Serotonin and Ribonucleic Acid and Collagen Metabolism of Fibroblasts *in Vitro*[1] (36512)

ROBERT J. BOUCEK, ATHAN J. SPEROPOULOS, AND NANCY L. NOBLE

Laboratories for Cardiovascular Research, Howard Hughes Medical Institute, and Departments of Medicine and Biochemistry, University of Miami School of Medicine, Miami, Florida 33136

After learning that serotonin enhances the growth of fibroblasts *in vitro* (1), we wanted to know if serotonin affected protein and collagen syntheses in fibroblasts. Circumstantial evidence in clinical medicine associates increased blood levels of serotonin with excessive collagen accretions on cardiovascular structures (2, 3). To examine for this possibility, the serotonin effect on the incorporation of ^{3}H-uridine (ribonucleic acid, RNA) and ^{3}H-proline (protein) and on the formation of radioactive hydroxyproline (collagen) by fibroblasts in culture was studied. It was learned that serotonin influences collagen synthesis but the effect seems to be mediated through an action of serotonin on RNA metabolism.

Materials and Methods. Mouse (3T6) and human embryo lung fibroblast lines were grown in Dulbecco's modification of Eagle's basal medium with 10% bovine calf serum and antibiotics (0.1 mg streptomycin and 100 units penicillin/ml). The culture, consisting of 5 ml of fresh medium containing 4×10^4 cell/ml, was placed in a 2-oz prescription glass bottle and flushed with a mixture of atmospheric air and carbon dioxide (90:10). The bottles were sealed with rubber stoppers and incubated at 34° in a light-tight incubator. Ascorbic acid at a concentration of 50 μg/ml (4, 5) was added after 24 hr of cell growth because inclusion of ascorbic acid with the subculture medium retarded cell growth. Serotonin bimaleate at a medium concentration of 10^{-6} *M* was used and its effect compared with that of an equimolar concentration of tryptamine.

The cells were harvested after first removing the medium and washing the attached cells with 5 ml of Hanks' solution. Five milliliters of a 0.6% trypsin:0.15% disodium EDTA solution were added, and after a 3- to 5-min period of agitation at 25°, the cells were transferred to a centrifuge tube and centrifuged at 900*g* for 10 min. The pellet of cells was washed with Hanks' solution, resuspended in 0.15 *M* NaCl, and counted in an electronic counter.

The effect of serotonin on RNA synthesis at different time periods of cell growth was studied in serial and individual cultures of mouse fibroblasts (3T6). The growth characteristics of these cells in our laboratory were reported previously (1). For the serial studies, a large number of flasks was inoculated from a pool of cells, each flask receiving approximately 20×10^5 cells. In one group, serotonin or tryptamine was included in the initial incubation medium with supplements of the amines injected into the culture during the growth phase of the culture. The second and third groups of cultures were treated with serotonin or tryptamine only after the logarithmic growth period. The medium was *not* changed at any time during the study.

The RNA was labeled by injecting 6-^{3}H-uridine (New England Nuclear Corp., Boston, MA; sp act, 10.4 Ci/mmole) into the culture flask 24 hr prior to harvesting the cell. RNA was extracted from a suspension of the cell pellet by the method described by

[1] This investigation was supported in part by U.S. Public Health Service Program Project Grant No. HL 11035 and Training Grant No. HL 05463 from the National Heart and Lung Institute.

Girard (6) for a large number of cells. The method was modified for use with a smaller number of cells and only the changes from the standard procedure are noted. Cells were suspended in 2 ml of cold 0.25 *M* sucrose and 0.25 ml each of 5% recrystallized sodium dodecyl sulfate solution and of 0.1 *M* disodium EDTA solution added. RNA was released into the aqueous phase by three successive extractions at 60° with 2, 1.3, and 1 ml of 88% phenol, respectively. The precipitated RNA was dissolved in either 50 or 100 μl of 0.05 *M* NaCl:1 mmole disodium EDTA, pH 6.2. Absorbancies at 260 and 280 mμ and radioactivity were measured on 5 or 10 μl of the RNA solution. The 280:260 ratio of the samples ranged from 0.521 to 0.578. The 260 values were used as a measure of RNA content.

The species of extracted RNA were separated on vertical 10% polyacrylamide gel electrophoretic column (6 × 0.75 cm) after the technique described by Peacock and Dingman (7). The mobilities of 30 and 18 sRNA extracted from the microsomal fraction of rat liver and pure transfer RNA (tRNA) (gift of Dr. K. Muench) were used as standard markers. Forty microliters of the RNA solution and 50 μl of 80% sucrose containing bromphenol blue were applied to a column, and a current of 14 mA (95 V) was applied for 2.5 hr. The temperature of the water jacket was maintained at 19–20°. The gel was removed and either sliced into 40 fractions for radioactivity measurements after dissolving in 0.2 ml of 1 *M* NaOH or fixed in acetic acid and stained with methylene blue.

