Incorporation of Heparin in Mammalian Cell Cultures (33947)

H. MONTES DE OCA,[1] AND C. P. DIETRICH
(Introduced by L. B. Jaques)

Department of Anatomy and Department of Physiology and Pharmacology, College of Medicine, University of Saskatchewan, Saskatoon, Canada

The effect of heparin in tissue culture cells has been a subject of much controversy. Inhibitory effect of heparin on mytosis has been reported by several authors (1, 2) while King *et al.* (3) were unable to detect any particular inhibition of this process in L cells. On the other hand these authors (3) reported that a considerable degradation of heparin took place when the compound was incubated with growing L cells.

Heparin is degraded by bacterial enzymes (4) and the products of this degradation have been characterized (5), but its degradation by mammalian systems is not yet completely established (6). A study of different cell lines in cultures in the presence of heparin would help in clarifying some of these apparent contradictions. The present paper describes the effect and fate of heparin in Hela, L, LE, and mouse embryo cells in culture.

Methods. Hela, L, and LE (7) cells were grown in stationary cultures in 199 media+ 20% horse serum (8). Mouse embryo cell cultures were prepared by trypsinization of 15–18-day-old mouse embryos according to the method of Rappaport (9) with slight modifications and cultivated in stationary cultures with Eagle's essential amino acid media (10) plus 10% calf serum (Industrial Biological Laboratories Inc., Rockville, Md.). The fourth generation of these cells were used for the experiments. In all cases, the medium was replaced by a fresh one containing the required amounts of heparin 24 hr after inoculation. Controls were done with fresh medium without heparin. The grown cells were counted in a Coulter counter after mechanical dispersion. In the case of Hela cells a count by hemocytometry was performed due to the excessive number of clumps present. The relative amount of cells was also estimated by the content of RNA fractions in the cells by densitometry after microelectrophoresis (see Fig. 2). All the experiments were performed in duplicate and at least two different times. The variation found was in the range of ±3%.

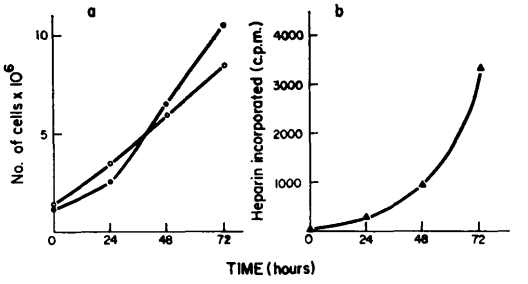

FIG. 1. Rate of incorporation of ^{35}S heparin in L cells: the L cells were inoculated and 24 hr later ^{35}S heparin was added in a fresh medium to a final concentration of 200 μg/ml. After incubation at the times indicated the cells were harvested at 50g for 5 min and washed three times with cold medium containing nonradioactive heparin at the same concentration. Aliquots of the third wash and cells were counted in the liquid scintillation spectrometer. The values obtained for the third wash were subtracted from the values obtained for the cells: (○), no. of cells grown in the absence of heparin; (●), no. of cells grown in the presence of heparin; and (▲), heparin incorporated in cells.

Chemicals. The ^{35}S Heparin was obtained from Calbiochem (Los Angeles, Calif.) with a specific activity ranging from 0.15 to 4.5 mCi/g in different batches. This compound had the same electrophoretic mobility as other commercial heparins and an anticoagulant activity of 160 IU/mg. It was also equally well degraded by *Flavobacterium* heparinases as other heparins (11). Nonlabeled heparin was obtained from Lederle Laboratories, Pearl River, N. Y., through the courtesy of Dr. E. H. Dearborn.

Analytical. The methods for the detection

[1] Present address: American Foundation for Biological Research, Rockville, Maryland.

