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INCORPORATION OF ^{35}S INTO GLYCOSAMINOGLYCANS OF OVARIAN FOLLICULAR AND LUTEAL TISSUE ISOLATED DURING THE GUINEA-PIG OESTROUS CYCLE

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Incorporation of ^{35}S into follicular chondroitin sulphates occurred in ovarian tissue isolated at all times during the oestrous cycle. Little incorporation into follicular dermatan and heparan sulphates was observed. Incorporation into luteal glycosaminoglycan occurred predominantly in tissue isolated at times of luteal growth and maturation. The relationship between these incorporation patterns and the different growth characteristics of the two ovarian tissues is discussed.

Many Metazoan cells possess an anionic pericellular envelope, of which sulphated glycosaminoglycans are characteristic components. At mammalian cell surfaces, the relative proportions of different glycosaminoglycans vary, depending upon the tissue examined, the stage of development of the tissue and the age of the animal; also, during the growth, passage and senescence of cultured cells, after transformation, during differentiation, and during tissue regeneration. Glycosaminoglycans of tumours and of cultured cells derived from tumours also differ from those of normal counterpart cells (1). The flexible chains of glycosaminoglycans, their large molecular domains, and their closely-adjoining and varied anionic groups make these compounds ideal candidates for functions involving the binding and release of small and macromolecular cations. Such observations suggest that pericellular glycosaminoglycans may influence aspects of cellular behaviour that depend upon the cellular reactions to extracellular signals.

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The mammalian ovary comprises tissue-organised cells, different components of which exhibit different growth characteristics, and respond differently to external signals (2). The present communication represents the first reported investigation of ovarian glycosaminoglycans during the normal oestrous cycle in an animal possessing a spontaneous luteal phase.

MATERIALS AND METHODS

Animals: Adult female Dunkin-Hartley strain guinea-pigs (650-1050g) were obtained from the Animal Breeding Unit, University of Nottingham, Sutton Bonington, Loughborough, Leics., U.K. The progress of the oestrous cycle in individual animals was monitored by daily examination of the vaginal membrane and of vaginal smears taken, by the lavage method, when the vagina was open. Only animals with established regular cycles were used. At the time of killing, the vagina was removed, sectioned, stained with haematoxylin and eosin and examined microscopically. The day of post-ovulatory influx of leucocytes immediately after maximal vaginal cornification was designated Day 1 after ovulation (3).
Materials: Chondroitin ABC lyase (EC 4.2.2.4) from Proteus vulgaris, chondroitin AC lyase (EC 4.2.2.5) from Arthrobacter aurescens, bovine serum albumin, penicillin, amino acids used in the incubation medium and glycosaminoglycans used as carriers during extraction of radio-labelled ovarian glycosaminoglycans were from Sigma Chemical Co., St. Louis, MO, U.S.A. Bovine plasma γ-globulin was from Bio-Rad Labs., Watford, Herts, U.K. Glycosaminoglycans used as reference preparations for the determination of the efficiency of the extraction procedure were given by Professor M.B. Mathews, Dept. of Pediatrics, University of Chicago, IL, U.S.A. Pronase from Streptomyces griseus was from BDH, Poole, Dorset, U.K. Sepraphore III cellulose acetate strips were from Gelman Instrument Co., Ann Arbor MI, U.S.A. $^{35}S[SO_4^{2-}]$ (25 Ci/mg) was from Amersham International, Bucks., U.K. Other reagents were of analytical grade.
Incorporation of radioisotope: Guinea-pigs were killed at particular times during the oestrous cycle by a blow to the head, followed by cervical dislocation. Ovaries were excised and adherent fat removed. They were then dissected to give two fractions: one comprising large and medium-sized follicles; the other corpora lutea. Experiments in which residual material, comprising interstitial tissue and small follicles, was incubated with $^{35}S[SO_4^{2-}]$ revealed incorporation that was lower than, but of the same pattern as, that exhibited by the fraction containing large and medium-sized follicles. Tissue fractions from both ovaries of individual animals were combined. Fractions were washed three times with phosphate-buffered saline, pH 7.4 (4), and then incubated, according to the method described by Gebauer et al. (5). In brief, fractions were incubated separately in 25 cm^3 Erlenmeyer flasks, each containing 5 cm^3 of phosphate-buffered saline supplemented with glucose (1 $mg.cm^{-3}$), bovine serum albumin (10 $mg.cm^{-3}$), penicillin (50 I.U. cm^{-3}) and amino acids at concentrations specified for F-10 medium (6). Gas (95% O_2 /5% CO_2, v/v) was passed continuously over the surface of the medium at a flow rate of 0.07 $cm^3.min^{-1}$, and flasks were incubated for 15 min at 37°C in a shaking water-bath (A. Gallenkamp and Co., East Kilbride, Strathclyde U.K.) operating at a speed of 90 shake-cycles.min^{-1}. 7.5 µCi of $^{35}S[SO_4^{2-}]$ was added to each flask, and incubation continued for a further 6h. Preliminary experiments under these incubation conditions showed that incorporation of ^{35}S into glycosaminoglycans reached a steady level at 2-4 h and remained steady for at least 12h.

