Roseman, S. (1970) *Chem. Phys. Lipids 5*, 270–297.
Shier, W. T., & Trotter, T. J., III (1976) *FEBS Lett. 65*, 165–168.
Wickner, W. (1979) *Annu. Rev. Biochem. 48*, 23–45.
Yamada, K., & Weston, J. A. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3492–3496.
Yurchenco, P. D., & Atkinson, P. H. (1977) *Biochemistry 16*, 944–953.

Synthesis of Glycosaminoglycans by Cultured Rabbit Smooth Muscle Cells†

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ABSTRACT: Rabbit aortic smooth muscle cells were evaluated for their ability to synthesize and accumulate glycosaminoglycans (GAGs). Because of the sensitivity of the microtechniques utilized, it is possible to determine the specific radioactivity of the GAGs obtained after radioactive incorporation of [^{35}S]SO_4^{2-} and [^{14}C]glucosamine. Data obtained at various incubation times indicate that the distribution of the GAGs secreted by the cells into the medium is different from that retained by the cell layer. Hyaluronic acid was shown to be the most abundantly produced GAG, and much of this GAG does not appear to be incorporated into the extracellular matrix. Also, a high percentage of the total chondroitin sulfate B synthesized was secreted into the medium. On the other hand, most of the heparan sulfate and chondroitin sulfate C/A synthesized seems to be associated with the cell layer. These results are consistent with those found in whole rabbit aorta.

Evidence for the biosynthesis of glycosaminoglycans (GAGs)[1] by vascular smooth muscle cells has recently been established (Wight & Ross, 1975). In these studies the GAGs were measured only in terms of radioactivity present in the culture medium and at a single incubation time. Cultured fibroblasts and arterial endothelial cells (Buonassisi, 1973) have been examined as well. More recently, kinetic data of total GAG synthesis have been evaluated (Gamse et al., 1978).

In the present report we describe the synthesis of GAGs by cultured rabbit aortic smooth muscle cells. The types of GAGs formed and their rates of synthesis and secretion were examined. The micromethod employed in these studies permits simultaneous determination of two essential aspects of GAG biosynthesis. It is possible to measure the total amount and distribution of the GAGs synthesized as well as to quantify the radioactivity of the [^{35}S]SO_4^{2-} or [^{14}C]glucosamine incorporated into each of these mucopolysaccharides during a prescribed pulse period. Such a procedure allows for long-term investigations as well as short-term pulse–chase experiments.

Although many studies on collagen and elastin biosynthesis have been carried out on vascular smooth muscle cells in culture (Ross, 1971; Faris et al., 1976; Burke et al., 1977), little is known of the role the GAGs play in the synthesis and secretion of these proteins. By developing methods to determine GAG distribution and synthesis in cell cultures, one should now be able to evaluate the influence of these mucopolysaccharides on connective tissue protein synthesis. The present communication suggests that such experiments are feasible and will be the subject of further investigations.

Materials and Methods

Preparation of Smooth Cell Cultures. Smooth muscle cells were isolated and grown from the medial layer of the aortic arch of weanling rabbits as described previously (Faris et al., 1976). Cells were seeded into second passage at a density of 1.5×10^6 cells/flask (75 cm^2). These cells were maintained in 20 mL of Dulbecco's Modified Eagle's Medium supplemented with fetal bovine serum (10%), penicillin (100 units/mL), and streptomycin (100 μg/mL). Flasks were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed 2 times/week. Previous electron microscopic studies revealed that cells prepared in this manner have the ultrastructure characteristics of smooth muscle cells (Mazurkowitz et al., 1980).

