The Influence of Norethynodrel on Sialic Acid Content of the Rat Submandibular Gland[1] (36199)

F. FAVA-DE-MORAES[2] AND BURTON L. BAKER

Department of Anatomy, The University of Michigan Medical School, Ann Arbor, Michigan 48104

Several hormones influence the structure and function of submandibular glands in the mouse and rat (1). Among the end points that have been studied is the intraglandular level of sialic acid. Sialic acid appears to be especially sensitive to alteration in the hormonal milieu of the body but the observations reported do not permit the development of a rational concept regarding hormonal regulation of its metabolism within the gland. Thus, an increase in sialic acid concentration is reported in the rat made diabetic (2) and after thyroparathyroidectomy (3); in the mouse after orchiectomy (4); and in the hamster after administration of estrogen (5). Pursuant to hypophysectomy sialic acid concentration of the submandibular gland is reduced and after adrenalectomy does not change (6).

No data are available concerning the influence of progestins or oral contraceptive agents on the sialic acid content of saliva or the salivary glands. Since human use of oral contraceptive agents is so widespread this study was designed to ascertain the effect of treatment with norethynodrel[3], the major component of the pill Enovid®, on sialic acid content in the submandibular gland. Sialic acid probably plays a major role in maintaining oral health since it promotes viscosity of the saliva (7), protects salivary glycoproteins against enzymatic degradation (8) and binds calcium ions tightly to mucins so they may form a protective layer over dental surfaces (9).

Material and Methods. Norethynodrel was suspended in Upjohn vehicle 98 (carboxymethylcellulose, polysorbate, and propylparaben) diluted 1:2 with 0.9% saline at concentrations to provide doses of 0.075 and 3 mg in 0.2 ml. Subcutaneous injections were given daily for 10 days; control rats received 0.2 ml of the vehicle.

Thirty-day-old Sprague-Dawley rats were used. Thirty rats were ovariectomized. Two months later they and 30 intact rats from the same shipment were arranged in groups of 10, and treated as follows: (A) intact, vehicle; (B) intact, 0.075 mg norethynodrel; (C) intact, 3 mg norethynodrel; (D) ovariectomized, vehicle; (E) ovariectomized, 0.075 mg norethynodrel; and (F) ovariectomized, 3 mg norethynodrel.

Teklad Rat-Mouse Diet and water were offered *ad lib.* to all rats from the time of their arrival in the laboratory. Food was removed 18 hr before termination of the experiment. The animals were killed by cervical dislocation 24 hr after the last injection. The uteri were weighed to provide an index of the estrogenic stimulation provided by norethynodrel treatment. The submandibular glands were excised and weighed. For quantification of sialic acid the glands were homogenized at 5% (w/v) in distilled water, and subjected to acid hydrolysis in 0.1 *N* H_2SO_4 (final concentration) at 80° for one hour (10). Proteins were precipitated with 5% trichloracetic acid at 4° for 15 min, followed by centrifugation at 2000 rpm. for 15 min. Sialic acid

[1] Supported in part by research grant HD03159-04 from the National Institutes of Health. The technical assistance of Mrs. Ya-Yen Yu and Miss Elizabeth Palczynski is appreciated. We thank Dr. David Aminoff for advice and a supply of *N*-acetylneuraminic acid, and the G. D. Searle Co. for norethynodrel.

[2] Department of Histology, Biomedical Science Institute, University of São Paulo, São Paulo, Brazil. Supported by a research grant from Fundação de Amparo a Pesquisa do Estado de São Paulo. Reprint requests should be sent to B. L. Baker, Department of Anatomy, University of Michigan, Ann Arbor, MI 48104.

[3] Enovid is made up of 98.5% norethynodrel (17α-ethnyl-17-hydroxy-$\Delta^{5(10)}$-estren-3-one) and 1.5% mestranol.

TABLE I. The Influence of Norethynodrel on Body Weight and Sialic Acid Content of the Submandibular Gland.