Extraction and estimation of glycosaminoglycans: After incubation, fractions were washed three times in ice-cold NaCl (0.9%, w/v) containing 1.4 x 10^{-3} mol.dm^{-3} sodium sulphate. To each fraction, 1.4 cm^3 of Tris/HCl buffer (0.2 mol.dm^{-3}, pH 8.0) was added, and the tissue was homogenised in a low-clearance glass/Teflon homogeniser fitted to a Tri-R StiR-R drive unit (Tri-R Instruments, Rockville Center, NY, U.S.A.; speed setting 7.5, 10 strokes). 200 mm^3 of homogenate, was taken for protein assay, 200 mm^3 for estimation of radioactivity, and 1cm^3 was incubated with Pronase (2 mg) for 24h. Pronase was inactivated by heating the mixture at 100^{0}C for 5 min. The digest was cooled and duplicate 100 mm^3 samples incubated under each of the following conditions: (a) with Tris/acetate buffer (100 mm^3 , 0.05 mol.dm^{-3}, pH 8.0) supplemented with NaCl (0.15 mol.dm^{-3}) and bovine serum albumin (0.1%, w/v) at 37^{0}C for 20h; (b) with supplemented Tris/acetate buffer containing chondroitin AC lyase (0.1 units) at 37^{0}C for 20h; (c) with supplemented Tris/acetate buffer containing chondroitin ABC lyase (0.1 units) at 37^{0}C for 20h; (d) as for (c), but with a further incubation for 6h at ambient temperature with 300 mm of acetic acid (16.5% v/v) containing sodium nitrite (2.5%, w/v).

After incubation, solutions were heated at 100^{0}C for 5 min, cooled, and then 200 µg of a mixture of equal parts by weight of heparin, chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate added to each sample. Cetyl-pyridinium chloride (to a final concentration of 1%, w/v) and NaCl (to a final concentration of 0.035 mol.dm^{-3}) were then added and the volume of each sample adjusted with distilled water to 1cm^3 . Samples were incubated at 4^{0}C for 8h, warmed to ambient temperature, and the precipitated cetyl-pyridinium chloride-glycosaminoglycan complexes collected by centrifugation for 5 min in a micro-centrifuge (Quickfit Instrumentation, Corning Stone, Staffs., U.K.) Precipitates were washed twice with ice-cold distilled water, and dissolved in 0.2 cm^3 of NaCl (2 mol.dm^{-3}). Glycosaminoglycans were then re-precipitated by the addition of 0.8 cm^3 of ethanol, followed by incubation at 0^{0}C for 2h. The precipitates were washed twice with ethanol, once with diethyl ether, and then air-dried. For radioactivity estimation, the pellet was re-suspended in 200 mm^3 of distilled water before being added to 5 cm^3 of scintillation fluid (2,5-biphenyloxazole, 0.4%, w/v; 1,4-bi(5-phenyloxazol-2-yl)-benzene, 0.005%, w/v; toluene, 68%, v/v; Triton X-100, 32%, v/v). Radioactivity was determined in a Tri-Carb scintillation counter (Packard Instruments, Caversham, Berks., U.K.) at a counting efficiency of 85%. Since chondroitin ABC lyase treatment degrades chondroitin and dermatan sulphates, chondroitin AC lyase treatment degrades chondroitin sulphates, and HNO_2 treatment degrades heparan sulphates, analysis of radioactivity recovered after incubation of Pronase-treated extracts under conditions (a)-(d) allowed calculation of radio-activity incorporated into individual glycosaminoglycan types (7,8). The efficiency of the glycosaminoglycan extraction procedure was estimated by the use of standard glycosaminoglycan preparations as previously described (7). The recoveries were: heparan sulphate 98%, dermatan sulphate 89%, chondroitin sulphates 94%

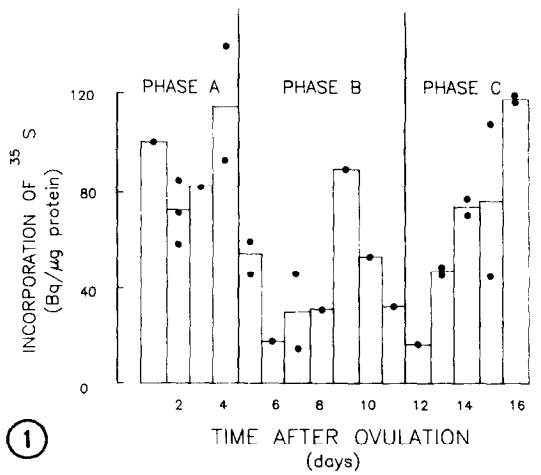
Protein was determined by the method of Bradford (9) using bovine plasma γ-globulin as standard.

