ruled out as an explanation of the mammary underdevelopment in the full-collared group.

The physiological irritant, formalin, far from retarding mammary development, increased it to the highest level, significantly exceeding the level of development in the group injected with distilled water. The stress effects of restraint also are thus ruled out as a cause of the underdevelopment.

We conclude that the mammary underdevelopment in the full-collared rats was caused by the prevention of self-licking and not by the method of prevention. Self-licking is thus shown to effectively stimulate mammary development during pregnancy, at a time when mammary growth and secretion were heretofore thought to be primarily under endogenous control.

Among sites that are licked on the ventral surface are the nipple areas and the genital and pelvic regions. The most likely route by which the licking stimulates the mammae, however, is by way of sensory receptors located in the nipples. This possible dependence of mammary development during pregnancy on stimulation of the nipples is consistent with what is known about the sensory conditions that maintain postpartum mammary function, and it suggests that a similar neuroendocrine mechanism and a similar hormone (or hormones) are involved. Grosvenor and Turner (*9*) implied a similar conception when they suggested that late in gestation a neural stimulus, possibly arising from the uterus, caused release of prolactin from the pituitary, which in turn caused prepartum lactation. Our results indicate that a behavioral event of pregnancy can provide the required stimulation.

LORRAINE L. ROTH
JAY S. ROSENBLATT
Institute of Animal Behavior,
Rutgers University,
Newark, New Jersey

References and Notes

1. A. T. Cowie and S. J. Folley, in *Sex and Internal Secretions*, W. C. Young, Ed. (Williams and Wilkins, Baltimore, ed. 3, 1961), p. 590.
2. Y. Shinde, L. Ota, A. Yokoyama, *J. Endocrinol.* **25**, 279 (1962); R. E. Munford, *ibid.* **28**, 1 (1963); *Dairy Sci. Abstr.* **26**, 293 (1964).
3. J. S. Rosenblatt and D. S. Lehrman, in *Maternal Behavior in Mammals*, H. L. Rheingold, Ed. (Wiley, New York, 1963), p. 8; L. L. Roth and J. S. Rosenblatt, paper read before Amer. Psychol. Assoc., Los Angeles (1964).
4. From Charles River Breeding Farm.
5. H. G. Birch, *Amer. J. Orthopsychiat.* **26**, 279 (1956).
6. M. N. Runner, *Anat. Record* **133**, 330 (1959); M. W. Weir and J. C. DeFries, *Psychol. Rept.* **13**, 365 (1963).
7. L. B. Schnürer, *Acta Endocrinol. Suppl. 80* **43**, 1 (1963).
8. G. K. Benson and S. J. Folley, *J. Endocrinol.* **16**, 189 (1957).
9. C. E. Grosvenor and C. W. Turner, *Endocrinology* **66**, 96 (1960).
10. Supported by NIH research grants MH-03398 and MH-08064 to J.S.R. and by PHS predoctoral fellowship MPM-19-140 to L.L.R. We thank W. R. Butler for technical assistance with the histological material and G. K. Benson and D. S. Lehrman for suggestions—particularly the former for making available his extensive knowledge of lactation physiology.

17 December 1965

Mucopolysaccharides: N-Acetylglucosamine- and Galactose-6-Sulfates from Keratosulfate

Abstract. *Galactose-6-sulfate and* N*-acetylglucosamine-6-sulfate were obtained pure from a partial acid hydrolyzate of corneal keratosulfate by paper chromatography and electrophoretic fractionation. These sugars were also present in hydrolyzates of skeletal keratosulfate. The distribution of the sulfate groups in the various keratosulfates might depend upon their source, and this distribution probably has important biological significance.*

Keratosulfate is a sulfated mucopolysaccharide composed of equimolar amounts of D-galactose and *N*-acetyl-D-glucosamine and of variable quantities of methylpentose and sialic acid (*1*). It has been isolated from cornea (*2*), nucleus pulposus (*3*), and cartilage of mammals and lower species (*4*). In these tissues keratosulfate is covalently bound to protein by at least two different types of bonds, either by *N*-glycosyl bonds to the amide groups of asparagine and glutamine or by *O*-glycosyl bonds to the hydroxyls of serine and threonine (*1*). The *O*-glycosidic bonds are alkali-labile and are split by β-elimination (*5*). The *N*-glycosyl bonds are mainly present in keratosulfate of cornea, the *O*-glycosyl linkages in skeletal keratosulfate. The structure of corneal keratosulfate was deduced from methylation and enzymatic digestion of the sulfated and desulfated polymer as *N*-acetyllactosamine-6-sulfate, polymerized by 1,3 bonding of the alternating hexosaminyl and D-galactosyl moieties (*6*).

