# Modification of Cyclocytidine-Induced Changes in [$^3$H]Thymidine Incorporation and Weight of Parotid Gland by Partial Sialoadenectomy (43317)

CHARLOTTE A. SCHNEYER,*[1] H. DAVID HALL,[†] AND MICHAEL G. HUMPHREYS-BEHER[‡]

*Laboratory of Exocrine Physiology,* University of Alabama at Birmingham, Birmingham, Alabama 35294, Department of Oral Surgery,[†] Vanderbilt University School of Medicine, Nashville, Tennessee 37232, and Department of Oral Biology,[‡] University of Florida, Gainesville, Florida 32610*

*Abstract.* **Cyclocytidine (CC), a potent antitumor agent, caused a 2.3- to 5.0-fold increase in [$^3$H]thymidine uptake of rat parotid gland after 3 days of daily administration of 500 mg/kg body wt. Gland weight also showed a 47–67% increase from that of controls. Ablation of the submandibular-sublingual glands prior to initiation of the CC regimen prevented the usual CC-induced increase in [$^3$H]thymidine uptake, but did not inhibit the increase in gland size. It is postulated that CC-induced parotid hyperplasia requires an initial release of growth factors from the submandibular gland; however, enlargement of the parotid gland by CC is independent of such factors.** [P.S.E.B.M. 1991, Vol 198]

Cyclocytidine (CC) is a potent antitumor agent (1), with side effects on organs such as the salivary glands and the heart (2). These side actions apparently result from chronic activation of β-adrenoceptors by norepinephrine (NE) released from sympathetic nerve endings by the CC (3). In the heart, this effect of CC causes increased size, and, in salivary glands, CC causes increased mitotic activity, as well as an increase in gland size (3). More recently, it has been shown that CC also acts on the submandibular gland to cause the release of nerve growth factor (NGF) (4). The mechanism of the CC-induced release of NGF has not been determined. However, since NGF is released by CC, it becomes pertinent to determine whether the NGF has any role in mediation of the CC-induced growth responses of the parotid gland. Therefore, the aim of the present work was, first, to establish what, if any, role the submandibular gland has in mediation of CC-induced parotid growth and, if it does, what the relative effects are on hyperplasia and hypertrophy of this organ.

## Materials and Methods

Long-Evans rats, 32–49 days of age, were used in these experiments. Animals were maintained on Purina chow and water *ad libitum* before and after removal of both submandibular-sublingual glands (partial sialoadenectomy [Des]) under ether anesthesia. CC, in a dose of 500 mg/kg body wt, was intraperitoneally administered daily for 3 days to groups of intact rats or to those that had had submandibular-sublingual glands removed 7 days earlier. On the morning of the fourth day, [$^3$H] thymidine (in a dose of 50 μCi/100 gm rat) was intraperitoneally injected into CC-treated, as well as control, rats. Five hours later, parotid glands were removed from rats under anesthesia (sodium pentobarbital in a dose of 50 mg/kg body wt), weighed rapidly on a torsion balance, and then placed in Tris-HCl buffer for monitoring of [$^3$H]thymidine incorporation. Samples (100 μl) of tissue (homogenized at 4°C) were removed for precipitation with trichloroacetic acid on glass fiber filters, followed by the addition of scintillation mixture for measuring [$^3$H]thymidine incorporation. Part of the sample was removed for a protein assay. Duplicate samples of untreated animals were used to determine basal rates of synthesis. Gland homogenates were prepared by centrifugation at 250*g* for 10 min (4°C) to remove connective tissue. Protein concentrations were

[1] To whom requests for reprints should be addressed at Laboratory of Exocrine Physiology, University of Alabama at Birmingham, UAB Station, Birmingham, AL 35294.

Received February 26, 1991. [P.S.E.B.M. 1991, Vol 198]
Accepted June 3, 1991.

0037-9727/91/1983-0806$3.00/0

determined by the Schacterle and Pollack (5) modification of the Lowry protein assay using bovine serum albumin as standard.

Samples of parotid gland were also placed in Bouin's solution, and tissues were prepared for histological examination. Tissues were cut at 6 $\mu$ and stained with hematoxylin and eosin. Acinar cell size was determined as described previously (6); since nuclear size was relatively constant, the number of nuclei per unit area served as an indicator of acinar cell size when counts of nuclei were made on sections of acinar cells only. A relative difference in the size of acinar cells became evident; thus, the size of acinar cells was inversely related to the number of nuclei per unit area; ten areas per slide were counted and three slides were examined (one slide per animal).

