & Salvesen, 1983). Its primary physiological role is the inhibition of neutrophil elastase, with an insufficiency leading to the development of pulmonary emphysema. α_1AT deficiency can be either hereditary or acquired (Laurell & Eriksson, 1963; Eriksson, 1964; Kueppers & Black, 1974; Morse, 1978a,b; Carrell et al., 1982; Gadek & Crystal, 1982; Brantly et al., 1988). Attempts are presently being made to provide a protective shield against excess neutrophil elastase

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[1] Abbreviations: α_1AT, α_1-antitrypsin; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; *E. coli*, *Escherichia coli*; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetate (tetrasodium salt); PTH, phenylthiohydantoin; ELISA, enzyme-linked immunosorbent assay; IMAC, immobilized metal affinity chromatography; rtPA, recombinant tissue type plasminogen activator; RID, radial immunodiffusion; AEX, anion-exchange chromatography.

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