Treatment of results: Results are expressed as radioactivity incorporated per unit mass of tissue protein. When they were expressed as radioactivity incorporated perunit mass of wet tissue, essentially similar patterns of incorporation were obtained. In the Figures, individual results, and means for the appropriate cycle day are presented. For analysis, results were considered as three groups, the first (A) comprising days 1-4 inclusive (the period of growth and differentiation of the corpus luteum, and of low and increasing levels of peripheral blood progesterone); the second (B) comprising days 5-11 inclusive (the period of maximal steroidogenesis); and the third (C) comprising days 12-16 inclusive (the period of functional and morphological regression of the corpus luteum). Results were examined using the Kruskal-Wallis analysis of variance of rates followed by individual comparisons between

groups using the Dunn test (10). Differences were accepted as real if significant at less than the 1% level.

RESULTS

The changing pattern of ^{35}S incorporation into total glycosaminoglycans extracted from entire ovaries excised at various times during the 16-day guinea-pig oestrous cycle is shown in Figure 1. Incorporation occurred in each of the post-ovulatory, pre-ovulatory and mid-cycle phases. Analysis of ^{35}S incorporation into total glycosaminoglycans of separated follicular and luteal tissue is shown in Figure 2. Incorporation into the two tissues is presented schematically as incorporation during two sequential oestrous cycles, since ovulation occurs between follicular development and differentiation into luteal tissue. Figure 2 shows that much of the post-ovulatory incorporation revealed in Figure 1 occurred in luteal tissue, while mid-cycle and pre-ovulatory incorporation was concentrated in follicular tissue. Figure 3 shows the differential incorporation of ^{35}S into the individual glycosaminoglycan classes of separated follicular and luteal tissues. Most of the radioactivity



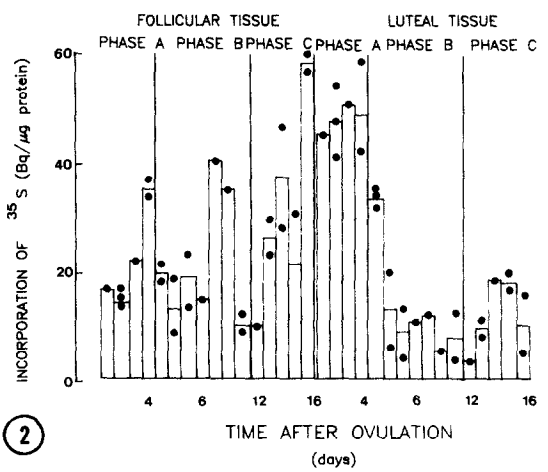


Figure 1 Incorporation of ^{35}S into total glycosaminoglycans of entire ovaries during the oestrous cycle.

Figure 2. Incorporation of ^{35}S into total glycosaminoglycans of follicular and luteal tissue during the oestrous cycle.

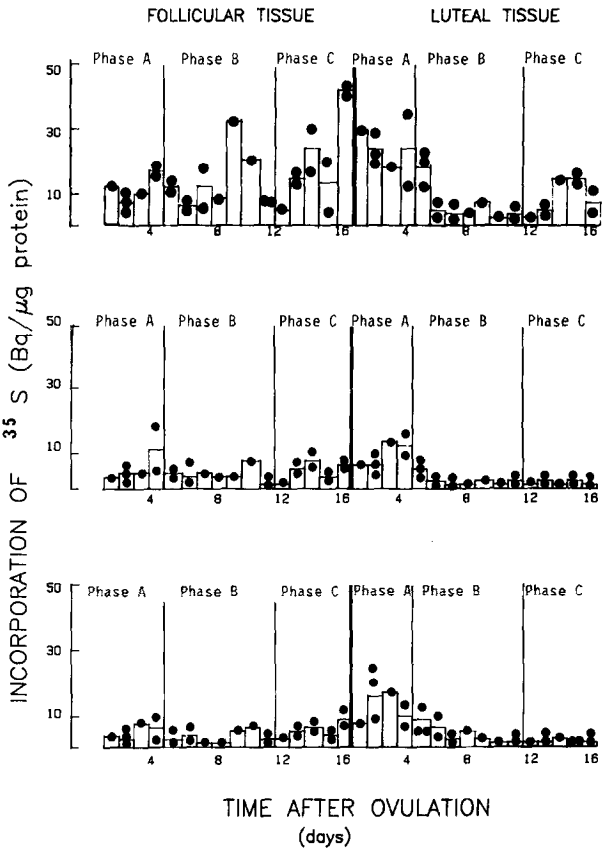


Figure 3. Incorporation of ^{35}S during the oestrous cycle into: I, chondroitin sulphates; II, dermatan sulphate; III, heparan sulphate of follicular and luteal tissues.