For determination of NE, tissues were first homogenized (using a Brinkman polytron) in 2 ml of 0.5 *M* cold perchloric acid, which contained sodium metabisulfite (10 mg/liter) as an antioxidant. Following centrifugation, the catecholamines in the protein-free supernatant were adsorbed with alumina at pH 8.6 in 3 *M* Tris buffer. After washing with water, the catecholamines were eluted with 0.1 *M* perchloric acid.

NE was analyzed using a modification of a method described by Krstulović (7) that employs high performance liquid chromatography and electrochemical detection. The separation was achieved with a Supelco $C_{18}Rp_3$-$\mu$m column and Water's pump. The mobile phase, a phosphate-citric acid buffer, contained sodium acetyl sulfate as an ion pair and methanol as a modifier. The eluting compounds were detected with a Bioanalytical Systems amperometric detector, set at a potential of +0.5 V. NE analyses were run in duplicate and were reproducible. Recovery studies showed an 80–90% recovery of NE, but an internal standard (dihydroxybenzlamine) was also run for each sample to correct for differences in each sample. A Student's *t* test was used for statistical analysis of the data.

## Results

The data in Figure 1 show effects of partial sialoadenectomy on CC-induced [$^3$H]thymidine incorporation into the rat parotid gland. The parotid glands of rats 32, 37, 38, 45, and 46 days of age showed a 2.3- to 5.0-fold increase in [$^3$H]thymidine uptake over those of controls when CC was administered daily for 3 days. In