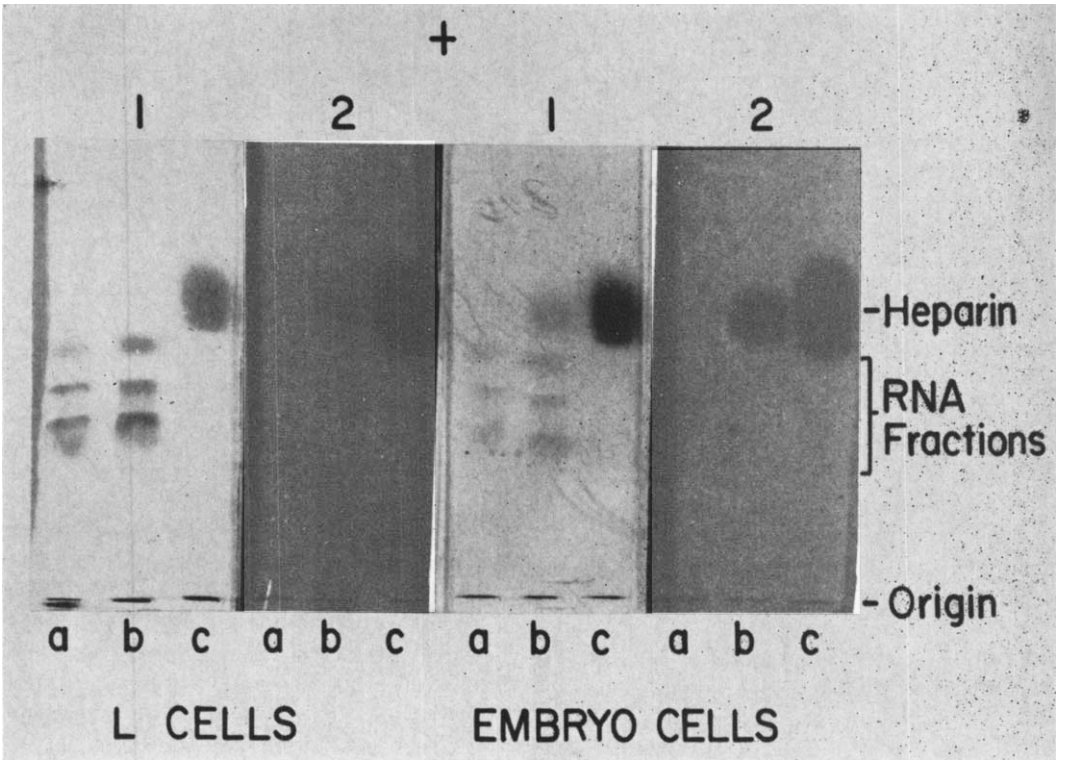

FIG. 2. Microelectrophoresis of extracts of L cells and mouse embryo cells after growth on heparin: L and mouse embryo cells were grown under the same conditions as described in Fig. 1 except that the cells were washed with medium without heparin. Mouse embryo cells (1×10^6) and L cells 2×10^6) were deproteinized with phenol, and the water phase was evaporated to dryness. The residue was resuspended in 10 μl, and 2-μl aliquots were applied to microelectrophoresis slides. After electrophoresis the slides were stained with toluidine blue (1) and radioautographed (2): (a) cells grown in the absence of heparin; (b) cells grown in the presence of heparin; and (c) supernatant medium containing ^{35}S heparin in which cells (b) were grown.

of heparin and/or its degradation products by chromatography, electrophoresis, and microelectrophoresis were those already described (5, 11, 12). For the isolation of heparin and its degradation products, the cells and supernatant media after growth on heparin were deproteinized by phenol (13). For the measurement of the ^{35}S heparin incorporated into the cells the following method was used. The cells after growth in the medium containing heparin were harvested and washed three times with heparin-free medium. The cells were then resuspended in 1 ml of Dulbecco's phosphate buffer solution (14) and an aliquot was mixed with 20 vol of solution of 0.5% (w/v) 2, 5 diphenyloxazole–10% (w/v) naphthalene in dioxan and counted in a Beckman LS-100 liquid scintillation spectrometer. The supernatant of the third wash was also counted by the same procedure and the values obtained subtracted from those of the cells.

