Circadian Uptake of ^{3}H-Thymidine in the Rat Incisor Under the Influence of Cortisone[1] (37426)

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An earlier study (1) reported that the administration of cortisone resulted in an increase in number of mitoses in the maxillary incisor of young rats during certain periods of the 24-hr cycle. On the basis of these results, it was postulated that a stimulation in cell proliferation could be one of the factors responsible for the acceleration in eruption rates observed in cortisone-treated rats (2–4). However, this study did not yield information regarding the effect of cortisone on the S phase of the cell cycle. It is possible that the stimulation in mitotic activity may have been the result of an increase in rate of DNA synthesis thus adding to the pool of mitotic cells. Hence, by utilizing ^{3}H-thymidine, it was decided to investigate the effect of cortisone on DNA synthesis in the incisor over a 24-hr period. Many tissues, including the rat incisor, have yet to be investigated with respect to the effect of cortisone on DNA synthesis.

Methods. Female, albino Sprague–Dawley rats, 30 days old, were acclimated in a controlled environment for a 10-day period. They were kept in constant light from 7 AM to 7 PM, complete darkness from 7 PM to 7 AM and at a constant temperature of 76 $\pm$ 2°F. Only the investigator entered the room and only at regularly scheduled intervals for feeding, watering and cage cleaning. Immediately following the acclimation period, the animals were treated as follows: beginning at 7 AM, 1 PM, 7 PM, and 1 AM, 4 adrenalectomized rats for each period received a single injection of 1.0 mg of cortisone (Cortone Acetate supplied by Merck and Co., West Point, Pa.). In addition, 4 adrenalectomized and 4 normal rats for each period received an equal volume of a 0.85% saline solution. Exactly 6 hr later, all animals received 0.6 μCi/g of ^{3}H-thymidine (sp. act. 18.3 Ci/mM; concentration 1 mCi/ml) (purchased from Amersham-Searle Corp., Arlington Heights, IL.), and exactly 3 hr later they were sacrificed. All injections were sc. Body weights were recorded prior to each injection and at time of sacrifice.

Immediately upon sacrifice, skulls were split midsagittally and maxillary incisors recovered and processed by procedure previously described (1). Midsagittal serial sections (5 μm) of incisors were then overstained with hematoxylin and prepared for a radioautographic study employing the liquid emulsion coating method (5). The slides were dipped in Kodak NTB2 liquid emulsion and stored at 4°, for an exposure time of 15 days, after which they were developed in Kodak Dektol (2:1 dilution) for 2 min and fixed for 3 min in a 24% sodium thiosulfate solution.

Counts of ^{3}H-thymidine labeled nuclei were made on 4 cell layers of cervical (labial) loop of incisor. These layers were stratum intermedium, preameloblasts, preodontoblasts and adjacent pulp. Successive circumscribed fields were counted beginning 70 μm from base of cervical loop and continuing for a total of 8 fields. Fields were defined by means of a ruled eyepiece micrometer and counts were made at a magnification of 1000×. The area of each oil-immersion field was 70 μm in length × 90 μm in width or 6300 μm^2. Four midsagittal sections were counted for each tooth and only every fourth section was counted. Care was exercised to avoid

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counting overlapping areas of fields more than once. By summing counts per field, the mean number of labeled cells per tissue section was calculated for each cell layer of each incisor. The differences between averages of the 3 groups of animals for each time period were statistically analyzed by means of Student t test. The Analysis of Variance (F test) was used to evaluate data with respect to rhythmicity of DNA synthesis. Differences were considered significant at 5% level of probability.

Results. The administration of cortisone to adrenalectomized rats resulted in a significant increase in average number of ^{3}H-thymidine labeled cells as compared to nontreated adrenalectomized animals. (Table I). This increase occurred in all 4 cell layers and during each of periods studied. Furthermore, the differences were statistically significant ($p < 0.01$ or < 0.001) in every instance with these exceptions: at 7 AM, the preodontoblasts ($p > 0.10$), at 7 PM, the stratum intermedium ($p > 0.05$); the preodontoblasts and pulp ($p > 0.10$).