Pulse-Label Experiments. Cells used for pulse experiments were grown for 19 days in the second passage [approximately (4–5) $\times 10^6$ cells/flask]. Before the cells were pulsed, the spent medium was aspirated off and the cell layers were washed 2 times with Ca^{2+}- and Mg^{2+}-free Puck's saline G followed by one wash with serum-free Dulbecco medium. The cells to be pulsed with [^{35}S]SO_4^{2-} were then preincubated for 1 h in the same medium. The cells to be pulsed with [^{14}C]glucosamine were preincubated for 1 h with serum-free medium containing 10 mM pyruvate in place of glucose. In each case this medium was replaced with 10 mL of serum-free medium per flask that contained 1 μCi/mL of either [^{35}S]SO_4^{2-} or [^{14}C]glucosamine (New England Nuclear, Boston, MA). Control flasks did not contain any radioactive labeled precursors.

At specified incubation times the spent radioactive medium from pairs of flasks containing the same isotope was pooled and the cells were washed 3 times with 2.0 mL of Puck's saline G. These wash solutions were added to the corresponding pooled medium, and the resulting solutions were dialyzed vs. cold 0.01 M Na_2SO_4 and then against water. In the case of [^{14}C]glucosamine, dialysis was carried out vs. water. The cell layers of each pair of flasks were harvested in 1.5 mL of Puck's saline G with the aid of a rubber policeman and pooled.

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[1] Abbreviations used: GAG, glycosaminoglycan; CSB, chondroitin sulfate B; CSC, chondroitin sulfate C; HA, hyaluronic acid; HS, heparan sulfate. CSC may also contain a minute amount of CSA.

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Isolation of Glycosaminoglycans. The dialyzed medium derived from two flasks of cells (~50 mL) was digested with a series of enzymes in the following manner. The medium was first treated with three separate additions of 6 mg of Pronase E (70 000 p.u.k./g; Kaken Biochemical Co., Tokyo, Japan) in 0.5 mL of 5 mM $Ca(OAc)_2$, pH 7.8, 45 °C, one addition initially and then one each after 3 and 6 h. The reaction mixture was incubated for a total of 24 h. The resulting solution was then dialyzed exhaustively against 0.005 M NaCl at 4 °C, concentrated to a volume of ~3.0 mL, and digested with papain. Papain treatment consisted of the addition of 3.0 mg of papain (Sigma Chemical Co., St. Louis, MO) in 0.01 M EDTA–0.01 M Cys·HCl–0.005 M NaCN buffer, pH 7.8, in three successive treatments at 3- to 4-h intervals. This reaction mixture was then kept at 60 °C for a total of 24 h. Subsequently, a three- to fourfold excess of $Ca(OAc)_2$ was added, followed by three separate additions at 3–6-h intervals of 3.0 mg of Pronase E (pH 7.8, 45 °C). After 24 h, the digest was centrifuged at low speed for 30 min and the residue was washed twice with 0.5 mL of 0.005 M $Ca(OAc)_2$. The supernatants and $Ca(OAc)_2$ washes were pooled, exhaustively dialyzed at 4 °C against 50 L of 0.005 M $Ca(OAc)_2$, lyophilized, and dissolved in water (usually 25 μL/flask) for the subsequent GAG analyses.

The cell layers were delipidated with several changes of acetone, dried in a vacuum desiccator overnight, and then suspended in Tris-HCl buffer (0.005 M, pH 7.8) containing 0.0005 M $MgCl_2$ so that the final volume was 2 mL. The average dry weight of the cell layers per flask was 9 mg. The cell layers were then treated at 37 °C for 6 h with 0.4 mg each of DNase and RNase (Sigma Chemical Co., St. Louis, MO) followed by papain and Pronase treatments using essentially the same conditions described for the medium. Papain was added at an enzyme/cell ratio of 1:100. After the enzyme treatments the final preparation was dissolved in 0.10 mL of water and analyzed as described below.

