

# PUTRESCINE TRANSPORT IS GREATLY INCREASED IN HUMAN FIBROBLASTS INITIATED TO PROLIFERATE

PIRKKO POHJANPELTO

From the Department of Virology, University of Helsinki, Helsinki, Finland

## ABSTRACT

Putrescine (diaminobutane) was previously found to stimulate proliferation of human fibroblasts in tissue culture, and a growth factor produced by these cells was identified as putrescine. In the present paper putrescine transport is studied. The rate of putrescine transport was dependent on temperature, and most of the labeled putrescine was retained by the cells after washing with excess unlabeled putrescine. The concentration of radioactivity after a [ $^{14}\text{C}$ ]putrescine pulse was 85 times higher in the cells than in the medium, and over 95% of the radioactivity in the cells was as unchanged putrescine. Butanol treatment removed 70% of the radioactivity from the cells. The calculated  $K_m$  was about the same for rapidly growing and for starved cultures, while  $V_{max}$  was higher for the former than for the latter cultures. Putrescine transport was inhibited to varying degrees by other polyamines, but not by amino acids or divalent cations. Stimulation of cell proliferation by serum was followed by an 18–100-fold increase in the rate of putrescine transport, which was not inhibitable with cyclic AMP, dibutyryl cyclic AMP, or prostaglandin  $\text{E}_1$ . Removal of serum resulted in a rapid decrease in the rate of putrescine transport. Insulin in low serum medium and trypsin in the absence of serum also accelerated putrescine transport. Moreover, the rate of putrescine transport was dependent on cell density. It was faster in sparsely populated than in densely populated cultures. SV40-transformed human fibroblasts responded to addition and removal of serum in the same way as the untransformed parent cell line.

Rapidly growing tissues contain high concentrations of polyamines, and stimulation of cell proliferation in vivo (for further references, see reference 18) or in vitro (8, 10) is followed very early by a rise in the activity of putrescine-synthesizing enzyme, ornithine decarboxylase. Putrescine is able to increase cell proliferation in cultures of human fibroblast (15) and in cultures of Chinese hamster fibroblasts growing in serum-less medium (5). Human fibroblasts, particularly if they are not of embryonic origin, release into the medium a growth factor which has been identified as putrescine (15). In agreement with the earlier findings

demonstrating increased synthesis of putrescine after initiation of cell proliferation, the concentration of putrescine causing maximal stimulation of cell proliferation, and the quantity of putrescine released into the medium, as measured by its growth-promoting activity, were highest for the cultures that contained the largest number of proliferating cells (14).

In the present report putrescine transport is studied. The results show that [ $^{14}\text{C}$ ]putrescine is concentrated in the cells and that most of it cannot be removed by washing with an excess of unlabeled putrescine. Factors affecting cellular growth were

found to have a critical influence on the rate of putrescine transport. Stimulation of cell proliferation by serum was followed by a rapid and extensive rise, and removal of serum by a decline in the rate of putrescine transport. In addition to serum, other growth factors, e.g., insulin in low serum medium and trypsin in the absence of serum, accelerated putrescine transport. Moreover, putrescine was transported more rapidly in nonconfluent, exponentially growing cultures than in densely populated, more stationary cultures. These results suggest that initiation of cell proliferation in human fibroblasts is accompanied by an increased rate of putrescine transport.

## MATERIALS AND METHODS

### Cells

Fibroblasts from human fetal skin, passages 1–24, WI38 from human fetal lung, passages 33–47, and WI38/VA13, the SV40-transformed line, were the cell lines used in the experiments. The two last mentioned cell lines were kindly provided by Dr. A. Vaheri. For the experiments the cells were grown on plastic petri dishes (diam 3.5 cm) in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum, 200 IU penicillin, and 50 µg/ml streptomycin.