In addition, cortisone-treated animals revealed more labeling than controls at 1 PM and 1 AM in all layers and at 7 AM in all layers except preodontoblasts. At 7 PM the control group showed more labeled cells than cortisone treated group in each of the layers with the exception of pulp. The most significant differences were noted at 1 PM (pulp, $p < 0.01$; other layers, $p < 0.001$). Except for the stratum intermedium at 7 AM and pulp at 1 AM ($p < 0.001$), all other differences between cortisone-treated adrenalectomized animals and controls were found to be not significant ($p > 0.10$).

The values of contols were consistently and significantly greater than those of untreated adrenalectomized animals except at 1 PM where the reverse occurred in all layers except pulp. These differences at 1 PM were statistically significant (preodontoblasts and pulp, $p < 0.05$; stratum intermedium, $p < 0.01$ and preameloblasts, $p < 0.001$). Other significant differences were: 7 AM preameloblasts, $p < 0.01$ and pulp, $p < 0.001$; 7 PM stratum intermedium, $p < 0.01$ and preameloblasts, $p < 0.001$ and at 1 AM preameloblasts, $p < 0.05$ and preodontoblasts $p = 0.05$.

When the average number of labeled cells per section are combined, irrespective of individual cell layers, one finds essentially a reflection or reinforcement of the results seen in individual layers, namely that the values of cortisone-treated adrenalectomized animals are significantly above those of un-

TABLE I. Average Number of ^{3}H-thymidine Labeled Cells in the Incisor of Cortisone Treated Adrenalectomized, Adrenalectomized and Control Rats.

Time 3H-thy. admin.	No. rats	Stratum intermedium	Pre ameloblasts	Pre odontoblasts	Pulp	Combined layers
7 AM	4AdC[a]	28.00[b] ± 0.91[c]	62.59 ± 2.05	19.97 ± 0.95	39.75 ± 1.39	150.31 ± 2.63
	4Ad	21.53 ± 0.90	52.84 ± 1.80	19.66 ± 0.93	31.66 ± 1.28	125.69 ± 2.99
	4C	22.31 ± 0.73	60.34 ± 1.81	20.25 ± 0.80	38.97 ± 1.60	141.88 ± 2.52
1 PM	4AdC	32.21 ± 1.33	81.04 ± 2.58	26.21 ± 1.26	46.29 ± 1.68	185.75 ± 4.53
	4Ad	25.09 ± 0.99	64.72 ± 1.41	20.81 ± 0.75	34.81 ± 1.50	145.44 ± 2.95
	4C	20.92 ± 1.05	55.54 ± 1.63	18.08 ± 0.86	39.79 ± 1.78	134.33 ± 3.68
7 PM	4AdC	20.59 ± 0.90	58.65 ± 1.77	16.47 ± 0.91	38.84 ± 2.27	134.56 ± 4.39
	4Ad	18.21 ± 1.03	51.00 ± 1.66	14.88 ± 0.86	35.08 ± 1.75	119.17 ± 3.58
	4C	22.14 ± 0.78	61.36 ± 1.55	16.75 ± 0.49	33.68 ± 1.58	133.93 ± 2.72
1 AM	4AdC	21.79 ± 1.14	58.50 ± 2.01	19.00 ± 1.22	41.14 ± 1.57	140.43 ± 4.49
	4Ad	17.09 ± 0.97	48.47 ± 1.44	16.22 ± 0.64	29.28 ± 1.05	111.06 ± 2.54
	4C	19.94 ± 1.36	54.25 ± 2.17	18.59 ± 1.01	29.16 ± 1.51	121.94 ± 4.19

[a] AdC = Adrenalectomized + Cortisone; Ad = Adrenalectomized; C = Control.
[b] Average per tissue section.
[c] Standard error.

treated adrenalectomized (at 7 PM, $p < 0.01$; other periods $p < 0.001$) and control rats (all periods $p < 0.01$ or less except at 7 PM where $p > 0.10$). Moreover, the combined averages of controls, with the exception of 1 PM are seen to be significantly greater than those of untreated animals (Table I).

