pharmacological, not physiological, antagonism of endogenous pain-attentuating processes. Thus during pregnancy there is activation of an endorphin system that is apparently quiescent in nonpregnant female rats treated the same way. Endorphins therefore appear to be an important component of intrinsic mechanisms that modulate responsiveness to aversive stimuli during pregnancy.

ALAN R. GINTZLER*
Department of Anatomy,
College of Physicians and Surgeons,
Columbia University, New York 10032

References and Notes

1. E. J. Simon, J. M. Hiller, I. Edelman, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1947 (1973); C. B. Pert and S. H. Snyder, *Science* **179**, 1011 (1973); L. Terenius, *Acta Pharmacol. Toxicol.* **33**, 377 (1973).
2. J. Hughes, *Brain Res.* **88**, 295 (1975); G. W. Pasternak, R. Goodman, S. Snyder, *Life Sci.* **16**, 1765 (1975); B. M. Cox, K. E. Opheim, H. Teschemacher, A. Goldstein, *ibid.*, p. 1777; C. Li and D. Chung, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1145 (1976).
3. J. Gautray, A. Jolivet, J. Uielh, R. Guillemin, *Am. J. Obstet. Gynecol.* **129**, 211 (1977); Y. Nakai, K. Nakao, S. Ohi, H. Imura, *Life Sci.* **23**, 2013 (1978); K. Csontos, M. Rust, V. Holt, W. Mahr, W. Kromer, H. J. Teschemacher, *ibid.* **24**, 835 (1979); J. C. Houck, C. Kimball, C. Chang, N. W. Pedigo, H. I. Yamamura, *Science* **207**, 78 (1980).
4. Cylindrical pellets (diameter, 4.5 mm; length, 9 mm) of naltrexone (30 mg), cholesterol (105 mg), and glyceryl tristearate (15 mg) were prepared as described by A. L. Misra, R. B. Pontanai, and N. L. Vadlamani [*Res. Commun. Chem. Pathol. Pharmacol.* **20**, 43 (1973)], and A. L. Misra and R. B. Pontanai [*Commun. J. Pharm. Pharmacol.* **30**, 325 (1978)]. Two 30-mg pellets were implanted subcutaneously under light ether anesthesia in the dorsal area of each rat 1 day before the start of testing. This was sufficient to block the analgesic effects of morphine in rats (10 mg/kg, intraperitoneally) for 2 to 3 months.
5. Tests were conducted in a standard chamber with a 30 by 24 cm floor grid composed of 14 bars (diameter, 6 cm) spaced 1.8 cm apart. Scrambled electric shock was delivered to the grids by a constant-current shock generator.
6. A. Spiaggia *et al.*, *Pharmacol. Biochem. Behav.* **10**, 761 (1979).
7. W. O. Evans, *Psychopharmacologia* **2**, 318 (1961).
8. I would like to thank B. Yoburn for many helpful discussions and assistance in the preparation of this manuscript and A. L. Misra for the naltrexone pellets. This research was supported by NIMH grant DA01772.
* Present address: Department of Biochemistry, Downstate Medical Center, Brooklyn, N.Y. 11203.

14 April 1980; revised 30 May 1980

Ornithine Decarboxylase Is Important in Intestinal Mucosal Maturation and Recovery from Injury in Rats

Abstract. *A transient increase in ornithine decarboxylase activity and polyamine biosynthesis occurs in the intestinal mucosa of the newborn rat in the third week after birth. During this period, there is a rapid conversion of the mucosa from a fetal to a mature adult status. A similar increase in ornithine decarboxylase activity also accompanies the rapid recovery of the mucosa 1 week after an injury is induced by chemotherapy in adult rats. In vivo, α-difluoromethyl ornithine, a highly selective, enzyme-activated, irreversible inhibitor, suppresses these increases in mucosal ornithine decarboxylase and delays both intestinal mucosal maturation and recovery from injury. Thus increased ornithine decarboxylase activity, with the resultant increase in polyamine content, may play an essential role in intestinal mucosal maturation and regeneration in the rat.*

The decarboxylation of ornithine by ornithine decarboxylase (ODC) (E.C. 4.1.1.17) leads to the formation of putrescine, and this reaction is the initial and rate-limiting step in the biosynthesis of polyamines (*1*, *2*). Marked increases in ODC activity and rapid accumulation of the tissue polyamines putrescine, spermidine, and spermine are characteristically associated with rapid growth (*2*, *3*). Attempts to document in vivo an essential role for this increased polyamine biosynthesis in cell growth and differentiation has proved difficult because many of the available ODC inhibitors are competitive, and hence reversible (*4*).