Group	Treatment	Daily dose (mg)	Mean body weight (g)			Mean submandibular wt. (2 glands)		Mean sialic acid (μmol)	
			Initial	Start of injections	Final	(mg)	Gland wt. / 100 g b.w.[b]	per 100 g gland	Total (2 glands)
A	Intact, veh.[c]	—	—	243 ± 16[a]	229 ± 20	370 ± 35	161 ± 16	231.0 ± 28.1	0.84 ± 0.09
B	Intact, nor.[d]	0.075	—	241 ± 17	228 ± 14	364 ± 42	159 ± 11	240.2 ± 15.4	0.88 ± 0.12
C	Intact, nor.	3.0	—	248 ± 12	217 ± 11	325 ± 22	150 ± 9	320.5 ± 33.3	1.03 ± 0.10
D	Ovar.[e], veh.	—	109 ± 16	309 ± 37	300 ± 38	431 ± 64	147 ± 17	190.1 ± 20.1	0.81 ± 0.16
E	Ovar., nor.	0.075	105 ± 15	316 ± 22	276 ± 24	415 ± 36	150 ± 12	235.0 ± 21.5	0.97 ± 0.12
F	Ovar., nor.	3.0	107 ± 12	328 ± 24	268 ± 21	363 ± 52	135 ± 13	383.3 ± 40.4	1.01 ± 0.24
			A vs B	P	NS[f]	NS	NS	NS	NS
			A vs C	P	NS	< 0.01	NS	< 0.001	< 0.001
			A vs D	P	< 0.001	< 0.02	NS	< 0.001	NS
			D vs E	P	NS	NS	NS	< 0.001	< 0.05
			D vs F	P	< 0.05	< 0.02	NS	< 0.001	< 0.05

[a] Standard deviation; [b] Body weight; [c] Vehicle; [d] Norethynodrel; [e] Ovariectomized; [f] Not significant.

of the supernate was estimated by the thiobarbituric acid method (11) as *N*-acetylneuraminic acid, using 3–4 aliquots for each homogenate. *N*-acetylneuraminic acid purified from pig submandibular gland was used as the standard.

Significance of the difference between mean values for the various groups was determined by the Student's *t* test.

Results. The loss in body weight of the intact and ovariectomized rats (Table I, Groups A and D) treated with the vehicle is accounted for by the terminal starvation period. Administration of norethynodrel to intact rats did not cause a significant loss in body weight (Groups A vs. B, A vs. C). In ovariectomized rats a reduction in body weight occurred only in those that received the 3 mg dose of norethynodrel (Groups D vs. F, $p < .05$).

The absolute weight of the submandibular gland was less after administration of the 3 mg dose of norethynodrel in both intact (Table I, Groups A vs. C, $p < .01$) and ovariectomized rats (Groups D vs. F, $p < .02$), while the 0.075 mg dose did not elicit this response. The minimal changes in weight of the submandibular glands were probably related to alteration in body weight since no significant differences were observed in the mean submandibular weight/body weight ratios between the vehicle- and norethynodrel-treated rats whether intact or ovariectomized. The loss in body weight probably resulted from the intrinsic estrogenic property of norethynodrel. That the compound was estrogenic is indicated by an increase in mean weight of the uterus from 75 mg for the vehicle-treated ovariectomized rats (Group D) to 117 mg at the 0.075 mg dose (Group E) and 237 mg at the 3 mg dose of norethynodrel (Group F). All differences between these values were significant at the 0.1% level.

Sialic Acid. The mean concentration of sialic acid in the submandibular glands was lower in ovariectomized (190.1 μmol/100 g of tissue) than in intact (231 μmol) rats treated with vehicle (Table I, Groups D and A). On the other hand, administration of norethynodrel to both intact and ovariectomized rats increased the concentration of sialic acid, the change being greater with the 3 mg than with the 0.075 mg dose. The percentage increase was slightly greater in ovariectomized animals.