A rhythmicity in uptake of ^{3}H-thymidine was noted in all 3 groups (Table I). Both the cortisone-treated and untreated adrenalectomized groups revealed significantly more labeled nuclei at 7 AM and 1 PM than at 7 PM and 1 AM in all layers ($p < 0.001$) except pulp ($p > 0.05$). When averages were combined, disregarding individual layers, both adrenalectomized groups revealed a highly significant circadian rhythm in rate of DNA synthesis by the cells of the cervical loop ($p < 0.001$). The control animals showed a cyclic activity in uptake of ^{3}H-thymidine which was, however, less pronounced and of a different nature than that of the adrenalectomized groups. At 7 AM and 7 PM, more activity was noted in the stratum intermedium ($p > 0.05$) and preameloblasts ($p > 0.001$). At 7 AM and 1 AM a greater uptake of the isotope was seen in the preodontoblasts ($p < 0.05$) while at 7 AM and 1 PM the pulp revealed the highest number of labeled cells ($p < 0.001$). Thus, each of these layers showed high activity at 7 AM but revealed a different pattern at other periods. When the averages of these layers were combined for a total number of labeled cells per tissue section, a circadian rhythm was revealed with significantly more activity at 7 AM and 1 PM ($p < 0.01$). Therefore, in control animals, even though the rhythmic activity of individual layers is apparently asynchronous, when these values are combined, the total activity of the cervical loop is similar to, though not so pronounced as that of experimental groups, whereas, the individual layers in the adrenalectomized groups showed essentially the same rhythm which was significantly reflected in the overall activity of the cervical loop.

Discussion. Despite the fact that the causes of tooth eruption have been under investigation for a long time, a totally satisfactory solution to this problem is still being sought. A great deal of evidence exists to show that the proliferation of cells at the base of the growing tooth is largely responsible for its eruption. While there is general agreement with the observation that these cells are actively proliferating during the eruptive process, there is no agreement as to whether they are the actual causative agent or are simply growing in response to this movement. Nevertheless, a number of studies have shown a positive correlation between eruption and cellular proliferation. Amputation at the tip of the mandibular incisor in the rabbit (6) and rat (7) results in an increase in eruption rate and number of mitoses in the apical tissue. In another study on the lower incisor of young mice (8), the number of ^{3}H-thymidine labeled cells in the apical tissues decreased immediately after functional occlusion was established. Utilizing ^{3}H-thymidine, the migration rate of ameloblasts in the mouse incisor (9) was 365 μm/day and the average eruption rate, measured grossly, 338 μm/day. While these and other similar studies do not conclusively establish cell division as the actual causative agent, they do lend support to this theory. The results of our study support the concept that cortisone promotes the rate of incisor eruption by stimulating proliferation of formative tissues at the apex of this organ. Adrenalectomy caused a marked reduction in uptake of ^{3}H-thymidine while a single injection of cortisone resulted in a highly significant increase in the number of these cells. These results present the interesting possibility that the action of cortisone is manifested primarily during the S (DNA synthesizing) phase of cell cycle. In addition, however, we previously reported (1) that cortisone influences the M (mitotic) phase of the generation cycle in normal rats, by causing an increase in number of colchicine arrested cells during certain periods of the day. Thus, even though the primary action of cortisone may be stimulation of DNA replication in preparation for cell division, resulting in a greater number of cells entering the M phase, additional studies are needed in which the effects of cortisone on generation cycle of incisor cell populations can be more precisely studied. Information is available on the generation time of preamelo-

blasts in mouse incisor (10), the rat molar (11), and in the rabbit molar (12), but none on the influence of cortisone on the cell cycle of these tissues.

Studies concerning the influence of cortisone on developing teeth are relatively few in number. Furthermore, the effect appears to be largely dependent on dosage administered. In newborn rats, given 40 mg/kg of cortisone on alternate days for 11 days, a hypoplasia of ameloblasts and odontoblasts was observed in developing teeth (13). Similarly, both upper and lower incisors of adult rats receiving 50 mg of cortisone daily for 8 days, revealed odontoblasts that were highly disorganized and incompletely differentiated (14). The predentin was wider than normal and irregular in appearance. However, the pulp showed an increase in number of cells and blood vessels. On the other hand, intraocular, guinea pig, tooth germ transplants initially showed an increase in growth under the influence of cortisone (15). The dosage was 2.5 mg/day for 8 days. Similarly a precocious development and premature differentiation of tissues in lower incisors of fetal and postnatal rats was observed after administration of cortisone (16). The dosages were 15–20 mg daily to pregnant rats for a period of 14–20 days and 0.1 mg daily for 2–14 days to the newborn of such rats. It seems, therefore, that minimal doses of cortisone may result in enhanced growth of developing teeth while doses which exceed physiological limits generally inhibit growth. While the general body effects of cortisone are known to be largely inhibitory, it is also known that cortisone stimulates incisor eruption in both fetal and postnatal rats. Thus, one might expect a positive influence on the growth of oral tissues when it is administered in amounts approximating endogenous levels.