### Determination of Transport of Radioactively Labeled Substances

[<sup>14</sup>C]Putrescine was added to half- or nearly confluent cultures to a final concentration of  $10^{-7}$  M, and incubated for 15 min at 37°C. For each determination a pool of three cultures was used. After washing twice with ice-cold Hanks' salt solution, the cells were detached with a mixture of trypsin (0.25%) and ethylenediaminetetraacetic acid (EDTA) (0.02%) and extracted at 0°C with 5% trichloroacetic acid (TCA). The extract was centrifuged, 1 ml of the supernate was mixed with 10 ml of Aquasol, and the radioactivity was determined by liquid scintillation. The precipitate showed no radioactivity above the background. In most experiments the pulse was terminated before washing by adding unlabeled putrescine at room temperature to a final concentration of about  $10^{-3}$  M. The increase in putrescine transport was found to be linear with time for at least 30 min. Fetal calf serum was found to inhibit putrescine transport.

[<sup>3</sup>H]Deoxyglucose and [<sup>3</sup>H]leucine transport were determined in the following way. Cells on the three plates were washed three times with warm Hanks' salt solution without glucose, then 1 ml of [<sup>3</sup>H]leucine ( $6 \times 10^{-8}$  M) or [<sup>3</sup>H]deoxyglucose ( $6 \times 10^{-8}$  M) was added. After 10 min of incubation at 37°C the cells were washed three times with ice-cold Hanks' salt solution, scraped into 0.3 N NaOH, and 0.2 ml of the suspension was mixed with 10

ml of Bray's solution and the radioactivity was determined (6). Transport of [<sup>3</sup>H]uridine (1 µCi/ml) and [<sup>3</sup>H]thymidine (1 µCi/ml) was determined in the same way, except that the labeled compound was added directly to the growth medium.

### Determination of DNA Synthesis

Cells were exposed to [<sup>3</sup>H]thymidine (2 µCi/ml) for 1 h, washed three times, and detached with a mixture of trypsin and EDTA. Cells from three cultures were pooled and ice-cold TCA was added to a final concentration of 10%. After 10 min of incubation in ice water the mixture was centrifuged and the precipitate dissolved in 0.5 ml of 0.3 N NaOH. The precipitation and dissolving was repeated once more. Finally, 0.2 ml of the solution was mixed with 10 ml of Aquasol and the radioactivity was determined by liquid scintillation.

### Butanol Treatment

Triplicate cultures were given a 15-min pulse of [<sup>14</sup>C]putrescine ( $10^{-7}$  M), chased with unlabeled putrescine, and detached with a mixture of trypsin and EDTA. After centrifugation the supernate was carefully removed and the cells were incubated with 3 ml of 100% butanol-1 for 15 min at room temperature. The cells were then centrifuged again, the pellet was extracted with 5% TCA at 0°C, and the radioactivity in the supernate was determined.

### Determination of Putrescine

After a [<sup>14</sup>C]putrescine pulse the cells were washed three times with Hanks' salt solution, detached with a mixture of trypsin and EDTA, centrifuged, and extracted with 5% TCA at 0°C. The supernate was then applied to paper chromatography (16), and the position of the radioactivity was compared with that of authentic putrescine. The paper chromatography was kindly performed by Dr. E. Hölttä.

### Determination of the Properties of the Transformed Cell Line

The presence of SV40 T antigen was demonstrated by the indirect immunofluorescence test, which was kindly performed by Dr. S. Stenman. The growth of the cells in soft agar was studied according to the method of Macpherson (12).

### Radiochemicals

[1,4-<sup>14</sup>C]putrescine dihydrochloride (20 mCi/mmol) was obtained from New England Nuclear Chemicals, GmbH, Frankfurt-am-Main, Germany. D-[2-deoxy-1-<sup>3</sup>H]glucose (8 Ci/mmol), [5-<sup>3</sup>H]uridine (25 Ci/mmol), [methyl-<sup>3</sup>H]thymidine (50 Ci/mmol), and L-[4,5-<sup>3</sup>H]leucine (400 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England.