Recently, a potent, enzyme-activated, irreversible ODC inhibitor, DL-α-difluoromethyl ornithine (DFMO; RMI 71782), has been developed at the Merrell Research Center (*5*). Other than the selective inhibition of ODC, DFMO has no acute pharmacologic activity, and is essentially nontoxic in normal mice and rats (*6*, *7*). DFMO suppresses induced formation of putrescine and arrests the growth of mouse L1210 leukemia cells and rat hepatoma cells in culture (*6*), and completely suppresses the sharp rise in uterine ODC activity accompanying murine embryogenesis, with resultant complete arrest of embryonic development (*7*). DFMO may thus be used to manipulate selectively the polyamine biosynthetic pathway and to delineate the functional role of ODC and the polyamines in tissue growth.

The rapidly proliferating and maturing epithelium of the small intestine offers a model for the study of cell growth and differentiation where polyamine biosynthesis may play a critical role. In the first 3 weeks after birth in the rat, the mucosal crypts elongate, cell proliferation increases, the villi lengthen, and mature mucosal cells bearing disaccharidases increase in number, with the most rapid increases in enzyme activity occurring during week 3 after birth (*8*). The intestinal mucosa of the adult rat also undergoes constant cell regeneration; the process from cell division through maturation and loss into the lumen is completed in 2 to 3 days. This cell growth pattern is altered by injury to the mucosa (*8*). Treatment with arabinosylcytosine (ara-C), a chemotherapy agent that acts specifically against cells in the proliferative phase, or S (synthesis) phase of the cell cycle, is followed by a loss of mature mucosal cells and an increased proliferation of the uninjured crypt cells. During the next 2 to 3 days, new mucosal cells bearing the disaccharidases appear and increase in number, and histologic and biochemical recovery is complete in 1 week (*9*). Our laboratory has previously shown that ODC and diamine oxidase (DAO) (E.C. 1.4.3.6), an enzyme which deaminates the polyamine putrescine, are found in increasing amounts during cell maturation in the intestinal mucosa (*10*). The ontogenic developmental pattern of DAO in the newborn rat is similar to that of the disaccharidases and can be used to monitor the maturity and integrity of the intestinal mucosa (*11*). We therefore studied the dynamics of polyamine biosynthesis and metabolism in the rat small intestine mucosal epithelium during maturation in the newborn and recovery from injury in the adult.

In newborn rats the most rapid increases in the activities of maltase and DAO, enzymes of the mature mucosa, occurred during week 3 after birth, when there was also a rapid increase in polyamine biosynthesis and mucosal ODC activity increased 15-fold from its low basal level (Fig. 1A). The ODC "spike" was accompanied by an increased activity of the second critical enzyme in the polyamine biosynthetic pathway, *S*-adenosylmethionine decarboxylase (SDC) (E.C. 4.1.1.50). SDC catalyzes the decarboxylation of *S*-adenosyl methionine, thus providing propylamino groups for the subsequent biosynthesis of spermidine and spermine. The increases in the polyamine biosynthetic enzyme activities were accompanied by increases in polyamine content (Fig. 1A). These enzyme activities and polyamine concentrations returned to their basal levels by day 24, when the mucosa had achieved its mature status.