The manner in which cortisone promotes growth of the incisor is, of course, still to be determined. However, our observation that apical tissues undergo a circadian rhythm in the synthesis of DNA may provide useful information. This observation should not be surprising since a rhythmic uptake of ^{3}H-thymidine has been reported in preameloblasts of lower rat incisors (17), and in the cheek and palate epithelium of rats (18). Furthermore, the present experiments show that the adrenals influence this cyclic activity since adrenalectomy modified the rhythm. Others have shown that adrenalectomy modifies the normal circadian mitotic rhythm in the corneal epithelium (19, 20) and in the epidermis (20) of rats. In addition, an overall reduction in the mitotic rate was noted in these tissues after the adrenalectomy. Despite the fact that cortisone amplified this rhythm, the cyclic activity pattern remained essentially the same in our experiments since both treated and untreated adrenalectomized groups showed significantly more labeling at 7 AM and 1 PM. This observation would seem to indicate that other adrenal hormones, possibly inhibitory in nature, may influence the normal mitotic rhythm since even with the administration of cortisone, the rhythm still differed from that of controls.

Closer inspection of data, however, shows that differences in rhythmic patterns are due primarily to the 1 PM period and that the pattern except for this period is quite similar. This observation would also seem to support the assumption that inhibitory hormones may be involved in the adrenal influence on the proliferative activity. Since the greatest effect of adrenalectomy was to inhibit uptake of ^{3}H-thymidine, one might expect that the major influence on DNA synthesis is mediated through the stimulatory system. However, the increase in labeled cells at 1 PM, observed after adrenalectomy, also indicates the presence of an inhibitory influence, removal of which resulted in increased activity at this time. From these observations, one might postulate a hormonal system, primarily stimulatory but also inhibitory in nature, which operates to regulate the proliferative activity of the incisor. The interplay of these hormones could conceivably result in the fluctuations observed during the 24-hr period.

Since it is well known that the hormones of the adrenal are secreted in a rhythmic manner, it seems reasonable to assume that the resulting variation in availability of these hormones to the proliferating pool of cells, would result in variations in the rate of mitotic activity. The rhythm of controls, which is less pronounced and of a different

nature than that of experimentals, could be the result of a rhythmic secretion and interaction of these hormones. Evidence for the existence of a mitosis-stimulating hormone has been reported by several investigators (21, 22).

Adrenalectomy modified but did not abolish the circadian rhythm indicating that the cells of the incisor possess some intrinsic regulating mechanism influenced by adrenal hormones. A mitotic inhibitor, presumably a glycoprotein has been identified in the cells of the epidermis which was named chalone (23, 24). Experiments *in vitro* on mouse epidermis have shown that chalone may not be capable of fully inhibiting mitosis in the absence of adrenalin (25). Evidence is available to show that hydrocortisone may serve to prolong inhibitory action of the chalone–adrenalin complex on the epidermis *in vitro* (26). On the basis of these and other observations, it was postulated that growth of certain mammalian tissues is controlled by a system of inhibitors and that the loss of this inhibition results in a stimulation of mitotic activity. The increase in cell division observed during wound healing has been attributed to a loss of chalone (23). Other studies indicate, however, that a substance must be present to inhibit chalone during wound healing, *i.e.*, a wound hormone (27). Besides, it has been postulated that growth-stimulating hormones such as the estrogens and androgens may do so by inactivating chalone in target tissues (24). These experiments suggest that cortisone may stimulate cell proliferation in the rat incisor by inhibiting the tissue chalone. Whether cortisone acts directly to stimulate the proliferating tissues or interferes with the inhibitory action of a tissue-specific chalone remains to be determined. The presence of a chalone has not been identified in the incisor and there is evidence that some tissues do not contain chalones.

