References and Notes

1. C. B. Huggins, *Proc. Soc. Exp. Biol. Med.* **27**, 349 (1930); *Proc. Amer. Phil. Soc.* **113**, 458 (1969).
2. A. J. Friedenstein and K. S. Lapykina, *Bull. Exp. Biol. Med.* **55**, 104 (1963); A. J. Friedenstein, *Clin. Orthopaed.* **59**, 21 (1968).
3. H. C. Anderson and P. R. Coulter, *J. Cell. Biol.* **33**, 165 (1967); M. R. Urist, T. A. Rowell, P. H. Hay, B. S. Strates, *Clin. Orthopaed.* **59**, 59 (1968).
4. C. B. Huggins, H. R. McCarroll, B. H. Blocksom, Jr., *Arch. Surg.* **32**, 915 (1936).
5. M. R. Urist, *Science* **150**, 893 (1965); G. Bang and M. R. Urist, *Arch. Surg.* **94**, 781 (1967); M. R. Urist, B. F. Silverman, K. Büring, F. L. Dubuc, J. M. Rosenberg, *Clin. Orthopaed.* **53**, 243 (1967); M. R. Urist, P. H. Hay, F. L. Dubuc, K. Büring, *ibid.* **64**, 194 (1969).
6. C. H. Fiske and Y. Subbarow, *J. Biol. Chem.* **66**, 375 (1925).
7. C. Huggins and S. Morii, *J. Exp. Med.* **114**, 741 (1961).
8. G. Gomori, *Microscopic Histochemistry, Principles and Practice* (Univ. of Chicago Press, Chicago, 1952), p. 175.
9. M. J. Griffin and R. P. Cox, *Proc. Nat. Acad. Sci. U.S.* **56**, 946 (1966).
10. M. D. Waters and G. K. Summer, *Biochim. Biophys. Acta* **177**, 650 (1969).
11. F. Griffith, *J. Hyg.* **27**, 113 (1928).
12. O. T. Avery, C. M. MacLeod, M. McCarty, *J. Exp. Med.* **79**, 137 (1944).

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5-Hydroxytryptamine: A Cytospecific Growth Stimulator of Cultured Fibroblasts

Abstract. *5-Hydroxytryptamine (serotonin) in micromolar amounts increased the growth of fibroblasts in culture while not affecting five other cell lines. Serotonin appeared to shorten the lag phase of cell growth. The effect was less when serotonin was added to the fibroblast culture after the initial 24-hour period. The two functional groups of the serotonin molecule were required for growth enhancement. Serotonin in millimolar concentrations was toxic to fibroblasts.*

In humans with the carcinoid syndrome, elevated concentrations of 5-hydroxytryptamine (serotonin) in the plasma are often causally related to excessive formation of connective tissue (*1*). Under experimental conditions, repeated injections of serotonin into joint spaces (*2*) or into the subcutaneous tissue (*3*) cause an overgrowth of connective tissue. Serotonin has been reported to increase the incorporation of radioactive thymidine into guinea pig skin slices in vitro (*4*). From these associations, the question was raised whether serotonin would directly stimulate the growth of fibroblasts in culture.

Two fibroblast lines were used, the mouse (3T6) and the human embryo lung. The origin and growth characteristics of these cells were described previously (*5*). The cells were maintained in Dulbecco's modification of Eagle's basal medium (*6*) with 10 percent bovine calf serum and antibiotics [streptomycin (0.1 mg) and penicillin (100 unit/ml)].

The HeLa (epithelioid carcinoma, cervix; human), KB (epidermoid carcinoma, oral; human), and BHK (kidney; golden hamster, *Mesocricetus auratus*) were obtained from the cell culture repository (American Type Culture Collection, Rockville, Maryland) and maintained in Eagle's minimum essential medium. The cell lines were adapted to the Dulbecco's modified Eagle's medium by two or three passages before being used.

Cell cultures were started by placing 5 ml of fresh medium containing 4×10^4 cell/ml into a 2-ounce glass bottle. The bottles were flushed with a mixture of oxygen and carbon dioxide (90 : 10), sealed with rubber stoppers, and then incubated at 34°C in a light-tight incubator. The cultures were grown for 1 to 7 days without a change in the culture medium. Under these experimental conditions, the fibroblasts were confluent at approximately 6 days.

The cells were harvested by first removing the medium; they were then washed with 5 ml of Hanks solution, and finally detached with 5 ml of a 0.05 percent trypsin and 2 percent ethylenediaminetetraacetate solution. In the trypsin step the cells were incubated with the trypsin solution at 34°C for 5 minutes, and the flask was mechanically agitated for 3 to 5 minutes. The cells were transferred to a test tube and centrifuged at 900*g*. The pellet was washed with Hanks solution, centrifuged, and resuspended in 0.9 percent sodium chloride solution. A cell count was made on a portion of the cell suspension with a Coulter counter. The counts were made in quadruplicate, and the variation around the mean was less than 3 percent. The bottles were checked by microscopic examination for cells that might have remained attached to the flasks.