A rapid increase in ODC activity and polyamine biosynthesis also accompanied mucosal regeneration and maturation during the first week of recovery

0036-8075/80/1010-0195$00.75/0

from mucosal injury in the adult rat. After injury is induced by ara-C there is a marked decrease in the activities of the enzymes of the mature mucosa, maltase and DAO. We found that these enzyme activities reached a nadir on day 4, and recovered by day 9. During the period of rapid recovery, the mucosal ODC activity increased 16-fold from its low basal levels; this increase was accompanied by increases in SDC and the polyamines (Fig. 1B). The enzyme activities and polyamine concentrations then returned to normal by day 9, when the mucosa had recovered.

To evaluate the importance of the changes in polyamine biosynthesis for maturation of the intestine in the newborn and recovery from injury in the adult, we conducted simultaneous experiments in rats given the ODC inhibitor DFMO as a 2 percent solution in their drinking water (*7*). We also used newborn rat pups that received by way of their mothers' milk the DFMO that was given to the mother in drinking water. We found that DFMO in vitro did not inhibit SDC, DAO, or the disaccharidases, and that DFMO given to normal adult rats for 2 weeks caused no histologic changes in the intestinal mucosa although basal ODC activity was suppressed by 60 percent.

In the newborn rats, DFMO produced a 65 percent inhibition of the ODC "spike" that normally occurs during week 3 after birth and abolished the increases in putrescine and spermidine concentrations. In contrast, both SDC activity and spermine content increased. These biochemical changes were accompanied by a delay in maturation of the intestinal mucosa. On day 24, mucosal maltase and DAO activities in the untreated animals reached normal adult levels (Fig. 1A); in contrast, neither enzyme activity reached normal adult levels until day 32 in rats treated with DFMO. This 8-day delay in biochemical maturation is accompanied by a 3-day delay in histologic maturation (Fig. 2, A and B). When the rats received DFMO continuously until day 40 (via the mother's milk from days 14 to 28) the mucosal enzyme activities did not reach normal adult levels until day 46, 22 days after the controls (data not shown).

In the adult rats given ara-C to induce mucosal injury, DFMO completely abolished the ODC spike and the increases in putrescine and spermidine concentrations normally seen during the first week of mucosal recovery from injury (Fig. 1B). Again, increases in SDC activity and spermine content occurred during treatment with DFMO. Compared to the