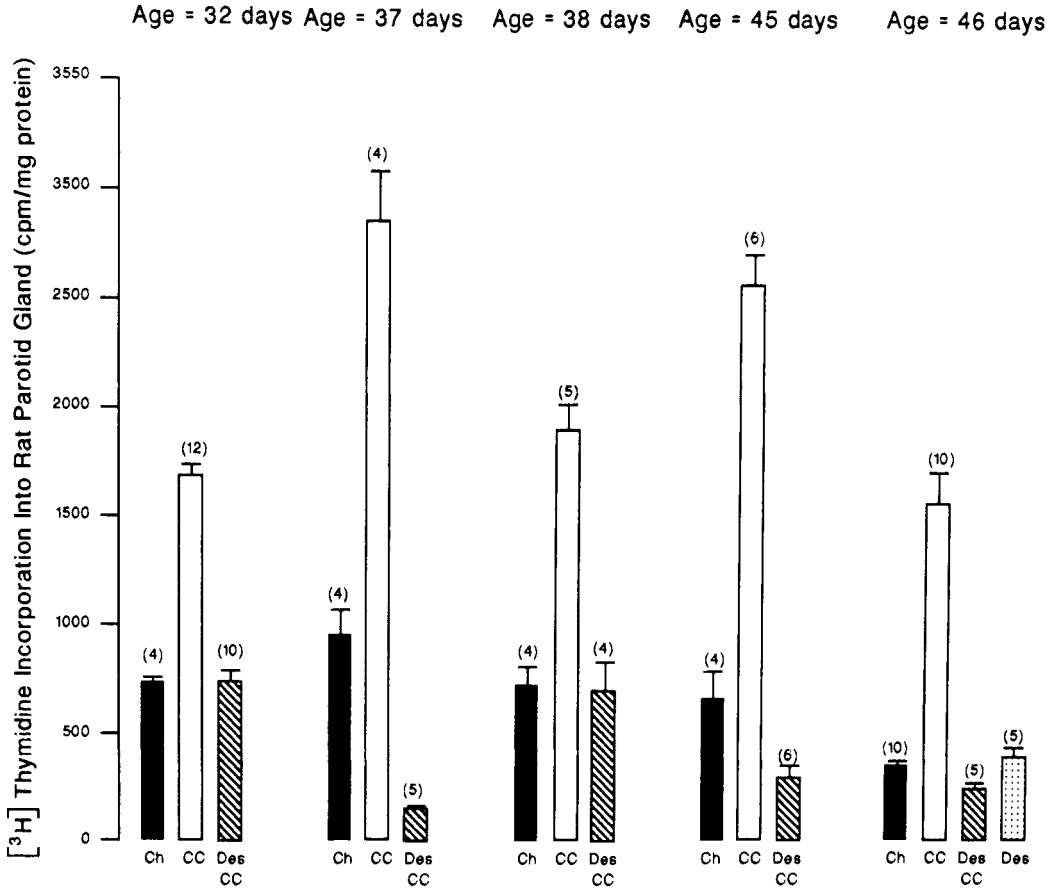


**Figure 1.** Values are means ± SE of [$^3$H]thymidine incorporation into DNA of rat parotid gland after administration of cyclocytidine (rats intraperitoneally injected daily for 3 days with CC in a dose of 500 mg/kg body wt) to intact animals (designated CC). CC values are significantly different from those of chow controls (Ch) ($P < 0.01$), and Des (partially sialadenectomized) CC values differ significantly from those of chow controls and also from CC values ($P < 0.01$). The number in parentheses indicates the number of rats; the ages of rats are given in days.

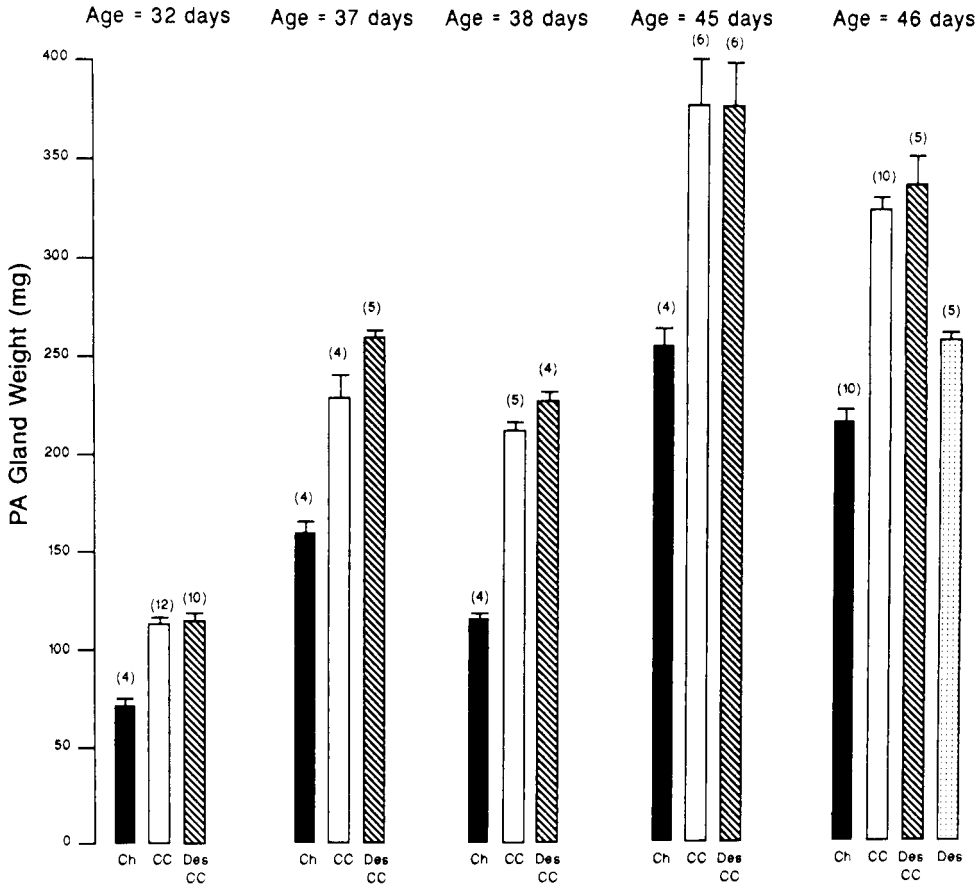


**Figure 2.** Values are means ± SE of parotid gland weight (mg) of rats of the same groups described in Figure 1. Symbols are also the same. Values for CC and Des CC do not differ significantly from each other ($P > 0.01$), but do differ significantly ($P < 0.01$) from Ch values.