Ovariectomy had no effect on the total amount of sialic acid in the submandibular gland (Table I, Groups A and D) probably because glandular enlargement compensated for the reduced concentration of sialic acid. However, treatment with the 3 mg dose of norethynodrel increased the total amount of sialic acid in both intact (Groups A vs. C) and ovariectomized rats (Groups D vs. F).

Discussion. Although sialic acid occurs commonly in cellular membranes and intercellular matrices its concentration is high only in epithelial cells that secrete glycoproteins. With respect to salivary glands, biochemical and histochemical observations indicate that sialic acid is localized primarily in the secretory acini (5, 12). Evidence regarding the influence of sex hormones on submandibular acini is conflicting, some investigators (13–16) having claimed that estrogens increase the size and functional activity of the acinar cells while others (17–20) found no alterations. Similarly the submandibular glands of female rats (21) and hamsters (22) stain more intensely with alcian blue than do those of males and since sialic acid is stained by alcian blue, these findings suggest that accumulation of sialic acid results from estrogenic stimulation. Shackleford and Klapper (5) suggested that estrogen promotes incorporation of sialic acid into the mucin molecule. Uncertainty also exists regarding the influence of progesterone on the submandibular glands, since some investigators (23, 24) have reported responses indicative of stimulation while others (16, 17, 25) observed no effects.

With respect to the action of norethynodrel on the submandibular gland, its capacity to increase the concentration and total content of sialic acid is significant particularly in light of the fall that occurred in absolute weight of the gland. Liu *et al.* (26) also demonstrated a reduction in weight of the submandibular glands of rats treated with norethynodrel; however, they reported an increase in glandular weight/body weight ratio which did not occur in our experiments.

Whether the estrogenic or progestational property of norethynodrel is responsible for the changes elicited in the submandibular

gland cannot be ascertained at present. Norethynodrel possesses some progestational activity when administered subcutaneously and exhibits 3–7% of the estrogenic activity of estrone depending on the bioassay used for comparison (27). The estrogenicity of norethynodrel is revealed also by its capacity to stimulate prolactin cells (28, 29) and, as observed in this study, to increase uterine weight in ovariectomized rats. In our investigations these effects were not due to contamination of the norethynodrel with other estrogens since the preparation was free of aromatic content (J. K. McGowan, personal communication).

Liu and his colleagues observed a reduced rate of increase in weight of submandibular glands in prepubertal rats treated with contraceptive agents that possess significant estrogenic activity (ethinyl estradiol (23), mestranol, norethynodrel (26)) and an increase after administration of a potent progestational agent (medroxyprogesterone) (23, 30). However, these observations cannot be correlated with the changes we observed in sialic acid content after treatment with norethynodrel because the total content increased in spite of a decrease in glandular weight. Nevertheless, the fall in sialic acid concentration in the submandibular gland after ovariectomy and the increase in sialic acid levels that appeared to result from the estrogenic activity of administered norethynodrel seem to indicate that estrogens are significant regulators of sialic acid in the submandibular gland. Furthermore, an important link to general oral biology may be indicated by the observation of Liu *et al.* (23, 26, 30, 31) that the incidence of dental caries rises in rats treated with either estrogenic or progestational types of contraceptive agents.

Summary. The influence of norethynodrel on the sialic acid content of the submandibular gland was studied. Seventy days after ovariectomy, the concentration of sialic acid in the gland was reduced greatly. Administration of norethynodrel at daily doses of 0.075 and 3 mg for 10 days to intact and ovariectomized rats increased the concentration and total amount of sialic acid in the gland, the response being greater at the higher dose. The evidence indicates that (a) the sialic acid content of the submandibular glands is dependent on ovarian secretions, and (b) norethynodrel has the capacity to increase the content of sialic acid in the submandibular gland, this response most likely being due to the estrogenic property of norethynodrel.