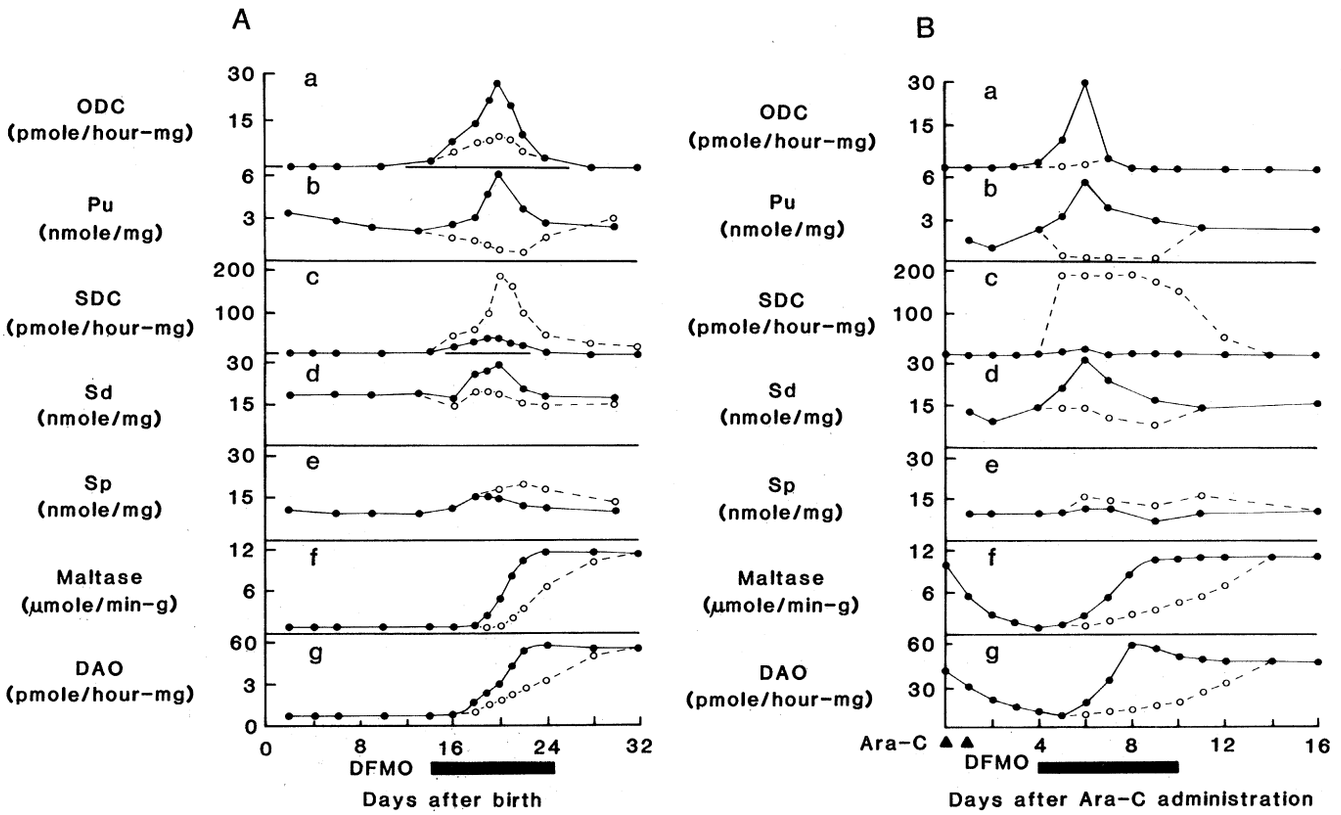

Fig. 1. Effects of DFMO on changes in polyamine biosynthetic enzyme activities, polyamine concentrations, and mature mucosal enzyme activities in rat intestinal mucosa during (A) mucosal maturation and (B) recovery from ara-C. (A) Newborn rats were killed at intervals of 1 to 3 days from day 0 to day 40 after birth and intestinal mucosa was removed for enzyme and polyamine measurements and histologic studies as described (*11*). Ornithine decarboxylase (*ODC*) and *S*-adenosylmethionine decarboxylase (*SDC*) activities were measured in mucosal homogenates by the measurements of $^{14}CO_2$ liberated from the respective substrates (*10*, *11*). Putrescine (*Pu*), spermidine (*Sd*), and spermine (*Sp*) were measured fluorometrically in acid extracts of mucosal homogenates (*12*). Disaccharidases were assayed by the method of Dahlqvist (*13*). Diamine oxidase (*DAO*) was assayed by a modification of the method of Beaven and Jacobsen (*14*) and protein determination was done by the phenol method of Lowry (*15*). Symbols: ●, untreated control animals; ○, animals whose mothers were given DFMO as a 2 percent solution in drinking water during days 14 to 24 after birth (mean daily drug intake of the mothers, 2.6 g/kg). (B) Adult female Lewis rat littermates (each about 200 g) were given ara-C (300 mg/kg per dose) subcutaneously every 8 hours for six doses, and killed at daily intervals from days 1 to 20. Intestinal mucosa was removed for enzyme and polyamine measurements and histologic studies. Symbols: ●, controls that did not receive DFMO; ○, animals receiving DFMO as a 2 percent solution in the drinking water during days 4 to 9 after ara-C treatment (mean daily intake, 2.2 g/kg). Values shown in both (A) and (B) are means of 10 to 15 determinations at each point for ODC, SDC, maltase, and DAO and three to five determinations for the polyamines. The differences in the enzyme activities and polyamine content between the controls and the DFMO-treated animals, when shown by separate symbols, are statistically significant ($P < .05$ to $P < .01$ by Student's unpaired *t*-test).

newborn rats receiving DFMO in their mother's milk, the adult rats given DFMO in their drinking water showed a greater inhibition of ODC and a higher increase in SDC, a result compatible with the different routes of DFMO administration.