Since the yields of the isolated methyl ethers were rather low, the methylation of bovine cornea keratosulfate was reinvestigated. Improved methods markedly increased the yields of methyl ethers, which, in general, confirmed the proposed structure. However, the evaluation of the experiment suggested that carbon No. 6 of galactose was partly blocked, indicating either branching at this position or substitution by another group.

After searching for optimum conditions we obtained, on hydrolysis of 1.0 g of keratosulfate for 1 hour with 0.5*N* H_2SO_4 at 100°C, two sulfated monosaccharides, which were isolated by paper chromatography and electrophoresis. Fraction I (8.0 mg) was galactose-6-sulfate, the other (fraction II, 13.2 mg) *N*-acetylglucosamine-6-sulfate. In addition, a number of sulfated oligosaccharides were present.

The properties of fraction I are as follows: $[\alpha]_D$ as barium salt was +42.5°, as ammonium salt was +52.0°. In paper chromatography its migration was 10 percent slower than that of synthetic glucose-6-sulfate. On paper electrophoresis at *p*H 6 it had the same mobility as glucose-6-sulfate. Its staining with aniline phthalate and tetrazolium chloride showed that the reducing group and carbon No. 2 were free.

The ratio of sulfate to galactose was 1.06. The reducing value (Schales and Schales) was 84 percent that of galactose. On infrared spectroscopy the substance absorbed at 1240 and 820 cm^{-1}, showing the absence of a sulfate on carbon No. 4. It consumed 3 moles of periodate rapidly in unbuffered solution and then consumed slowly an additional mole. Negligible amounts of formaldehyde were released on oxidation in bicarbonate buffer (*p*H 7.5) for 24 hours (*7*).

The properties of fraction II were as follows: $[\alpha]_D$ as ammonium salt was +47.9°. Chromatographically and electrophoretically it had the same mobility as authentic *N*-acetylglucosamine-6-sulfate. It stained with aniline phthalate and gave a positive reaction for *N*-acetylhexosamine. The ninhydrin reaction was negative. On complete hydrolysis glucosamine was the only carbohydrate present. The ratio of sulfate to *N*-acetylglucosamine was 0.97, that of sulfate to glucosamine was 0.98. The reducing value (Schales and Schales) was 98 percent that of *N*-acetylglucosamine. On infrared spectroscopy the substance absorbed at 1240 and 820 cm^{-1}, indicating a primary sulfate ester group.

On periodate oxidation in bicarbonate buffer it liberated formaldehyde only in traces.

The data strongly indicate that fraction I is galactose-6-sulfate and that fraction II is *N*-acetylglucosamine-6-sulfate. Paper chromatography and electrophoresis of a similarly hydrolyzed sample of keratosulfate from cartilage of human rib likewise yielded galactose-6-sulfate. Judged from the intensity of the spots, the content of galactose-6-sulfate in this fraction appears to be higher than that of keratosulfate of cornea. The isolation of *N*-acetylglucosamine-6-sulfate is in agreement with the structure previously proposed.

The presence of galactose-6-sulfate is another instance of the variations in the mucopolysaccharides in general and in keratosulfate in particular, and is another example of the apparent randomness of the biosynthesis of these compounds.

The isolation of galactose-6-sulfate from keratosulfate of cornea is noteworthy in that the ratio of sulfate to hexosamine in cornea is always close to one, while keratosulfate of senile human cartilage, as well as that of elasmobranch cartilage, is oversulfated; that is, the ratio of sulfate to hexosamine is greater than one (*1*). In these sources the extra sulfate can be expected to be in the galactosyl moiety. Galactose sulfate in mammalian tissue occurs in the sulfatides of brain and other tissues (*8*), and recently galactose-6-sulfate was demonstrated in neuraminlactose sulfate from the mammary glands of rats (*9*).

The finding of sulfate ester groups in both galactosyl and hexosaminyl moieties of keratosulfate may explain the anomalous behavior of this polymer toward quaternary ammonium and pyridinium salts (*10*), which may be caused by the proximity of sulfate groups in neighboring instead of in alternating glycosyl groups, so that the compound behaves similarly to heparin.

In view of our finding, we should not assume that the keratosulfate fractions that are isolated from different normal and abnormal tissues, such as degenerating cartilage, nucleus pulposus, and tissue in Marfan's syndrome, are identical.