1. Baker, B. L., Clapp, H. W., and Light, J. A., *in* "Salivary Glands and Their Secretions" (L. Sreebny and J. Meyer, eds.), p. 63. Pergamon Press, New York (1964).

2. Nicolau, J., Rosa, R., and Fava-De-Moraes, F., Acta Physiol. Lat. Amer. **19**, 106 (1969).

3. Nicolau, J., Fava-De-Moraes, F., and Zucas, S. M., An. Acad. Brasil. Cienc. **41**, 133 (1969).

4. Devalle, J. J., Curbelo, H. M., Houssay, A. B., Tocci, A. A., and Gamper, C. H., Acta Physiol. Lat. Amer. **18**, 42 (1968).

5. Shackleford, J. M., and Klapper, C. E., Anat. Rec. **142**, 495 (1962).

6. Taylor, P. W., Jr., Richardson, K. C., Roddy, P. M., and Titus, E., J. Pharmacol. Exp. Ther. **156**, 483 (1967).

7. Gottschalk, A., "Glycoproteins. Their Composition, Structure and Function" (B. B. A. Library, ed.), Vol. 5. Elsevier Publ. Co., Amsterdam (1966).

8. Tappel, A. L., *in* "Lysosomes in Biology and Pathology" (J. T. Dingle and H. B. Fell, eds.), Vol. 2, p. 207. North Holland Publ. Co. (1969).

9. Bertolini, N., and Bettelheim, F., *in* "The Carbohydrates—Chemistry and Biochemistry" (W. P. Pigman and D. Herton, eds.), Vol. 2B, p. 677. Academic Press, New York (1970).

10. Svennerholm, L., Acta Chem. Scand. **12**, 547 (1958).

11. Warren, L., J. Biol. Chem. **234**, 1971 (1959).

12. Spicer, S. S., and Duvenci, J., Anat. Rec. **149**, 333 (1964).

13. Lacassagne, A., C. R. Soc. Biol. **133**, 180 (1940).

14. Harvey, H., Physiol. Zool. **25**, 205 (1952).

15. Pignataro, O., Pathologica **51**, 9 (1959).

16. Raynaud, J., Bull. Biol. Fr. Belg. **94**, 399 (1960).

17. Shafer, W. G., and Muhler, J. C., Ann. N.Y. Acad. Sci. **85**, 215 (1960).

18. Desclin, J., Jr., C. R. Acad. Sci. **264**, 749 (1967).

19. Liu, F. T. Y., J. Dent. Res. **46**, 471 (1967).

20. Liu, F. T. Y., Proc. Soc. Exp. Biol. Med. **124**, 591 (1967).

21. Turolla, E., and Martinazzi, M., Acta Histochem. **15**, 16 (1963).

22. Kronman, J. H., and Chauncey, H. H., J. Oral Ther. Pharmacol. **1**, 392 (1965).

23. Liu, F. T. Y., and Lin, H. S., J. Dent. Res.

48, 477 (1969).

24. Travill, A., Anat. Rec. **155**, 217 (1966).

25. Desclin, J., Jr., C. R. Acad. Sci. **264**, 129, 367 (1967).

26. Liu, F. T. Y., J. Dent. Res. **47**, 1047 (1968).

27. Saunders, F. J., Fed. Proc. **29**, 1211 (1970).

28. Baker, B. L., and Zanotti, D. B., Endocrinology 78, 1037 (1966).

29. Baker, B. L., and Yu, Y. Y., Proc. Soc. Exp. Biol. Med. **134**, 107 (1970).

30. Liu, F. T. Y., Lofgren, R., and Hoots, I., J. Dent. Res. **45**, 1236 (1966).

31. Liu, F. T. Y., and Lin, H. S., Proc. Soc. Exp. Biol. Med. **131**, 331 (1969).

Received Sept. 27, 1971. P.S.E.B.M., 1972, Vol. 139.