**Table I.** Norepinephrine Concentration of Parotid Gland of Immature Rats after Daily Administration of Cyclocytidine for 3 Days[a]

| Treatment of rats | Age (days) | Gland weight (mg) | Norepinephrine | |
|---|---|---|---|---|
| | | | Concentration (ng/g)[b] | Total (ng/gland) |
| Control | 35 | 157 ± 9 | 728 ± 29 (5) | 114 |
| Cyclocytidine | | 252 ± 10 | 284 ± 6 (5) | 72 |
| Control | 44 | 165 ± 2 | 631 ± 22 (4) | 103 |
| Cyclocytidine | | 330 ± 26 | 185 ± 3 (3) | 61 |
| Des-Cyclocytidine | | 297 ± 10 | 185 ± 18 (4) | 55 |
| Control | 49 | 220 ± 3 | 735 ± 23 (4) | 162 |
| Cyclocytidine | | 339 ± 10 | 271 ± 5 (4) | 92 |
| Des-Cyclocytidine | | 329 ± 11 | 250 ± 35 (5) | 82 |

[a] Values are means ± SE. The number of rats is indicated in parentheses. Cyclocytidine (500 mg/kg body wt) was administered intraperitoneally daily for 3 days. All values for cyclocytidine and Des-cyclocytidine differ significantly ($P < 0.01$) from control. Values for cyclocytidine and Des-cyclocytidine do not differ significantly from each other ($P > 0.01$).
[b] Nanogram per gram of wet weight of parotid.

rats from which the submandibular-sublingual glands were removed prior to initiation of the CC injections, there was no increase in [$^3$H]thymidine of the parotid gland after CC treatment, and values were the same or even less than those of control animals (Fig. 1).

Gland weight showed a different pattern of change from that seen with [$^3$H]thymidine uptake. As shown by data in Figure 2, the administration of CC caused an increase of 47–67% in gland weight when a comparison was made with controls. The increases observed when CC was given to rats following removal of the submandibular-sublingual glands were similar to the values of intact rats given CC. The size of the parotid gland was increased chiefly as a consequence of an

increase in the size of acinar cells. The size of the parotid acinar cells, inversely related to the number of nuclei per unit area, varied with the experimental permutations, as followed: Chow Con, 11 ± 0.9 (three rats); CC, 6 ± 0.4 (three rats); Des CC, 6 ± 0.5 (three rats). The total protein also served as an indication of change in the size of the gland, and the representative concentrations (mg/ml) for the two age groups were as follows (number of rats in parentheses) age, 32 days: Con, 11.1 ± 0.4 ($n$ = 5); CC, 13.4 ± 0.3 ($n$ = 8); Des CC, 11.4 ± 0.3 ($n$ = 10); age, 46 days: Con, 11 ± 0.5 ($n$ = 11); CC, 13.8 ± 0.3 ($n$ = 10); Des CC, 12.9 ± 0.4 ($n$ = 7).

The NE levels of the parotid gland (measured in three age groups after 3 days of CC administration) were markedly reduced from those of controls. The NE concentrations of the CC-treated parotid gland of 35-day-old rats were reduced 61% from that of controls; those of 44-day-old rats were reduced 71%, and those of 49-day-old rats were reduced 63% (Table I). CC caused a decrease in the NE of the parotid gland in rats without submandibular glands of 71% and 66% for 44- and 49-day age groups. Since CC caused an increase in the size of the gland, comparisons of total NE reductions were also calculated. Values for total glandular NE of CC-treated rats showed reductions of 37–43% from controls.

The acute effects of CC were also examined. NE of the parotid gland 1 hr after CC administration was decreased 51% from that of controls; the value for NE of CC-treated rats was 412 ± 44 ng/g (six rats), and that of controls was 837 ± 20 ng/g (nine rats) (age = 32 days).