## Chemicals

Putrescine (1,4-diaminobutane-dihydrochloride), *purum*, L-ornithine monohydrochloride, *purissimum*, L-arginine, *purissimum*, L-lysine, *purum*, L-leucine, *purissimum*, L-asparagine monohydrate, *purissimum*, and L-serine, *purissimum*, were purchased from Fluka AG Buchs SG, Switzerland. L-Tyrosine was obtained from S. A. F. Hoffman-La Roche & Co., Basel, Switzerland. Trypsin (pancreas protease) 8 m Anson-E/mg was obtained from Merck, Darmstadt, Germany, and pork insulin, three-times crystallized, 25.8 IU/mg from Novo Industri AS, Copenhagen, Denmark.

## RESULTS

### General Characteristics of Putrescine Transport

When exponentially growing cultures were given a 15-min pulse of [ $^{14}\text{C}$ ]putrescine and then washed three times with Hanks' salt solution, the cells retained 6% of the total radioactivity. Of this, only 15% was removed by washing at room temperature with a 10,000-fold excess of unlabeled putrescine. When the volume of the cells was calculated by using the cell counter, the concentration of radioactivity was found to be 85 times higher in the cells than in the medium. More than 95% of the label was in unchanged, 1,4-diaminobutane as indicated by paper chromatography of cold TCA extracts of the cells. After 30 min of exposure to [ $^{14}\text{C}$ ]putrescine the corresponding percentage was over 90%.

Butanol has sometimes been used to disrupt permeability barriers in bacteria without causing complete lysis of the cells (for additional references see reference 20). Tabor and Tabor took advantage of the fact and demonstrated that most of the polyamines taken up by *Escherichia coli* were released from the bacteria during butanol treatment (20). They concluded that if butanol affected only the permeability barriers the concentration gradient obtained by polyamines was a true one and not just an expression of the binding to intracellular polyanions. Because human fibroblasts grown on a solid surface retained their morphology after 20 min of incubation with 100% butanol, it was considered feasible to try butanol treatment on these cells. A [ $^{14}\text{C}$ ]putrescine pulse was given to the cultures. The cells were then detached from the plates and incubated with 100% butanol-1 for 15 min. The cells thus treated contained 70% less radioactivity than sister cultures not incubated with butanol.

The rate of putrescine transport was dependent

on temperature. About 30 times more putrescine was transported at 37°C than at 5°C. If the cells were killed by heating for 60 min at 65°C, or by incubating in distilled water for 60 min, the remnants of the cells contained only 1.2% and 2.2%, respectively, of the radioactivity present in the nontreated control cultures.

### Rate of Putrescine Transport at Different Putrescine Concentrations

Fig. 1 shows the rate of putrescine transport in exponentially growing, nonsynchronized cultures at different putrescine concentrations. A Lineweaver-Burk plot of the data gave a straight line ( $r = 0.9988$ ). Calculation of the  $K_m$  from 18 experiments gave a mean value of  $1.1 \times 10^{-6}$  M. The  $K_m$  values for exponentially growing cultures and for cultures starved of serum overnight were approximately the same, but maximal velocities differed. For the former cultures it was  $2.3 \times$

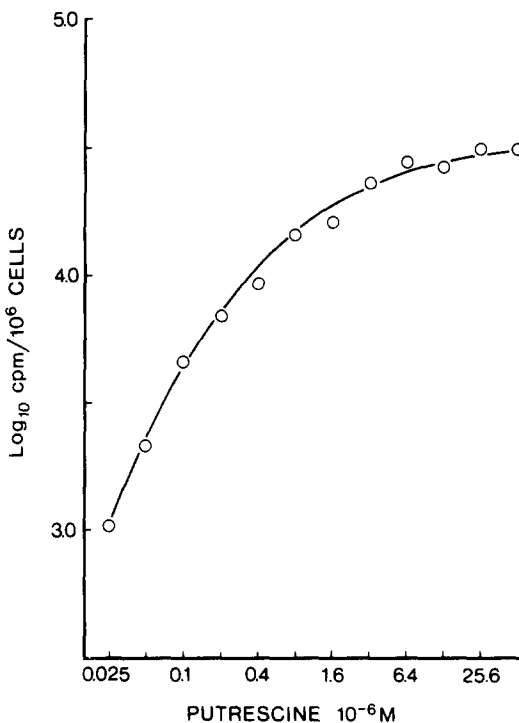


FIGURE 1 Rate of putrescine transport at different concentrations of putrescine. 15-min pulses of [ $^{14}\text{C}$ ]putrescine at different concentrations were given to cultures containing  $1.5 \times 10^4$  cells/cm<sup>2</sup>, and the radioactivity in the TCA-soluble fraction of the cells was determined.