Protein synthesis was studied by injecting the cultures with 3,4-^{3}H-proline (New England Nuclear Corp., Boston, MA, sp act. 5.26 Ci/mmole) 24 hr before harvesting the cells. Protein of the cell fraction and of the incubation medium was separately precipitated with 4 vol of cold absolute ethanol and kept at 4° overnight (8). The protein precipitates were washed with cold absolute ethanol containing 0.01 *M* L-proline until washes contained no label. The precipitates were hydrolyzed with 2 ml of 6 *N* HCl at 100° for 16 hr, the HCl removed by evaporation, and the residue dissolved in water. Total radioactivity was measured on a portion of the water solution. The remainder was placed on a Dowex 50 X 8 column (H^+, 200–400 mesh,) 1 × 20 cm) and eluted with 1.5 *N* HCl at room temperature (9). The hydroxyproline-containing eluate fractions were combined, freed of HCl, and the radioactivity determined.

Radioactivity measurements were made in the Packard Tri-Carb liquid scintillation spectrometer. Labeled RNA samples were added to 14 ml of a solution of toluene and absolute methyl alcohol (9:1) containing 7 g of 2,5-diphenyloxazole (PPO) and 0.35 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP)/liter. Polyacrylamide gel slices and other labeled samples were added to 14 ml of a phosphor solution of toluene:dioxane:absolute ethyl alcohol (4:4:2.4) containing 80 g naphthalene, 7 g PPO, and 0.3 g dimethyl POPOP/liter. Values, corrected for quenching and background, are expressed as dpm and related either to cell number or to RNA content.

Results. The groups of cultures studied serially revealed a serotonin effect on RNA synthesis (Table I). Serotonin when included with the subculture incubation medium with supplements given during the cell growth phase (Expt. A) reduced the uptake of ^{3}H-uridine.

On the other hand, serotonin added for the first time after the logarithmic growth phase stimulated ^{3}H-uridine incorporation (Expt. B, Table I). This effect was confirmed in two additional experiments, with an increase of 50 ± 14(5) and 42 ± 12(4)% over the control (mean ± SE given with number of cultures in parentheses). Serotonin added to the culture as the cells reached confluency (Expt. C, Table I) had no significant effect on ^{3}H-uridine incorporation. The serotonin effects on RNA synthesis are apparent both when the radioactivity is expressed on the basis of cell number and as specific activity (dpm/260 unit).

TABLE I. Effect of Serotonin on Fibroblast (3T6) Growth and ^{3}H-Uridine Incorporation.[a]

Culture time (day)	Cell no. (10^6) Control	Cell no. (10^6) Treated	^{3}H-Uridine (dpm/10^6 cells) Control	^{3}H-Uridine (dpm/10^6 cells) Treated	^{3}H-Uridine incorporation (dpm/260 unit) Control	^{3}H-Uridine incorporation (dpm/260 unit) Treated
Expt. A						
2	0.45[c]	0.74[d]	87,545	66,780	116,719	98,163
(1 μCi)[b]	± 0.03	± 0.06	± 15,053	± 4124	(1)	(1)
3	0.96	2.24[d]	80,079	51,293	144,209	77,137
(2 μCi)	± 0.14	± 0.20	± 12,099	± 5269	(2)	(2)
4	2.45	4.67[d]	204,807	121,373[d]	466,041	293,381[e]
(8 μCi)	± 0.29	± 0.44	± 15,466	± 11,804	± 71,943	± 15,317
7	6.11	9.63[d]	91,669	48,164[d]	270,316	149,864[e]
(14 μCi)	± 0.45	± 0.55	± 2293	± 7104	± 31,195	± 21,434
Expt. B						
6	7.64	9.25[d]	22,525	31,688[c]	46,932	64,056[e]
(10 μCi)	± 0.09	± 0.28	± 1050	± 3479	± 3750	± 5320
Expt. C						
7	9.08	10.65	18,367	16,631	86,436	104,276
(12 μCi)	± 0.19	± 0.76	± 1373	± 1880	± 4528	± 8873

[a] Experiment A, serotonin included in the initial incubation medium with supplements at 1, 2, 3, or 6 days of culture: Expt. B, serotonin added after 5 days of cell growth: Expt. C, serotonin added after 6 days of cell growth. ^{3}H-uridine was added 24 hr before harvesting the cells.