Determination of the Amount and Radioactivity of the Isolated Glycosaminoglycans. Aliquots (5–25 μL) of each of the solutions prepared above were applied to cellulose acetate membranes (15 × 15 cm, Sepraphore III, Gelman Instrument Co., Ann Arbor, MI) and subjected to two-dimensional electrophoresis as described earlier (Hata & Nagai, 1973; Stevens et al., 1976). A minimum of five analyses was performed for each determination. Briefly, the first direction of the electrophoresis was carried out in 0.1 M pyridine formate buffer, pH 3.2, while the second dimension was performed in 0.1 M $Ba(OAc)_2$. Subsequently, the cellulose acetate plates were stained with alcian blue. The alcian blue stained spot of each glycosaminoglycan was cut out and quantitated spectrophotometrically as described previously (Hata & Nagai, 1973; Stevens et al., 1976). The spot was dissolved in 10 mL of Bray's solution (Hata & Nagai, 1978), and the radioactivity of each spot was measured with a Packard Tricarb liquid scintillation spectrometer, Model 3253.

Identification of the Glycosaminoglycans. For the identification of the GAGs the following criteria were used: (1) comparison of the electrophoretic mobilities, including the use of standards added to our unknowns; (2) enzymatic studies, including leech hyaluronidase (Biotrics Inc., Arlington, MA), ovine testicular hyaluronidase (Sigma Chemical Co., St. Louis, MO), chondroitinases ABC and AC-II (Dr. T. Okuyama, Seikagaku Kogyo Co., Ltd., Tokyo, Japan), heparatinase (Dr. A. Linker, Salt Lake City, UT); (3) the use of HNO_2 for the specific degradation of HS (Cifonelli, 1968). All procedures were carried out as described previously (Stevens et al., 1976). Subsequently, the resulting reaction mixtures were evaluated by two-dimensional electrophoresis.

Autoradiography. Autoradiography was performed after two-dimensional electrophoresis of labeled GAGs on cellulose acetate plates which were stained with alcian blue, dried, and impregnated with PPO (2,5-diphenyloxazole) (Bonner & Lasky, 1974). The plates were placed on preexposed X-ray film (Lasky & Mills, 1975) and kept at −70 °C for several weeks before developing.

Results and Discussion

(A) Identification of the GAGs. The two-dimensional electrophoretic pattern of the GAGs of the cell layer (Figure 1A) revealed the presence of CSB, CSC, HA, and HS as judged by electrophoretic mobilities in two directions. Heparin and keratan sulfate were not detected. Further proof of the identity of GAGs was obtained when authentic GAGs were added individually to the GAG mixture prior to electrophoresis. The samples were also digested separately with highly specific enzymes and electrophoresed as described under Materials and Methods. Data obtained were consistent with the presence of CSB, CSC, HA, and HS. Leech hyaluronidase removed only HA. The fact that the HA spot was completely eliminated indicated the absence of chondroitin as well. Ovine testicular hyaluronidase removed HA and CSC, chondroitinase AC degraded HA and CSC, chondroitinase ABC hydrolyzed HA, CSB, and CSC, heparatinase removed HS, and HNO_2 degraded HS. The medium also contained CSB, CSC, HA, and HS (Figure 1B) which were identified by the same procedures.

(B) Quantitation of the GAGs. In Figure 2A the amount of GAGs per flask is plotted as a function of the incubation time. The predominant GAG in the cell layer was CSC, followed by HS. Hyaluronic acid and CSB remained essentially constant over the whole period of incubation and were lower in content than the other two GAGs when examined at 48 h. The relative percentages of the GAGs were 16% for CSB, 40% for CSC, 13% for HA, and 31% for HS. These values compare well with those obtained from the intimal–medial layer of the aortic arch of weanling rabbit (19% for CSB, 42% for CSC, 14% for HA, and 25% for HS).

In the medium the only GAG detected in significant amounts after a period of 6 h was HA (Figure 2B), although trace amounts of the other GAGs were observed. The amount of HA increased rapidly, whereas the concentrations of CSB and CSC rose slightly and then leveled off after 24 h. Heparan sulfate was always present in small amounts. The relative percentages of these GAGs at 48 h of incubation were found to be 64, 23, 11, and 2% for HA, CSB, CSC, and HS, respectively.