TABLE I  
Effect of Polyamines, Amino Acids, and Divalent Cations on the Rate of Putrescine Transport

| Compound added to the medium  | cp 10 min<br>× 10 <sup>2</sup> | Inhibition<br>% |
|---|--------------------------------|-----------------|
| <b>A</b>  |                                |                 |
| No additions  | 96                             | —               |
| Cadaverine C <sub>4</sub> H <sub>14</sub> N <sub>2</sub> 10 <sup>-6</sup> M               | 14                             | 86              |
| 10 <sup>-6</sup> M  | 43                             | 55              |
| Spermidine C <sub>7</sub> H <sub>18</sub> N <sub>4</sub> 10 <sup>-6</sup> M               | <1                             | >99             |
| 10 <sup>-6</sup> M  | 2                              | 98              |
| 10 <sup>-7</sup> M  | 13                             | 86              |
| Spermine C <sub>10</sub> H <sub>24</sub> N <sub>4</sub> 10 <sup>-6</sup> M                | <1                             | >99             |
| 10 <sup>-6</sup> M  | <1                             | >99             |
| 10 <sup>-7</sup> M  | 5                              | 95              |
| Ornithine C <sub>5</sub> H <sub>12</sub> O <sub>2</sub> N <sub>2</sub> 10 <sup>-6</sup> M | 121                            | —               |
| Arginine C <sub>6</sub> H <sub>14</sub> O <sub>2</sub> N <sub>4</sub> 10 <sup>-6</sup> M  | 76                             | 21              |
| Lysine C <sub>6</sub> H <sub>12</sub> O <sub>2</sub> N <sub>2</sub> 10 <sup>-6</sup> M    | 106                            | —               |
| Leucine C <sub>6</sub> H <sub>12</sub> O <sub>2</sub> N 10 <sup>-6</sup> M                | 98                             | —               |
| Serine C <sub>3</sub> H <sub>7</sub> O <sub>2</sub> N 10 <sup>-6</sup> M                  | 77                             | 20              |
| Asparagine C <sub>4</sub> H <sub>11</sub> O <sub>2</sub> N 10 <sup>-6</sup> M             | 76                             | 21              |
| Tyrosine C <sub>9</sub> H <sub>9</sub> O <sub>3</sub> N 10 <sup>-6</sup> M                | 98                             | —               |
| <b>B</b>  |                                |                 |
| CaCl <sub>2</sub> 20 mg/l   | 11                             | —               |
| CaCl <sub>2</sub> 200 mg/l  | 14                             | —               |
| CaCl <sub>2</sub> 800 mg/l  | 89                             | —               |
| MgSO <sub>4</sub> 20 mg/l   | 15                             | —               |
| MgSO <sub>4</sub> 200 mg/l  | 19                             | —               |
| MgSO <sub>4</sub> 800 mg/l  | 121                            | —               |
| CaCl <sub>2</sub> 20 mg/l + MgSO <sub>4</sub> 20 mg/l                                     | 21                             | —               |
| CaCl <sub>2</sub> 200 mg/l + MgSO <sub>4</sub> 200 mg/l*                                  | 98                             | —               |
| CaCl <sub>2</sub> 800 mg/l + MgSO <sub>4</sub> 800 mg/l                                   | 124                            | —               |

Cells were incubated overnight in fresh medium containing 10% serum. After rinsing once in serum-less medium, either ordinary MEM (A) or MEM without Ca<sup>++</sup> and Mg<sup>++</sup> (B) was added together with the compound to be tested. Then 15-min pulses of [<sup>14</sup>C]putrescine (10<sup>-7</sup> M) were given and the radioactivity in the TCA-soluble fraction of the cells was determined.