## Discussion

Present data confirm the previous observation that CC administered over a period of several days causes enlargement of the rat parotid gland (1, 2). The data also provide new evidence showing that marked cell proliferation occurs following CC treatment, since [$^3$H] thymidine levels of the parotid gland showed a 2.3- to 5.0-fold increase over those of controls. The thymidine increase usually induced by CC was, however, completely prevented when the submandibular-sublingual glands were removed prior to initiation of CC treatment, but the increase in gland size was not. Thus, the data indicate that the presence of the submandibular gland is essential for the induction of DNA synthesis by CC, but it is not essential for a CC-induced increase in gland size. Earlier work attributed the CC-induced growth responses of the parotid gland to chronic activation of β-adrenoceptors by NE released from the sympathetic nerve endings by CC (3, 8). Present data support the view that CC causes NE release inasmuch as NE of the parotid gland is reduced from controls by 51% 1 hr after a single injection and by as much as 61–71% after the 3-day CC regimen. In fact, total glandular NE was also reduced by 37–43%. This change in total NE is important, since the size of the CC-treated glands was increased from controls. Thus, even though glandular measurements of NE are only an indirect indicator of NE released from the sympathetic nerves, these changes do support the view that CC causes NE release. Thus, present data show that the proliferative response induced by CC involves the NE-induced activation of β-adrenoceptors on the parotid gland (9), but also requires the presence of the submandibular gland. It is well known that growth factors (NGF and epidermal growth factor, for example) are produced almost exclusively by the submandibular gland (10, 11); NGF, e.g., after being produced, is then taken up by adrenergic nerve terminals, after which it is transported to the perikaryon to exert effects on various neuronal functions (12). It has also been shown that CC induces the release of NGF from the submandibular gland (4). Thus, NGF (and epidermal growth factor as well) may be released with NE from sympathetic nerve endings by CC, and the [$^3$H]thymidine increase in the parotid gland is facilitated by the action of both at the appropriate parotid receptors. Thus, chronic β-adrenoceptor activation and the presence of the submandibular gland are essential to the induction of parotid mitogenesis by CC.

On the other hand, enlargement of the parotid gland, and specifically of its acinar cells, does not depend on the submandibular growth factors, since the hypertrophic response to CC occurs even in the absence of the submandibular gland. This was evident by measurements of total protein, as well as of gland weight and cell size. The hypertrophy thus must be attributed to direct activation of adrenergic receptors by NE released from sympathetic nerve endings on the parotid gland by CC (9). Moreover, the similarities in the reduction of NE of the parotid gland of intact CC-treated rats and the partially sialoadenectomized rats treated with CC bear out this point.

This work was supported by National Institute of Dental Research (NIDR) Grant DE 02110 (C. A. S.) and NIDR Grant 087798 and RCDA DE 00291 (M. H. B.). The authors would like to thank Nancita R. Lomax of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, for kindly supplying the cyclocytidine used in this study.

1. Haskell CM, Cline MJ. The treatment of acute leukemia. Radiat Drug Ther **8**:1–7, 1974.
2. Schneyer CA, Galbraith WM, Mellett LB. Unexpected β adrenergic effects of the antitumor agent, cyclocytidine, on rat salivary glands. Proc Soc Exp Biol Med **148**:1206–1211, 1975.
3. Schneyer CA, Galbraith WM. Inhibition of cyclocytidine-induced enlargement of parotid and heart by propranolol or sympathectomy. Proc Soc Exp Biol Med **150**:394–400, 1975.
4. Aloe L, Cozzari C, Levi-Montalcini R. Cyclocytidine-induced

release of nerve growth factor from mouse submandibular glands enhances regeneration of sympathetic fibers in adult mice. Brain Res **332**:259–265, 1985.
5. Schacterle GR, Pollack RL. A simplified method for the quantitative assay of small amounts of protein in biologic material. Anal Biochem **51**:654–655, 1973.
6. Schneyer CA, Hall HD. Autonomic regulation of postnatal changes in cell number and size of rat parotid. Am J Physiol **219**:1268–1272, 1970.
7. Krstulović AM. Investigations of catecholamine metabolism using high performance liquid chromatography. Analytical methodology and clinical applications. J Chromatogr **229**:1–34, 1982.
8. Schneyer CA, Yu JH. Developmental status of sympathetic innervation in relation to calcium accumulation by submandibular gland following reserpine, surgical sympathectomy or cyclocytidine. Proc Soc Exp Biol Med **179**:143–146, 1985.
9. Schneyer CA, Yu JH. Calcium concentration of saliva and salivary glands of rat after cyclocytidine. Proc Soc Exp Biol Med **175**:16–20, 1984.
10. Levi-Montalcini R, Angeletti PU. Nerve growth factor. Physiol Rev **48**:534–569, 1968.
11. Murphy RA, Watson AY, Metz J, Forssmann WG. The mouse submandibular gland: An exocrine organ for growth factors. J Histochem Cytochem **28**:890–902, 1980.
12. Iversen LL. Uptake processes for biogenic amines. In: Iversen LL, Iversen SD, Snyder SH, Eds. Handbook of Psychopharmacology. New York: Plenum Press, Vol **3**: pp381–442, 1975.