The high proportion of HA in the medium and the low proportion of this GAG in the cell layer suggest a high secretion rate of HA but a relatively poor efficiency of incorporation into the insoluble extracellular matrix. Since HA is known to be a potent inhibitor of proteoglycan synthesis (Wiebkin & Muir, 1977; Handley & Lowther, 1976), this may be one explanation why most of the HA in cell cultures is secreted into the medium. In contrast, the high percentage of CSC in the cell layer and the relatively low percentage in the medium might suggest a low rate of formation of this GAG but a relatively efficient incorporation into the extracellular matrix. The [^{14}C]glucosamine study described below corroborates these findings. Thus, the percent distribution of the GAGs secreted by the cells is different from that retained extracellularly. Kresse et al. (1975) studied fibroblast-like cells from the intima of bovine aorta and also emphasized striking

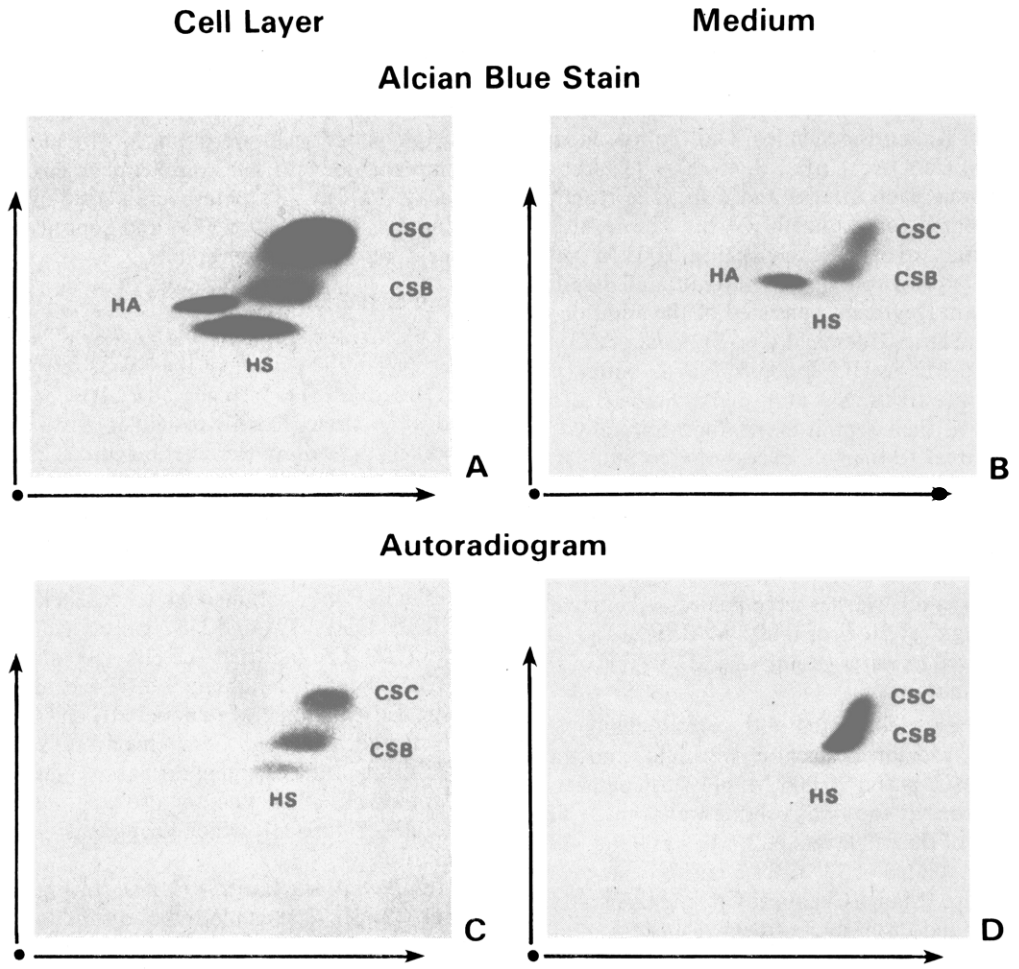

FIGURE 1: Two-dimensional electrophoresis of the glycosaminoglycans produced by cultured smooth muscle cells of the rabbit aorta. The electrophoretic distribution of the GAGs of the cell layer and the medium is seen in (A) and (B), respectively. These GAGs were derived from a 24-h culture and were stained with alcian blue after electrophoresis. The corresponding autoradiograms of these electrophoretic plates are shown in (C) and (D), respectively. Since the cells were pulsed with $[^{35}S]SO_4^{2-}$, CSB, CSC, and HS, but not HA, contained this label. The first and second directions of the electrophoresis are indicated by the horizontal and vertical arrows, respectively (for further information, see the text).

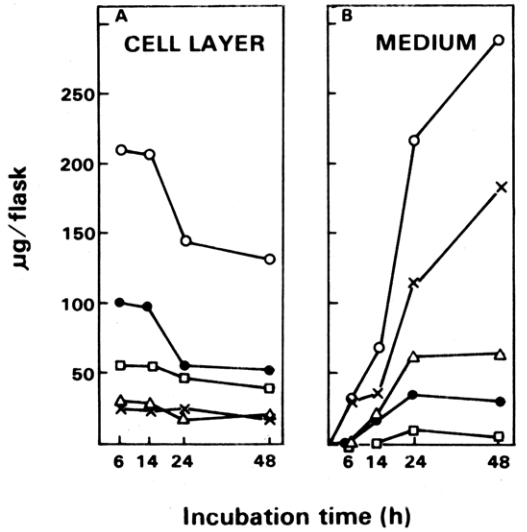