Table 1. Effect of serotonin ($10^{-6}M$) given at different periods of cultured fibroblast growth. The cells were harvested at either 4 or 6 days. The values are the mean of six experiments.

Days	Percent increase
0	73
1	56
2	26
3	21
5	26

Serotonin bimaleate, when present in the culture medium throughout the period of growth, influenced fibroblast growth. At high concentrations (10^{-2} and $10^{-3}M$) serotonin was toxic; a concentration of $10^{-3}M$ significantly reduced cell numbers by more than 80 percent (Fig. 1). Serotonin concentrations of 10^{-6} or $10^{-7}M$ significantly increased fibroblast growth by nearly 100 percent. Our studies on fibroblast growth with serotonin were at a concentration of $10^{-6}M$.

Serotonin given at zero time increased the number of fibroblasts after 24 hours by 58 percent; control, $2.45 \pm 0.16 \times 10^5$ and treated, $3.86 \pm 0.16 \times 10^5$ cells ($n = 6$). Supplemented injections of serotonin at 3, 5, or 6 days caused a significant increase of cell

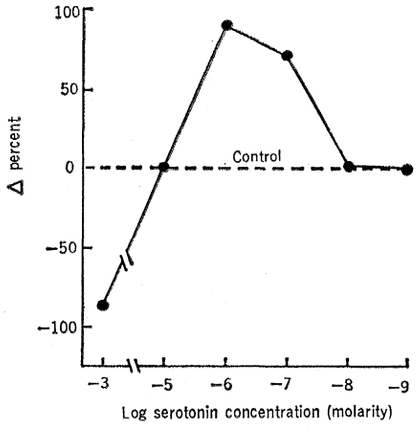

Fig. 1. Change in human fibroblast (96-hour cultures) numbers as a function of serotonin concentration. Control cultures were given concentrations of maleic acid equivalent to the amount in serotonin bimaleate. Maleic acid at this concentration had no effect upon cell growth. Compounds were included in the initial incubation medium, and supplements were added at 72 hours. The cells were harvested after 96 hours. Values are the mean of at least six experiments.

numbers throughout the entire incubation period (Fig. 2). Between 2 and 6 days, the number of treated cells was about 70 percent greater than the untreated; after 6 days, the increase was approximately 30 percent. Serotonin given only with the incubation medium did not affect cell numbers after 6 days of culture; control, $9.67 \pm 0.22 \times 10^6$; and treated, $10.43 \pm 0.49 \times 10^6$ cells ($n = 6$).

Delaying the time of the serotonin addition to the cell culture until 1, 2, 3, or 5 days (Table 1) reduced the stimulatory effect. Serotonin added to the culture when the cells were in the stationary phase of growth had no effect on cell numbers.

There were qualitative differences in the growth rate of the two fibroblasts. The human fibroblasts had a longer lag phase than the 3T6 fibroblasts, and their numbers after 7 days were considerably less than those of the 3T6, $2.99 \pm 0.34 \times 10^6$ as compared to $7.38 \pm 0.33 \times 10^6$. However, the response to serotonin was similar in the two strains of fibroblasts.

In an attempt to understand the mechanism of the serotonin action, we examined for an effect on the lag phase of human fibroblasts. Serotonin significantly increased the number of cells attached to the culture flask after 12 hours when compared to the control (less than 48 percent). During the 12- to 18-hour period, the increase in numbers of attached cells with serotonin was nearly 40 percent, while the controls were 25 percent. This difference was greater when the growth from 12 to 24 hours was considered; the increase with serotonin was more than 80 percent while the control was 30 percent. Clearly, serotonin shortened the lag phase of the cell culture.

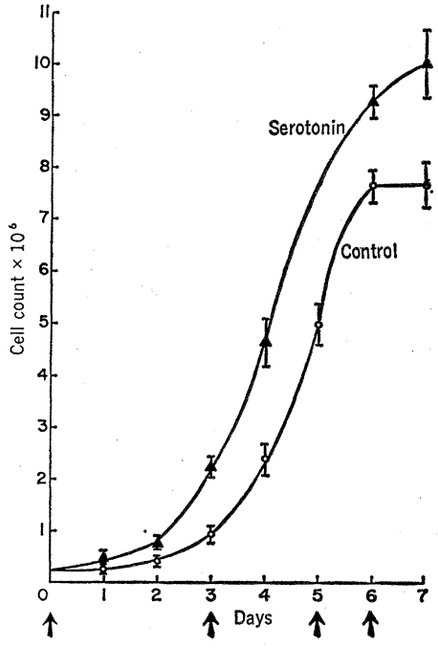

Fig. 2. Influence of repeated additions of serotonin ($10^{-6}M$) on mouse fibroblast (3T6) growth. Serotonin additions are indicated by the vertical arrows along the abscissa. Serotonin was included in the incubation medium at zero time, and supplemental serotonin was added to all flasks at 3 days. Cells obtained later were given an additional dose of serotonin on the day before harvesting. Values represent the mean and the S.E. of at least six experiments.