The DFMO suppression of the increases in ODC activity and putrescine and spermidine content is accompanied by a marked delay in mucosal recovery, as measured by the delayed return of the disaccharidase and DAO activities to normal preinjury levels (Fig. 1B). On day 9 after ara-C injury, when the mucosal disaccharidase and DAO activities of the non-DFMO treated control animals had returned to preinjury levels, disaccharidase and DAO activities were only 25 percent of control. Both the disaccharidase and DAO activities eventually returned to normal by day 14, a full 6 days after the controls, and 4 days after the DFMO was stopped. This marked delay in biochemical recovery of the mucosa was also accompanied by a 3-day delay in histologic recovery (Fig. 2, C and D). When DFMO was given continuously until day 16, the mucosal enzyme activities did not recover until day 20, 12 days after the controls (data not shown).

The resistance of the mucosa to complete cessation of growth and recovery after ODC inhibition might be explained by the concomitant SDC increase seen with DFMO suppression of the ODC spike in both the newborn and adult rats. This SDC increase might also account for the small but significant increase in spermine concentration. This apparent compensatory response of the polyamine biosynthetic pathway to ODC inhibition has been observed in other instances where DFMO has been used (*7*). Despite the increase in SDC, the suppression of the ODC spike during normal mucosal maturation in the newborn and mucosal recovery in the adult rat after injury results in marked delay of both mucosal maturation and recovery. Since DFMO has no known biological or pharmacological effect other than the specific inactivation of ODC, the results suggest an important role for the increase in ODC, and the resultant increase in putrescine and spermidine content, in proper intestinal mucosal maturation in the newborn rat and mucosal recovery after injury in the adult rat.

The present results further emphasize the functional role of the polyamine biosynthesis pathway in cell growth processes in general and in intestinal mucosal cell growth processes in particular. The data also pinpoint areas where the precise nature of this role might be further investigated. Usually, increases in ODC activity and polyamine synthesis