V. P. Bhavanandan
Karl Meyer
Department of Medicine, Columbia University College of Physicians and Surgeons, New York 10032

18 MARCH 1966

References and Notes

1. N. Seno, K. Meyer, B. Anderson, P. Hoffman, *J. Biol. Chem.* **240**, 1005 (1965).
2. K. Meyer, A. Linker, E. A. Davidson, B. Weissmann, *ibid.* **205**, 611 (1953).
3. S. Gardell and S. Rastgeldi, *Acta Chem. Scand.* **8**, 362 (1954).
4. K. Meyer, P. Hoffman, A. Linker, *Science* **128**, 896 (1958); T. Furuhashi, *J. Biochem. (Tokyo)* **50**, 546 (1961); M. B. Mathews and J. A. Cifonelli, *J. Biol. Chem.* **240**, 4140 (1965).
5. B. Anderson, N. Seno, P. Sampson, J. G. Riley, P. Hoffman, K. Meyer, *J. Biol. Chem.* **239**, 2716 (1964).
6. S. Hirano, P. Hoffman, K. Meyer, *J. Org. Chem.* **26**, 5064 (1961).
7. W. R. Frisell, L. A. Meech, C. G. Mackenzie, *J. Biol. Chem.* **207**, 709 (1954).
8. T. Taketomi and T. Yamakawa, *J. Biochem. (Tokyo)* **52**, 226 (1962); P. Stoffyn and A. Stoffyn, *Biochim. Biophys. Acta* **70**, 218 (1963).
9. L. C. Ryan, R. Carubelli, R. Caputto, R. E. Trucco, *Biochim. Biophys. Acta*, previews 85056 (1965).
10. J. E. Scott and J. Dorling, *Histochemie* **5**, 221 (1965).
11. Supported by USPHS grant AM-00570.

16 December 1965

Rubella Virus: Growth and Cytopathic Effect in Primary Cultures of Cells of Rabbit Embryos

Abstract. *Primary cultures of rabbit (New Zealand white) embryo cells support growth of rubella virus. Distinct cytopathic changes are discernible within 6 to 8 days after inoculation. This cell system has been successful for the recovery of rubella virus from clinical materials and the demonstration of neutralizing antibody in patient serum.*

We have found that primary cultures of rabbit embryo cells not only support rubella virus growth, but also develop marked specific cytopathic changes. These changes develop on primary isolation of virus from clinical materials and also are produced by virus previously adapted to other cell lines. In our hands, this cell system has provided a technique which is direct, sensitive, and reproducible for demonstration of rubella virus.

Weller and Neva first described subtle cytopathic effects of rubella virus in primary human amnion cell cultures (*1*). Other investigators have since reported cytopathic effects in several cell systems. McCarthy *et al.* observed cytopathic effects in a transformed line of rabbit kidney (RK-13) and, later, reported cytopathic effects in primary cultures of rabbit kidney (*2*). Günalp observed destructive changes in monolayers of a line of green monkey kidney (GMK-AH1) (*3*). These cytopathic changes have been described as subtle and may be difficult to detect on primary isolation of rubella virus, but they become more manifest after serial passage of the virus in the cell line under investigation. Leerhøy recently reported a cytopathic effect of strains of RK-13–adapted rubella virus in an established line of rabbit cornea cells (*4*). The effects described by Leerhøy are distinct and usually discernible within 4 days after inoculation; no reference was made to the utilization of this cell line for primary isolations from clinical materials.

Because of the difficulty in detecting cytopathic effects in early passages of rubella virus in primary human amnion and RK-13 cells, the indirect method described by Parkman *et al.* is most often used for diagnostic work (*5*). Although this method has proved successful for isolation and identification of rubella virus, it is somewhat laborious.

In the study now reported, four strains of rubella virus were used. The human amnion–adapted Bell strain was supplied by F. A. Neva (*6*); the green monkey kidney–adapted M33 strain was supplied by E. L. Buescher (*6*). Two additional strains (AE and LB) that we recovered from patients during the 1964 rubella epidemic were isolated in rabbit embryo cell cultures from original clinical materials. These strains were also isolated and identified by the indirect technique in green monkey kidney cells. Identification of the isolates was made with specific rubella antiserum to the Bell strain and antiserum supplied by J. L. Sever (*6*).

Rabbit embryos (New Zealand white), aged 17 to 21 days, were used for preparation of monolayer cultures. Whole embryos were minced to a fine pulp, and cells were dispersed from the tissue fragments with 0.25 percent trypsin (Difco 1:250) in Hanks balanced salt solution at *p*H 7.4. The cell concentration for planting was adjusted with growth medium to 10^6 cells per milliliter and 1-ml portions were planted in screw-cap Leighton tubes (16 by 120 mm). Growth medium consisted of Medium 199 (*7*) supplemented with 13 percent heat-inactivated horse serum, antibiotics (100 μg of streptomycin, 30 units of mycostatin, and 100 units of penicillin per milliliter), and sodium bicarbonate (0.35 g/liter). For maintenance medium, the serum concentration was reduced to 2 percent. All cultures were allowed to grow in the stationary position at 35°C for 3 to 4 days prior to inoculation. Twenty-four hours before inoculation, growth