Results. Uptake of ^{35}S heparin by 4 cell lines. Figure 1a summarizes the results obtained on the rate of growth of L cells in the presence and absence of radioactive heparin. There seems to be a slight inhibition of the growth in the presence of heparin at early stages of incubation. At later stages a stimulation of growth occurs. This stimulation (about 15%) was reproducible in all the experiments performed. Similar effects were observed with Hela, LE, and mouse embryo cells in the presence of heparin. The uptake

of radioactive heparin in the same experiment with L cells is illustrated in Fig. 1b. There is a definite lag phase in the uptake of this compound by the cells up to 48 hr. After this time a considerable increase of incorporation is observed. This lag phase of incorporation is more pronounced than the lag phase of the cell growth. Incorporation of heparin was observed in all four lines of cells tested but the rate and extent of incorporation differed from one line to the other as shown in Table I. The LE, L, and Hela cells incorporate heparin to a remarkably similar extent. The incorporation of heparin in one million cells is in the order of 1 μg in 72 hr. The embryo cells on the other hand incorporate heparin in much higher amounts. The value obtained in different experiments varied from 13 to 20 μg of heparin/million cells for the same period. These results can be more clearly appreciated by quantitative microelectrophoresis (Fig. 2). Embryo and L cells after incubation with radioactive heparin were extensively washed and then deproteinized with phenol. An aliquot of the water phase was then subjected to microelectrophoresis and the slide stained with toluidine blue and autoradiographed. The amount of the L cells was twice that of the embryo cells, as shown by the content of RNA fractions stained with toluidine blue in the microelectrophoresis slides. Nevertheless the amount of heparin is in much higher concentration in the embryo cells as detected by toluidine blue dye and autoradiography. The Hela and LE cells gave results similar to those of the L cells.

TABLE I. Comparison of Amount of Heparin Incorporated in Four Cell Lines.[a]

Cell line	No. of cells ($\times 10^6$)	Total ^{35}S heparin incorporated (cpm)	Heparin incorporated ($\mu g/\times 10^6$ cells)
LE	4.6	1.400	1.31
L	9.05	2.300	0.85
Hela	2.01	790	1.19
Mouse embryo	1.7	7.400	14.5

[a] LE, L, Hela, and mouse embryo cells were grown in medium containing 200 μg of heparin/ml for 72 hr at 37°.

TABLE II. Release of Heparin by Mouse Embryo Cells.

System	Total heparin content (μg)	Δ (μg)
I. Cells grown in heparin for 48 hr	15.0	
II. Cells of system I further grown for 48 hr in absence of heparin	1.2	—13.8
III. Supernatant of cells from system II	13.6	
IV. Third wash supernatant from the cells of system I	0.1	+13.5

Microscopic analysis of cells incubated with heparin. The different lines of cells were incubated with heparin for different periods of time and stained with toluidine blue. After 24 hr of incubation only very few small metachoromatic granules were observed. The granules grew in size and in number as the incubation proceeded. At 72 hr maximum incorporation of heparin and deposition of the granules was observed. Granules of different sizes were evenly distributed through the cells. In some cells an unusual concentration of granules could be observed in the perinuclear region. The granular formation and the metachromatic stain were undistinguishable from those present in mast cells. It was also possible to observe different phases of mitosis in many cells containing the granules. Controls, grown in the absence of heparin did not display in any instance these metachromatic granules.

Release of heparin from mouse embryo cells previously grown in heparin. (Table II) Mouse embryo cells were grown in ^{35}S heparin (100 μg/ml) with high specific activity (4.5 mCi/g). After 48 hr growth, the cells were washed extensively with fresh medium until almost no radioactivity could be detected in the supernatant. Measurement of an aliquot of the cells indicated that 15 μg of heparin were incorporated per million cells. These cells were then incubated for further 48 hr in medium free of heparin. After incubation the cells were harvested and the supernatant and precipitated cells were counted.