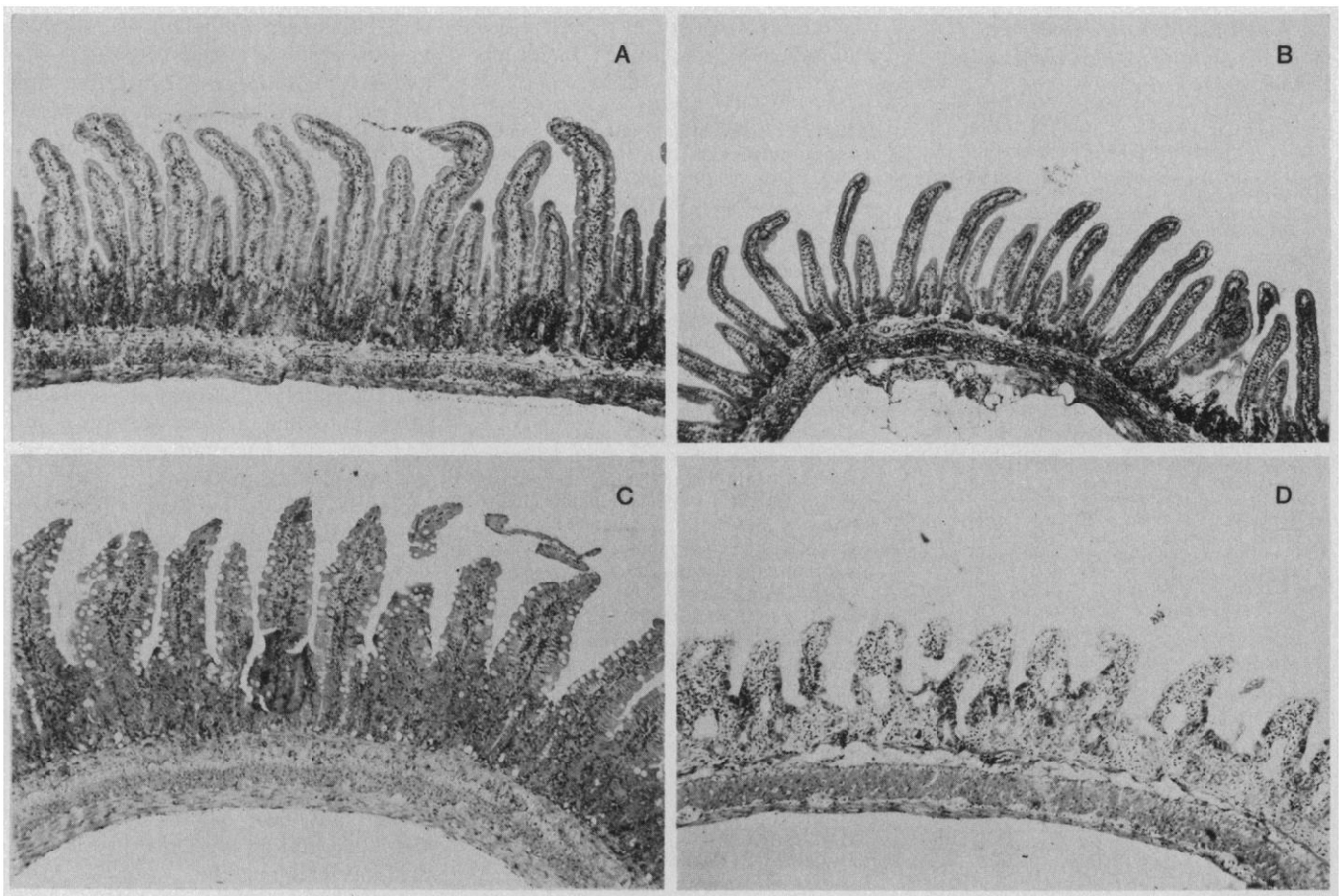

Fig. 2. Effects of DFMO on histologic changes in the rat intestinal mucosa during maturation in the newborn and recovery from injury in the adult (×75). Intestinal segments were obtained from rats as described in the legend to Fig. 1. The 1-cm segment closest to the ligament of Treitz was fixed (buffered Formalin) and embedded (paraffin) and serial sections were stained (hematoxylin-eosin). (A and B) A representative section of the intestinal mucosa of a normal 22-day-old rat (A) is compared to that of a 22-day-old rat whose mother was given DFMO (B). The overall thickness of the mucosa of the DFMO-treated animal is less, the villi shorter, and the staining of the supranuclear cytoplasm less dense than the controls ($N = 36$, $P < .05$ by Wilcoxon's unpaired rank sum test). After another 3 days, the histologic features of the mucosa of the DFMO-treated animals resembled that of the 22-day-old control animals. (C and D) The intestinal mucosa of an adult rat 6 days after ara-C–induced injury (C) is compared to that of a similarly injured animal given DFMO (D). The mucosa of the DFMO-treated animals shows less recovery from the mucosal damage and has fewer mitoses in each crypt ($N = 48$, $P < .05$ by Wilcoxon's unpaired rank sum test). After another 3 days, the recovery of the DFMO-treated animals approached that in the control animals.

are associated with initiation of rapid cell proliferation or cellular response to hormonal stimulation (*1*-*3*). The association of increased polyamine biosynthesis during the period of enzymic maturation of the newborn rat intestinal mucosa is especially intriguing in this regard. Is this process of mucosal maturation in the newborn rat accompanied by the proliferation of a new population of cells, or by hormonally mediated maturation and gene reprogramming of an existing population of cells? The type of manipulation of polyamine biosynthesis used in our present studies could help clarify this aspect of cellular differentiation.