[b] Amount of uridine injected into each flask.

[c] Values are means ± SE of at least 5 cultures; number of cultures <5 listed in parentheses.

[d] $p < .01$; p, probability using the Student's table of t.

[e] $p < .05$.

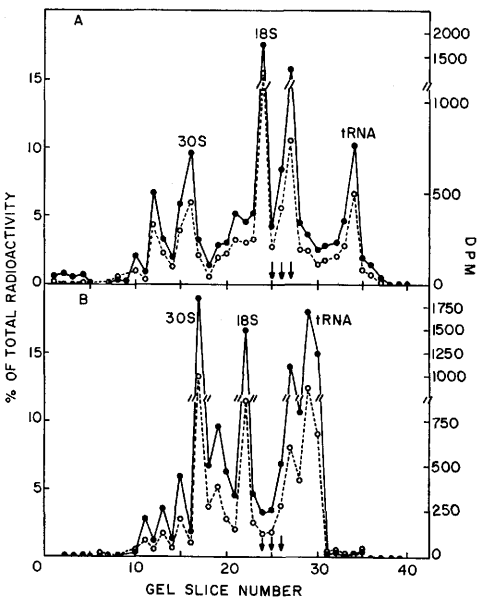

FIG. 1. Electropherogram of RNA extracted from the control (A) and serotonin-treated (B) cells of Expt. B, Table I. Radioactivity of the gel column is shown as percentage of the total radioactivity (O) and as dpm per gel slice (●). Bromphenol blue indicated by three vertical arrows along the abscissa. Equivalent amounts of RNA (260 OD units) from the control and treated cells, containing 24,259 and 32,172 dpm, respectively, were applied.

A serotonin stimulation of ^{3}H-uridine incorporation into RNA that migrates to areas coincident with standards of 30 sRNA and tRNA is shown in Fig. 1. An increased synthesis of these species of RNA occurred only in cultures showing a serotonin-mediated stimulation of RNA. Interestingly, a stimulation of ribosomal RNA and tRNA by serotonin in cultures resembles the effect reported by Rhode and Ellem (10) that follows a change in incubation medium.

One of the objectives of this study was to determine any effect of serotonin on collagen metabolism. For these studies, both the slow-growing human lung diploid and mouse (3T6) aneuploid fibroblasts were used. (With the diploid cells, changes in incuba-

TABLE II. Effect of Serotonin on Cell Growth, Proline Incorporation and Conversion to Hydroxyproline in the Combined Protein Fractions of Cells and Incubation Medium of Fibroblasts in Culture.[a]

Fibroblast	^{3}H-Proline (dpm/10^6 cells)	^{3}H-Hydroxyproline (dpm/10^6 cells)
Human embryo lung		
Expt. A		
Control	13,360,000 ± 86,320[b]	18,650 ± 5866
Serotonin	10,020,000 ± 764,700	12,510 ± 1406
% change	− 25	− 33
Expt. B		
Control	3,544,000 ± 544,100	2774 ± 1032
Serotonin	2,122,000 ± 336,600	1263 ± 113
% change	− 40	− 54
Mouse (3T6)		
Expt. C		
Control	61,740 ± 5090	3992 ± 266
Serotonin	55,010 ± 6540	3540 ± 429
% change	− 10	− 13
Expt. D		
Control	150,300 ± 24,500	14,400 ± 1884
Serotonin	264,200 ± 41,000	16,800 ± 749
% change	+ 76	+ 25

[a] Expts. A, B, and C: serotonin added while cells were rapidly growing; Expt. D: serotonin added as cell growth slowed. Serotonin and ^{3}H-proline (1 μCi/ml medium) were added 24 hr prior to harvest.

[b] Values are means ± SE of at least 3 cultures.

tion medium were routinely made every third day.) Serotonin added to rapidly growing diploid or aneuploid cells seemed to reduce ^{3}H-proline incorporation (Expts. A, B, and C, Table II), while serotonin added when cell growth was slow (Expt. D) caused a stimulation of ^{3}H-proline incorporation.

In general, a serotonin-induced change in ^{3}H-proline incorporation was accompanied by an equivalent change in ^{3}H-hydroxyproline formation (Table II), indicating the lack of a specific effect of serotonin on collagen synthesis since hydroxyproline is a natural marker of collagen. The human diploid fibroblast incorporated considerably more ^{3}H-proline than the aneuploid mouse fibroblast, while the synthesis of ^{3}H-hydroxyproline was essentially similar in the two cell lines.