The supernatant contained most of the heparin (13.6 μg/10^6 cells) while the washed cells contained only 1.2 μg of heparin/10^6 cells.

Analysis of the products after incubations The radioactivity incorporated in the four cell lines, the radioactivity released by the cells after preincubation on heparin as well as the supernatants of these experiments were extensively analyzed by chromatography, electrophoresis, and microelectrophoresis. Radioautograms and sensitive color reactions for the probable degradation products were performed on these chromatograms. The only radioactive material present in all these analyses was native heparin. ^{35}S inorganic sulfate or degradation products of heparin similar to those obtained from *Flavobacterium heparinum* enzymes (5, 11) could not be detected. The microelectrophoresis technique did not indicate any desulfated heparin [see Fig. 1, Ref. (12)]. In another group of experiments ^{35}S heparin with high specific activity (4.5 mCi/g) was incubated for 24 hr at 37° with mouse embryo cells and L cells previously grown in the presence and absence of heparin. Also under these conditions no degradation could be observed. The limit of detection of degradation products, by these various methods is in the order of 1% of the total heparin added.

Effect of antibiotics on the incorporation of heparin in L cells. In order to obtain some information on the mechanism by which heparin penetrates the cell and is released afterwards, antibiotics known to act as specific metabolic inhibitors were used. The cells were grown in the absence of heparin as described above. After 24 hr, medium containing ^{35}S heparin (200 μg/ml) and antibiotics (20 μg/ml) were added to the culture. The measurement of ^{35}S heparin and cell count were performed as described in Fig. 1. The results of the action of puromycin, mytomicin, and actinomycin are shown in Table III. All the antibiotics used promoted inhibition of growth of the L cell. Puromycin was found to be the strongest growth inhibitor. Conversely puromycin and actinomycin had a stimulatory effect on the incorporation of heparin by the L cells while mytomicin produced no detectable effect on the incorporation of heparin.

TABLE III. Incorporation of ^{35}S Heparin in L Cells in the Presence of Antibiotics.

Antibiotic added	Total no. of cells ($\times 10^6$)	Heparin incorporated (μg/$\times 10^6$ cells)
None	6.0	0.20
Actinomycin	2.6	0.53
Mitomycin	3.0	0.20
Puromycin	0.8	0.58

Discussion. The results obtained in the present work indicate that there is no inhibition by heparin of the growth of the four cell lines. These results are in agreement with those reported by King *et al.* (3). Nevertheless we were not able to observe even with very sensitive methods any degradation of heparin which was reported by those authors. The method used by King *et al.* was the measurement of the loss of metachromatic activity of heparin when incubated with the L cells. It is quite well known that many metabolites, protein and pH changes interfere with this type of reaction (15). It is probable therefore that the results obtained by King and coworkers arose from such interfering products. An interesting observation derived from the present study is the lag phase in the incorporation of heparin by these cells. It is possible that an active mechanism (and not simple diffusion) participates in this process. The results of the action of actinomycin and puromycin seem to agree with this proposition. The stimulatory effect of these two antibiotics could be due to an impairment of a membrane function in these cells or to inhibition of an actual mechanism for excretion of heparin by the cells. The existence of such a mechanism is suggested by the fact that heparin is released by cells previously grown in this compound.

Summary. The incorporation and fate of heparin in L, LE, Hela, and mouse embryo tissue culture cells was studied. The results were as follows: (a) heparin at the concentrations used had a stimulatory effect of about 15% in the cell growth; (b) incorporation of heparin in L, LE, and Hela cells was

in the order of 1 μg of heparin/million cells and in the mouse embryo cells in the order of 12–20 μg/million cells; (c) analysis by chromatography and electrophoresis of radioactive material incorporated or released after incubation with heparin of the four cell lines revealed that no degradation of the compound occurred; (d) microscopic observations indicated that heparin is deposited in the cells in the form of granules; (e) the incorporation of heparin by the cells was increased by puromycin and actinomycin and was not affected by mitomycin. A possible mechanism by which heparin penetrates and is excreted by the cells is discussed.

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