FIGURE 2: Glycosaminoglycan content of cultured rabbit aortic smooth muscle cells. The GAG levels in the cell layer and the medium are seen in (A) and (B), respectively. Note that at the zero time of this experiment the cells had already been grown for 19 days in the second passage. The following code was used: CSB, △; CSC, ●; HA, ×; HS, □; total GAG, ○ (for further information, see the text).

qualitative and quantitative differences in sulfated GAG production and secretion among different cell lines.

Incorporation of $[^{35}S]SO_4^{2-}$ *into GAGs.* The incorporation of $[^{35}S]SO_4^{2-}$ into the GAGs is illustrated in parts C and D of Figure 1. In the cell layer, the incorporation of this label measures the GAGs synthesized during the pulse period only, whereas the total GAG content represents the amount of GAGs detected after 19–21 days in culture. The data obtained from studying the GAGs of the medium, both the amount and the incorporated label described below, reflect the accumulation of these mucopolysaccharides during the pulse period.

An autoradiogram of the two-dimensional electrophoretogram of the $[^{35}S]SO_4^{2-}$-labeled GAGs of the cell layer revealed three components (Figure 1C) which were identified as CSB, CSC, and HS. HA does not incorporate $[^{35}S]SO_4^{2-}$. The major radioactive labeled component was CSB (45%). CSC accounted for 34% and HS, the minor component, accounted for 20% of the radioactivity. This ratio remained almost constant throughout the incubation period.

While CSC was shown to be the most predominant GAG in the cell layer when judged on a weight basis, CSB displayed the greatest incorporation of radioactivity (Figure 3A) The sensitivity of the methods employed has made it possible to obtain values for the specific radioactivity of the various GAGs (Table I). The specific radioactivity of CSB was highest at all times during the incubation and was found to be 3 times as high as that of CSC.