was detected in the chondroitin sulphate fraction. Incorporation into follicular chondroitin sulphates occurred in tissue isolated at all times throughout the cycle. For all glycosaminoglycans, the incorporation into developing luteal tissue (Group A) was significantly higher than that seen in steroidogenically active or regressing luteal tissue (Groups B and C).

DISCUSSION

Incorporation of ^{35}S into rat ovarian glycosaminoglycans *in vivo* and into rat and porcine ovarian glycosaminoglycans *in vitro* was stimulated following treatment of animal or tissue with follicle-stimulating hormone (11,12). Incorporation of [^{3}H]-glucosamine into glycosaminoglycans of porcine

follicular granulosa cells was also stimulated when cells were incubated with follicle-stimulating hormone (13). Conversely, addition of luteinizing hormone or progesterone to rat ovarian slices suppressed incorporation of ^{35}S into follicular glycosaminoglycans (5). Such experiments, involving administratio of exogenous hormones to animals, tissue or cells, suggest that ovarian glycosaminoglycans play a role in follicular development and ovulation, and that their production may normally be influenced by changes in steroid and gonadotrophic hormone concentrations. Our results represent the first reported examination of ovarian glycosaminoglycans throughout the oestrous cycle of an untreated mammal with a spontaneous luteal phase. The complex pattern of incorporation into follicular chondroitin sulphates perhaps reflects the multiphasic pattern of follicular development present in the guinea-pig (14). The fall in incorporation into luteal glycosaminoglycans following the rise during days 1-4(group A) coincides with a marked increase in serum progesterone concentration (Clinton et al., unpublished observations), and the possibly causal nature of this relationship is being studied.

Increased amounts and synthesis of cellular chondroitin sulphates are frequently associated with rapidly proliferating mitotic cells, and increased amounts and synthesis of cellular dermatan and heparan sulphates are frequently associated with cells approaching a stationary phase (1). Since follicular growth largely reflects rapid mitosis of granulosa cells, while luteal maturation involves a deceleration of mitosis followed by cellular hypertrophy (15), the patterns of incorporation reported here may reflect the different growth characteristics of the two tissues. Ovarian tissues and cells thus can provide a useful, experimentally manipulable model system in which glycosaminoglycans of proliferating and differentiating cells may be investigated.

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REFERENCES

1. Long, W.F., and Williamson, F.B. (1979) IRCS Med. Sci. 7, 429-434.
2. Hutchinson, J.S.M. (1979) The Hypothalamo-Pituitary Control of the Ovary. Eden Press, Quebec.
3. Donovan, B.T., and Traczyk, W. (1962) J. Physiol. 161, 227-236.
4. Dulbecco, R., and Vogt, M. (1954) J. Exp. Med. 99, 167-182.
5. Gebauer, H., Lindner, H.R., and Amsterdam, A. (1978) Biol. Reprod. 18, 350-358.
6. Ham, R.G. (1963) Exp. Cell. Res. 29, 515-526.
7. Edward, M., Long, W.F., Watson, H.H.K., and Williamson, F.B. (1980) Biochem. J. 188, 769-773.
8. Watson, H.H.K., Edward, M., Williamson, F.B., and Long, W.F. (1980) Biochem. Soc. Trans. 8, 134-136.
9. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
10. Dunn, O.J. (1977) Basic Statistics : A Primer for the Biomedical Sciences. 2nd Edition, Wiley, N.Y.
11. Ax, R.L., LaBarbera, A.R., and Ryan, R.J. (1978) Advan. Exp. Med. Biol. 112, 77-82.
12. Mueller, P.L., Schreiber, J.R., Lucky, A.W., Schulman, J.D., Rodbard, D., and Ross, G.T. (1978) Endocrinology 102, 824-831.
13. Ax, R.L., and Ryan R.J. (1979) J. Clin. Endocrinol. Metab. 49, 646-648.
14. Bland, K.P. (1980) J. Reprod. Fert. 60, 73-76.
15. Brambell, F.W.R. (1960) in Marshall's Physiology of Reproduction. A.S. Parkes (Ed.) 1; Longmans, Green, and Co., London, pp. 397-542.