Evidence that serotonin has no effect on collagen metabolism in general was obtained from studies on the distribution of ^{3}H-hydroxyproline in the cell culture system (Table III). The amount of ^{3}H-hydroxyproline in the alcohol-precipitable material of the cell pellet fraction, reflecting collagen aggregation, and in the alcohol-precipitable fraction of the incubation medium, representing newly synthesized collagen plus peptides

TABLE III. Effect of Serotonin on the Distribution of ^{3}H-Hydroxyproline Between the Alcohol-Precipitable Fraction of the Cell Pellets and of the Media of Fibroblast (3T6 and Human) Cultures Shown in Table II.[a]

	^{3}H-Hydroxyproline			
	Cell pellet (%)		Medium (%)	
	Control	Treated	Control	Treated
I	10 ± 2	9 ± 3	90 ± 2	91 ± 3
II	8	12	92	88

[a] Serotonin added while cells were rapidly growing, mean ± SE of 4 experiments of 3 cultures each (I); serotonin added as cell growth slowed, mean of one experiment of 3 cultures (II).

from collagen degradation, was not altered by serotonin nor was the time of cell culture when serotonin was added significant in this regard.

Discussion. Serotonin affects protein synthesis of fibroblasts probably by influencing the synthesis of RNA. The consistently observed effects on either cell growth (DNA) and/or RNA synthesis when cells are growing suggest a modulating effect of the amine on polynucleotide metabolism. In mammalian cells, Warashina (11) reports a stimulation of oxidative phosphorylation by isolated mitochondria from rat liver at a concentration of 10^{-6} *M* serotonin, identical to that used in the studies on fibroblasts. From the literature there are abundant reports associating serotonin with energy metabolism of cells, evidence that was recently summarized by Quay (12). An attractive explanation for a serotonin effect on RNA metabolism might involve the stimulation of adenosine triphosphate (ATP) formation. And augmented cell level of ATP could stimulate nuclear RNA synthesis (13) and the ribonucleoside diphosphate reductase system (14). The depression of RNA synthesis at one stage of cell culture (rapid cell growth) and a stimulation at another stage (slow cell growth) would merely reflect a limited pool size of the ribonucleotides.

Summary. Serotonin when added to mouse (3T6) and human embryo lung fibroblasts in culture at a medium concentration of 10^{-6} *M* affects RNA and collagen syntheses. The effect differs depending upon the stage of cell culture when serotonin is added. During rapid cell growth, serotonin reduces the uptake of ^{3}H-uridine into RNA and ^{3}H-proline into protein, while serotonin added to the cells after the logarithmic growth phase stimulates the synthesis of RNA and protein. In stimulating RNA synthesis, serotonin seems to affect predominantly the species of RNA that are involved in protein synthesis, the 30s and tRNA. At no time did serotonin affect protein or collagen synthesis without a parallel response in RNA synthesis.

The authors are pleased to acknowledge the review of the manuscript and helpful comments of Dr. Antero So.

1. Boucek, R. J., and Alvarez, T. R., Science **167**, 898 (1970).

2. Lembeck, F., Nature (London) **172**, 910 (1953).

3. Thorson, A., Biork, G., Bjormas, G., and Waldenstrom, J., Amer. Heart J. **47**, 795 (1954).

4. Jeffrey, J. J., and Martin, G. R., Biochim. Biophys. Acta **121**, 269 (1966).

5. Schafer, I. A., Silverman, L., Sullivan, J. C., and Robertson, W. V. B., J. Cell Biol. **34**, 83 (1967).

6. Girard, M., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 12A, p. 581. Academic Press, New York (1967).

7. Peacock, A. C., and Dingman, C. W., Biochemistry **6**, 1818 (1967).

8. Priest, R. E., and Davies, L. M., Lab. Invest. **21**, 138 (1969).

9. Robertson, W. Van B., Hewitt, J., and Herman, C., J. Biol. Chem. **234**, 105 (1959).

10. Rhode, S. L., III, and Ellem, K. A. O., Exp. Cell Res. **53**, 184 (1968).

11. Warashina, Y., Hoppe-Seylers Z. Physiol. Chem. **348**, 139 (1967).

12. Quay, W. B., *in* "Advances in Pharmacology" (S. Garattini and P. A. Shore, eds.), Vol. 6, p. 283. Academic Press, New York (1968).

13. McEwen, B. S., Allfrey, V. G., and Mirsky, A. E., Biochim. Biophys. Acta **91**, 23 (1964).

14. Reichard, P., Eur. J. Biochem. **3**, 259 (1968).

Received Feb. 11, 1972. P.S.E.B.M., 1972, Vol. 140.