When the medium from the $[^{35}S]SO_4^{2-}$-pulsed cells was examined, small but distinct amounts of radioactivity in CSB,

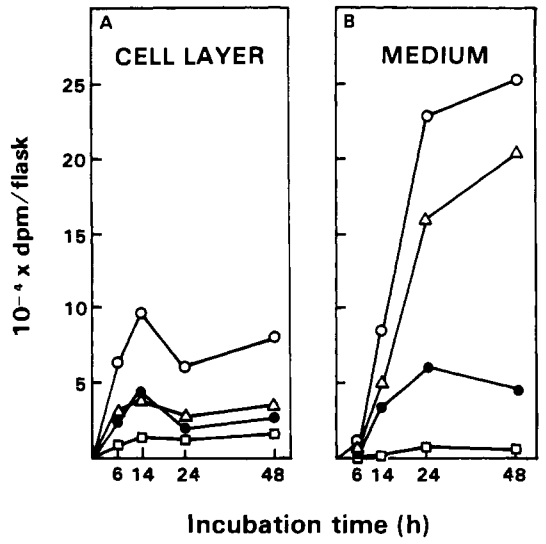

FIGURE 3: Incorporation of $[^{35}S]SO_4^{2-}$ into the glycosaminoglycans of the cultured rabbit aortic smooth muscle cells. The radioactive distribution of the GAGs in the cell layer and medium is seen in (A) and (B), respectively. The following code was used: CSB, △; CSC, ●; HS, □; total GAG, ○ (for further information, see the text).

Table I: Specific Radioactivities[a] of Sulfated GAGs of Cultured Rabbit Aortic Smooth Muscle Cells

	incubn time (h)	HS	CSB	CSC
cell layer	6	15 ± 0.6	106 ± 2.5	25 ± 1.3
	14	24 ± 1.1	142 ± 3.6	46 ± 2.0
	24	27 ± 1.8	153 ± 4.6	51 ± 2.0
	48	41 ± 2.8	182 ± 6.6	53 ± 1.1
medium	6	*b*	*b*	*b*
	14	136 ± 1.3[c]	272 ± 9.7[c]	216 ± 8.0[c]
	24	66 ± 4.3	257 ± 10	181 ± 7.8
	48	75 ± 4.5	311 ± 9.9	151 ± 6.8

[a] Specific radioactivities are expressed in dpm × 10^{-4} per mg of GAG. Each value was the result of 10 analyses. Standard error is also indicated. [b] Could not be determined. [c] Difficult to determine because of the small amounts of both weight and radioactivity.

CSC, and HS were observed after 6 h of incubation (Figure 3B). During the initial phase of the pulse period (14 h) CSC accounted for 40% of the total radioactivity. The rate of accumulation of each of these radioactive GAGs in the medium was highest during the first 24-h period of the pulse. The major radioactive sulfated GAG was always CSB; it accounted for ~55% of the radioactivity at 14 h of incubation, increasing to 80% at 48 h. The radioactivity of HS was less than 5%. The distribution of the radioactive GAGs in the medium after 24 h resembled that of arterial smooth muscle cells from the pigtail monkey which were cultured for the same period of time (Wight & Ross, 1975).

Incorporation of $[^{14}C]$*Glucosamine.* The $[^{14}C]$glucosamine studies were also carried out with rabbit smooth muscle cells that had been maintained for 19 days in the second passage as described above. However, the total GAG concentration in these cells at the start of the experiment was ~60% of that observed in the cell layers used in the $[^{35}S]SO_4^{2-}$ experiment, pointing out that cell culture variation must be considered in all experiments. The results from these experiments again show that more radioactivity was associated with the GAGs in the medium than with the GAGs of the cell layer. Also of significance is the fact that a high percentage (~50%) of the total radioactivity is incorporated into HA of the medium. The specific activity of HA was considerably higher than that of most of the other GAGs (Table II). It is important to realize that $[^{35}S]SO_4^{2-}$ does not account for total GAG production. To obtain more realistic values, we must carry out combinations of $[^{35}S]SO_4^{2-}$ and $[^{14}C]$glucosamine pulses.