GORDON D. LUK
Department of Medicine and
Oncology Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205
LAURENCE J. MARTON
Brain Tumor Research Center, University of California School of Medicine, San Francisco 94143
STEPHEN B. BAYLIN
Oncology Center and *Department of Medicine, Johns Hopkins University School of Medicine*

References and Notes

1. H. G. Williams-Ashman and Z. N. Canellakis, *Perspect. Biol. Med.* **1979-I**, 421 (1979); D. H. Russell and B. G. M. Durie, *Progress in Cancer Research and Therapy* (Raven, New York, 1978), vol. 8; C. W. Tabor and H. Tabor, *Annu. Rev. Biochem.* **45**, 285 (1976).
2. J. Jänne, H. Pösö, A. Raina, *Biochim. Biophys. Acta* **473**, 241 (1978).
3. S. H. Snyder and D. H. Russell, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **29**, 1575 (1970); D. Russell and S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **60**, 1420 (1968).
4. S. I. Harik, M. D. Hollenberg, S. H. Snyder, *Nature (London)* **249**, 250 (1974); P. S. Mamont, P. Böhlen, P. P. McCann, P. Bey, F. Schuber, C. Tardif, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1626 (1976); P. P. McCann, C. Tardif, M. C. Duchesne, P. S. Mamont, *Biochem. Biophys. Res. Commun.* **76**, 893 (1977); N. Seiler, C. Danzin, J. Prakash, J. Koch-Weser, in *Enzyme-Activated Irreversible Inhibitors*, N. Seiler, M. J. Jung, J. Koch-Weser, Eds. (Elsevier/North-Holland, Amsterdam, 1978), p. 55.
5. B. W. Metcalf, P. Bey, C. Danzin, M. J. Jung, P. Casara, J. P. Vevert, *J. Am. Chem. Soc.* **100**, 2551 (1978); P. Bey, in *Enzyme-Activated Irreversible Inhibitors*, N. Seiler, M. J. Jung, J. Koch-Weser, Eds. (Elsevier/North-Holland, Amsterdam, 1978), p. 27.
6. P. S. Mamont, M. C. Duchesne, A. M. Joder-Ohlenbusch, J. Grove, in *Enzyme-Activated Irreversible Inhibitors*, N. Seiler, M. J. Jung, J. Koch-Weser, Eds. (Elsevier/North-Holland, Amsterdam, 1978), p. 43.
7. J. R. Fozard, M. L. Part, N. J. Prakash, J. Grove, P. J. Schechter, A. Sjoerdsma, J. Koch-Weser, *Science* **208**, 505 (1980).
8. R. K. Crane, in *Handbook of Physiology*, section 6, *The Alimentary Canal*, C. F. Code and W. Heidel, Eds. (Williams & Wilkins, Baltimore, 1968), vol. 5, p. 2535; O. Koldovsky, *Development of the Functions of the Small Intestine in Mammals and Man* (Karger, New York, 1969); S. J. Henning and N. Kretchmer, *Enzyme* **15**, 3 (1973); G. L. Eastwood, *Gastroenterology* **72**, 962 (1977).
9. R. S. Verbin, G. Diluiso, H. Liang, E. Farber, *Cancer Res.* **32**, 1476 (1972); L. Lenaz, S. S. Steinberg, F. S. Philips, *ibid.* **29**, 1790 (1969); W. B. Leach, W. R. Laster, Jr., J. G. Mayo, D. P. Griswold, Jr., F. M. Schabel, Jr., *ibid.* **29**, 529 (1969).
10. S. B. Baylin, S. A. Stevens, K. M. M. Shakir, *Biochim. Biophys. Acta* **541**, 415 (1978).
11. G. D. Luk, T. M. Bayless, S. B. Baylin, *J. Clin. Invest.* **66**, 66 (1980).
12. L. J. Marton and P. L. Y. Lee, *Clin. Chem.* **21**, 1721 (1975).
13. A. Dahlqvist, *Anal. Biochem.* **7**, 18 (1964).
14. M. A. Beaven and S. Jacobsen, *J. Pharmacol. Exp. Ther.* **176**, 52 (1971).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
16. We thank A. Sjoerdsma and P. McCann, Merrell Research Center, for the DFMO; M. A. Beaven, National Institutes of Health, for the [β-^{3}H]-histamine; J. H. Yardley for help with the histology; T. R. Hendrix and A. H. Owens, Jr., for advice; and G. Goodwin, W. Lubrich, J. Messersmith, and K. Wieman for technical assistance. This work was supported in part by grants 5-R01-18404, 1-P50-HL-19157-01, 5-T32-AM-07192-03, RR-5378, CA-15515, and CA-13525 from NIH. L.J.M. (CA-00112) and S.B.B. (CA-00027) are supported by NIH research career development awards.