Summary. A single injection of cortisone in adrenalectomized rats resulted in a significant increase in number of ^{3}H-thymidine labeled cells in the cervical loop of maxillary incisors at 7 AM, 1 PM, 7 PM, and 1 AM when compared to untreated adrenalectomized animals. The increase was most pronounced at 1 PM and least significant at 7 PM. Controls showed consistently and significantly fewer labeled nuclei than cortisone-treated adrenalectomized rats at all periods except 7 PM. Again, the most significant increase occurred at 1 PM. In addition, control values were consistently and significantly higher than those of untreated adrenalectomized rats for all periods except those of the 1 PM period. A circadian rhythm in uptake of ^{3}H-thymidine was noted in all groups. Both treated and untreated adrenalectomized animals revealed significantly more labeled cells at 7 AM and 1 PM. Control rats showed a cyclic activity which varied between individual cell layers and was less pronounced than in adrenalectomized rats. The results provide further evidence to support the concept that the manner in which cortisone accelerates rate of eruption is by stimulation of proliferative activity in the tissues of incisor. It is suggested that the chief effect of the adrenals on growth of these tissues is stimulatory in nature but that inhibitory influences also exist which may result in fluctuations in rate of DNA synthesis over a 24-hr period.

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1. Domm, L. V., and Kiely, M. L., Proc. Soc. Exp. Biol. Med. **129**, 912 (1968).

2. Leroy, P., and Domm, L. V., Ann. Endocrinol. **16**, 773 (1955).

3. Garren, L., and Greep, R. O., Endocrinology **66**, 625 (1960).

4. Domm, L. V., and Wellband, W. A., Proc. Soc. Exp. Biol. Med. **107**, 268 (1961).

5. Kopriwa, B. M., and Leblond, C. P., J. Histochem. Cytochem. **10**, 269 (1962).

6. Ness, A. R., and Smale, D. E., Proc. Roy. Soc. London B **151**, 106 (1959).

7. Michaeli, Y., and Weinreb, M. M., J. Dent. Res. **47**, 633 (1968).

8. Grewe, J. M., and Felts, W. J. L., J. Dent. Res. **47**, 65 (1968).

9. Hwang, W. S., and Tonna, E. A., J. Dent. Res. **44**, (Suppl), 42 (1965).

10. Hwang, W. S., Cronkite, E. P., and Tonna, E. A., J. Dent. Res. **45**, 350 (1966).

11. Stallard, R. E., Diab, M. A., and Zander, H. A., J. Dent. Res. **45**, 315 (1966).

12. Starkey, W. E., Brit. Dent. J. **115**, 143 (1963).

13. Nakamoto, T., and Wilson, R. H., Arch. Oral Biol. **13**, 1107 (1968).

14. Anneroth, G., and Bloom, G., J. Dent. Res. **45**, 229 (1966).

15. Fleming, H. S., J. Dent. Res. **32**, 101 (1953).

16. Goldsmith, E. D., and Ross, L., Acta Endocrinol. **22**, 23 (1956).

17. Gasser, R. F., Scheving, L. E., and Pauly, J. E., J. Dent. Res. **51**, 740 (1972).

18. England, M. C., Jr., and Burke, G. W., Jr., J. Dent. Res. **47**, 478 (1968).

19. Scheving, L. E., and Pauly, J. E., *in* "The Cellular Aspects of Biorhythms" (H. von Mayersbach, ed.), p. 167. Springer Publishing Co., Berlin (1967).

20. Alov, I. A., Bull. Exp. Biol. Med. **48**, 1418 (1959).

21. Abercrombie, M., *in* "Advances in Biology of Skin" (W. Montagna and R. E. Billingham, eds.), Vol. 5, p. 95. Pergamon Press, Oxford (1964).

22. Argyris, T. S., *in* "Advances in Morphogenesis" (M. Abercrombie, J. Brachet and T. J. King, eds.), Vol. 7, p. 1. Academic Press, New York (1968).

23. Bullough, W. S., and Laurence, E. B., Proc. Roy. Soc. London B **151**, 517 (1960).

24. Bullough, W. S., Biol. Rev. **37**, 307 (1962).

25. Bullough, W. S., and Laurence, E. B., Exp. Cell Res. **33**, 176 (1964).

26. Bullough, W. S., and Laurence, E. B., Cell Tissue Kinet. **1**, 5 (1968).

27. Argyris, T. S., Amer. Zool. **12**, 137 (1972).

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