Table II: Specific Radioactivities[a] of $[^{14}C]$Glucosamine-Labeled GAGs of the Medium of Cultured Rabbit Smooth Muscle Cells

incubn time (h)	HA	HS	CSB	CSC
6	69 ± 3.0	19 ± 1.4	89 ± 3.2	58 ± 2.5
14	94 ± 3.0	29 ± 1.0	69 ± 1.8	55 ± 1.6
24	86 ± 1.9	26 ± 1.4	62 ± 0.6	50 ± 2.1
48	79 ± 3.3	17 ± 0.9	43 ± 0.5	33 ± 0.7

[a] Specific activities are expressed in dpm × 10^{-6} per mg of GAG. Each value was the result of 10 analyses. Standard error is also indicated.

These studies serve to confirm and extend previous observations on the synthesis and characterization of the glycosaminoglycans of vascular smooth muscle cells. In earlier investigations using cell cultures either the total GAG content was determined (Buonassisi, 1973) or the GAG distribution at a single time point was quantitated (Wight & Ross, 1975). The sensitivity of the procedure described is such that relatively few cells are required to evaluate the synthetic machinery for the production of GAGs. In addition, the distribution of the various GAGs in the medium and cell layer present after long-term accumulation in culture and/or produced by short-term pulse–chase experiments can now be determined by these methods.

The data presented in this paper reveal that HA is the most abundantly produced GAG and suggest that much of this GAG is not incorporated into the extracellular matrix of the cultured cell. One role of HA in connective tissue synthesis may be in directing the formation of the collagen and elastin fibers. Once these fibers are laid down, the hyaluronate may no longer be required. On the other hand, CSC and CSB may also play a significant role in the stabilization of the connective tissue fibers that have been formed. Smooth muscle cells were selected for this study because they have been shown by chemical and histological means to be capable of synthesizing both collagen and elastin in vitro. These cells are thought to be responsible for the connective tissue synthesis in large mammalian blood vessels which relates significantly to the pathogenesis of atherosclerosis. Although one must be cautious in relating in vitro cell culture studies to in vivo studies, the present data are consistent with our in vivo observations using weanling rabbit aorta in which CSC was found in the highest porportion. Future experiments must focus on the interactions between collagen deposition, collagen stabilization, and the glycosaminoglycans.

Acknowledgments

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References

Bonner, W. M., & Lasky, R. A. (1974) *Eur. J. Biochem. 46*, 83.

Buonassisi, V. (1973) *Exp. Cell Res. 76*, 363.

Burke, J. M., Baltan, G., Ross, R., & Bornstein, P. (1977) *Biochemistry 16*, 3243.

Cifonelli, J. A. (1968) *Carbohydr. Res. 8*, 233.

Faris, B., Salcedo, L. L., Cook, V., Johnson, L., Foster, J. A., & Franzblau, C. (1976) *Biochim. Biophys. Acta 418*, 93.

Gamse, G., Fromme, H. G., & Kresse, H. (1978) *Biochim. Biophys. Acta 544*, 514.
Handley, C. J., & Lowther, D. A. (1976) *Biochim. Biophys. Acta 444*, 69.
Hata, R., & Nagai, Y. (1973) *Anal. Biochem. 52*, 652.
Hata, R., & Nagai, Y. (1978) *Biochim. Biophys. Acta 543*, 149.
Kresse, H., von Figura, K., Buddecke, E., & Fromme, H. G. (1975) *Hoppe-Seyler's Z. Physiol. Chem. 356*, 929.
Lasky, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335.
Mazurkowitz, J., Vaughan, D. W., & Franzblau, C. (1980) *In Vitro* (in press).
Ross, R. (1971) *J. Cell Biol. 50*, 172.
Stevens, R. L., Colombo, M., Gonzales, J. J., Hollander, W., & Schmid, K. (1976) *J. Clin. Invest. 58*, 470.
Wiebkin, O. W., & Muir, H. (1977) *J. Cell Sci. 27*, 199.
Wight, T. N., & Ross, R. (1975) *J. Cell Biol. 67*, 675.