16 April 1980; revised 27 June 1980

Epidermal Growth Factor Is a Major Growth-Promoting Agent in Human Milk

Abstract. *Human milk stimulates DNA synthesis in cell cultures in which growth has been arrested. The mitogenic activity of milk is neutralized by the addition of antibody to human epidermal growth factor. The results identify epidermal growth factor as a major growth-promoting agent in breast milk.*

Epidermal growth factor (EGF) is a small polypeptide mitogen (molecular weight ≃ 6000) detected in many mammalian species and isolated from mice (*1*) and humans (*2*). Although recent studies have focused on the mechanism by which EGF promotes the proliferation of cultured cells, the growth factor produces significant biological effects in the intact mammal, particularly the fetus and newborn. These effects include enhanced proliferation and differentiation (keratinization) of the epidermis (*1*, *3*), increased growth and maturation of the fetal pulmonary epithelium (*4*), stimulation of ornithine decarboxylase activity and DNA synthesis in the digestive tract (*5*), and acceleration of the healing of wounds of the corneal epithelium (*6*). Apparently unrelated to these mitogenic effects is the fact that EGF inhibits histamine- or pentagastrin-induced secretion of gastric acid (*7*).

Epidermal growth factor is present in many human body fluids including plasma, saliva, urine, amniotic fluid, and milk (*8*). Breast milk is important to the developing infant not only as a source of gross nutrients (protein, carbohydrate, and fat) and micronutrients (minerals and vitamins), but also as a source of molecules (immunoglobulins and hormones) that may have a more specialized role in ontogeny. Since human milk is mitogenic for cultured cells (*9*), we performed experiments to determine whether the growth-promoting activity of milk can be attributed to the presence of EGF. We added specific antibodies to human EGF to samples of human milk and tested the samples for their capacity to stimulate DNA synthesis in diploid human fibroblasts in vitro by measuring the incorporation of [^{3}H]thymidine into acid-insoluble material.

In the presence of purified EGF [10 ng/ml, a concentration above that which produces maximal mitogenic activity (*10*)] or milk (5 percent by volume), the incorporation of [^{3}H]thymidine was increased approximately 11-fold over that incorporated by control (unstimulated) cells (Table 1). Adding antibodies to EGF completely blocked the mitogenic

Table 1. Influence of antibody to human EGF on the stimulation of DNA synthesis in human fibroblasts by human milk. Confluent monolayers of foreskin fibroblasts, cultured in Eagle's minimum essential medium (Dulbecco's modification) containing 10 percent calf serum and gentamycin (*10*), were transferred to fresh medium supplemented with 2 percent calf serum and gentamycin. After 2 days the indicated additions were made, and 20 hours later [*methyl*-^{3}H]thymidine was added to each dish. The cells were allowed to incorporate the isotope for 4 hours before the labeling was terminated. The amount of acid-insoluble radioactivity was then determined (*10*). Rabbit antibody to human EGF was prepared (*2*) and the gamma globulin fraction was purified by diethylaminoethane chromatography (*10*). The control gamma globulin, from a rabbit immunized with renin, was prepared in an identical manner. The human milk, donated 5 weeks after the mother gave birth, was centrifuged and sterilized by filtration through a 0.2-μm mesh filter.

Additions	[^{3}H]Thymidine incorporated (count/min per dish)
None	198
EGF (10 ng/ml)	2132
EGF (10 ng/ml) + antibody to EGF (37 μg/ml)	225
Antibody to EGF (37 μg/ml)	169
Control IgG (42 μg/ml)	172
Milk (10 percent)	4582
Milk (5 percent)	2228
Milk (1 percent)	528
Milk (5 percent) + antibody to EGF (37 μg/ml)	343
Milk (5 percent) + control IgG (42 μg/ml)	2001
Calf serum (10 percent)	3023

0036-8075/80/1010-0198$00.50/0