Serotonin has some unique properties for increasing fibroblast growth. For example, the requirements of the molecule itself were specific, as seen in the results with serotonin analogs shown in Table 2. Tryptamine, without the functional hydroxyl group on the benzene ring, did not increase cell numbers; 5-hydroxyindoleacetic acid, 5-hydroxytryptophan, or substituents onto the functional groups of serotonin, methoxytryptamine, and *N*-acetyl serotonin retarded growth.

Another example of the uniqueness of serotonin for fibroblast growth was seen when other cell lines were tested. Serotonin did not influence the growth of HeLa, KB, and BHK cells or of tryptophan-dependent and -independent strains of *Bacillus subtilis*.

There have been few studies on the effect of serotonin on cell division. Pukhal'skaya and Shirokova (*7*) reported a transient and reversible decrease in the number of prophases in a culture of HeLa cells by serotonin (21 μg/ml). Kobayashi *et al.* (*8*) observed an increased mitotic rate of ascites tumor cells, bone marrow, and thymus cells in rats and mice given intraperitoneal injections of serotonin (2.5 mg/100 g). Quay (*9*), in a review on the physiology of serotonin, suggested that there may be two cellular sites of serotonin action: an association in some manner with the conduction or contractile system of cytoplasmic microtubules which, in the dividing cell, might influence the separation of mitotic centers and an involvement in some unspecified manner with nucleotides.

The complete mechanism for the serotonin action on fibroblast growth remains to be determined, but it is clear that the serotonin molecule has a unique effectiveness for enhancing fibroblast growth under tissue culture conditions. It is not possible to extend the findings of serotonin and fibroblast growth under conditions in vitro to the more complex situations occurring in the clinical setting of the carcinoid syndrome. However, if the attraction between serotonin and the fibroblast can be supported by observations in vivo, then serotonin may be a cytohormone for growing fibroblasts.

Table 2. Relative effect of serotonin analogs ($10^{-6}M$) on human fibroblast (96-hour culture) growth. Compounds were included in the initial incubation medium with a supplement added after 72 hours. These are the combined results of two sets of experiments; the values for the control were set at 1.0×10^6 cells and the experimental points were normalized for comparison purposes. Values are the mean ± S.E. ($n = 6$).

Condition	Cell numbers (10^6)	Δ Percent
Control (maleic acid)	1.00 ± .04	
Serotonin	1.95 ± .13*	+95
Tryptamine	1.10 ± .11	+10
5-Hydroxytryptophan	0.80 ± .06†	−20
5-Hydroxyindoleacetic acid	0.80 ± .06†	−20
5-Methoxytryptamine	0.50 ± .03*	−50
N-Acetyl-5-hydroxytryptamine	0.70 ± .04*	−30

* $P < .001$. † $P < .05$.

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References and Notes

1. F. Lembeck, *Nature* **172**, 910 (1953); A. Thorson, G. Biork, G. Bjormas, J. Waldenstrom, *Amer. Heart J.* **47**, 795 (1954); A. Sjoerdsma, H. Weissbach, S. Udenfriend, *Amer. J. Med.* **20**, 520 (1956); M. Eder and A. Schauer, *Beitr. Pathol. Anat. Allg. Pathol.* **121**, 375 (1959).
2. C. J. Smyth, O. B. Gum, P. K. Hamilton, *J. Lab. Clin. Med.* **58**, 958 (1961).
3. R. A. MacDonald, *Amer. J. Pathol.* **35**, 297 (1959).
4. P. R. Mann, *Nature* **216**, 715 (1967).
5. B. Goldberg and H. Green, *J. Mol. Biol.* **26**, 1 (1967); M. Macek, J. Hurych, M. Chvapil, *Cytologia (Tokyo)* **32**, 426 (1967).
6. R. Dulbecco and M. Vogt, *J. Exp. Med.* **99**, 167 (1954).
7. E. C. Pukhal'skaya and T. G. Shirokova, *Byull. Eksp. Biol. Med.* **65**, 345 (1968).
8. J. Kobayashi, T. Yoshizaki, T. Kitajima, F. Kawamura, *Tokushima J. Exp. Med.* **12**, 45 (1965).
9. W. B. Quay, *Advan. Pharmacol.* **6**, 283 (1968).
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