Saponin–Cholesterol Interaction in the Multibilayers of Egg Yolk Lecithin As Studied by Deuterium Nuclear Magnetic Resonance: Digitonin and Its Analogues†

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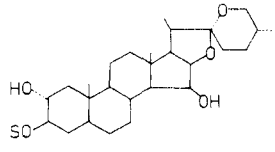
ABSTRACT: In order to gain an understanding of the hemolytic activity of digitonin, a ^2H NMR study was attempted with [26,26,26,27,27,27-$^2H_6$]cholesterol (Chol-d_6) and [18,18,18-$^2H_3$]stearic acid (SA-d_3) as probes for the terminal methyl portions of cholesterol and lipids, respectively, for the multibilayers of egg yolk lecithin containing digitonin or its analogues. It was found by ^2H NMR spectroscopy of the SA-d_3 probe that digitonin caused disordering of lipids noticeably, especially in the presence of cholesterol. The interaction between digitonin and cholesterol was characterized by the following three stages which depend upon the digitonin/cholesterol ratio: (1) "aggregated" species (the mole ratio being between 0 and 0.35), (2) the intermediate complex (between 0.35 and 0.9), and (3) the equimolecular (rigid) complex. The molecular ordering of Chol-d_6 was distorted by digitonin in the case of lower digitonin/cholesterol ratios, which is characterized as the aggregated species arising from cholesterol rapidly exchanging between free and complexed states with digitonin. At an elevated temperature (47 °C), however, these species were converted to the more rigid complex as in (2) or (3). ^2H NMR spectra of Chol-d_6 at the digitonin/cholesterol ratio 1.0 gave a quadrupole splitting as large as 14 kHz, which is very close to that of polycrystalline Chol-d_6. Cholesterol, in this case, is rather immobilized as a result of formation of the rigid complex with digitonin in the bilayers. In the case of the intermediate complex, the rigid complex was present together with the aggregated species. Saponins with a reduced number of terminal sugar moieties with lower hemolytic activity exhibited no distinct feature to form the rigid complex. Thus, the configuration of the terminal sugar moiety should play a specific role in forming the rigid complex which might be related to the hemolytic activity.

The saponins are steroid or triterpene glycosides that have the distinctive property of forming a soapy lather in water and have hemolytic activity (Tschesche & Wulff, 1973; Agarwal & Rastogi, 1974). Another characteristic is the formation of an insoluble equimolecular complex with cholesterol (Steiner & Holtzem, 1955). Digitonin (I) surpasses all other saponins in yielding complexes of great insolubility, called "digitonides" (Fieser & Fieser, 1959; Brooks, 1970). Digitonin has been used for the determination of cholesterol content in blood plasma, bile, and tissues and also is widely used to disperse membrane-bound proteins. In the early days, it was suggested that saponin hemolysis might be caused by the formation of a complex with cholesterol in the erythrocyte membrane. This postulate, however, is still controversial (Steiner & Holtzem, 1955). Therefore, elucidation of the specific interaction in model membrane systems might be very useful in understanding the hemolytic action in the plasma membrane.



I, S = Xyl-Glu-Gal-Glu-Gal
II, S = Xyl-Glu-Gal-Gal
III, S = Glu-Gal-

Recently, ^2H NMR[1] has proved to be a very powerful nonperturbing tool with which to examine molecular organization and molecular motion in model and biological membranes (Mantsch et al., 1977; Seelig, 1977; Stockton et al., 1977; Davis et al., 1979; Kang et al., 1979a). It is a natural extension to employ selectively deuterated cholesterol as a ^2H probe molecule since cholesterol is assumed to be the target for saponins. It should be mentioned that modification of even the side chain in cholesterol significantly alters the behavior

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[1] Abbreviations used: NMR, nuclear magnetic resonance; Chol-d_6, [26,26,26,27,27,27-$^2H_6$]cholesterol; SA-d_3, [18,18,18-$^2H_3$]stearic acid; EPR, electron paramagnetic resonance.

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