\* Corresponds to the ordinary MEM.

10<sup>-7</sup> mmol/15 min/10<sup>6</sup> cells, and for the latter 2.6 × 10<sup>-8</sup> mmol/15 min/10<sup>6</sup> cells.

### Effect of Polyamines and Divalent Cations on the Rate of Putrescine Transport

**POLYAMINES:** Cadaverine, spermidine, and spermine inhibited putrescine transport in varying degrees, as Table I illustrates. Because calf serum contains polyaminoxidase, the [<sup>14</sup>C]putrescine pulses in this experiment were given in serum-less medium. At the same molar concentration as putrescine, spermine inhibited putrescine transport by 95% and spermidine inhibited it by 86%, in 10-fold excess, the corresponding values were over 99% and 98%. Cadaverine was less effective. In 100-fold excess, it reduced putrescine transport by

86%. Ornithine, a precursor of putrescine, had no inhibitory effect in 100-fold excess, nor did 100-fold excess of other amino acids (arginine, lysine, leucine, asparagine, serine, and tyrosine) significantly inhibit putrescine transport.

Because putrescine often behaves like divalent cations, the effect of different concentrations of Ca<sup>++</sup> and Mg<sup>++</sup> on putrescine transport was tested. Table I shows that reducing the concentration of these cations resulted in a fall, and increasing the concentration resulted in a rise in the rate of putrescine transport. This experiment indicates that divalent cations do not compete with putrescine transport.

### Effect of Stimulation of Cell Proliferation on the Rate of Putrescine Transport

**SERUM:** The addition of serum to cultures starved of serum overnight resulted in a rapid and striking increase in the rate of putrescine transport. Fig. 2 shows a representative experiment. In 30 min, putrescine transport had increased fivefold

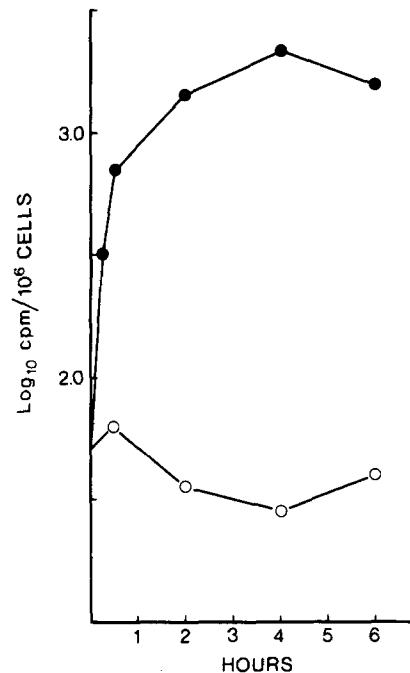


FIGURE 2 Effect of serum on the rate of putrescine transport. Cells were rinsed twice and incubated without serum for 18 h. Then 10% serum was added to half of the cultures, 15-min pulses of [<sup>14</sup>C]putrescine (10<sup>-7</sup> M) were given at different time intervals, and the radioactivity in the TCA-soluble fraction of the cells was determined. O, 0% serum. ●, 10% serum.

and in 4 h, 40-fold. In 10 repeated experiments the maximal increase in the rate of putrescine transport varied between 18- and 100-fold. In order to determine how early the rate of putrescine transport started to rise, [ $^{14}\text{C}$ ]putrescine and serum were given simultaneously and an excess of unlabeled putrescine was added 15 min later. During the first 15 min, the rate of putrescine transport increased two- to four-fold.

If cells were transferred from medium containing 10% serum to a serum-less one, the rate of putrescine transport declined rapidly. Fig. 3 shows a representative experiment. In 1 h the rate of putrescine transport had dropped to one-half and in 4 h to one-fortieth of the original. In 12 repeated experiments, the fall in the rate of putrescine

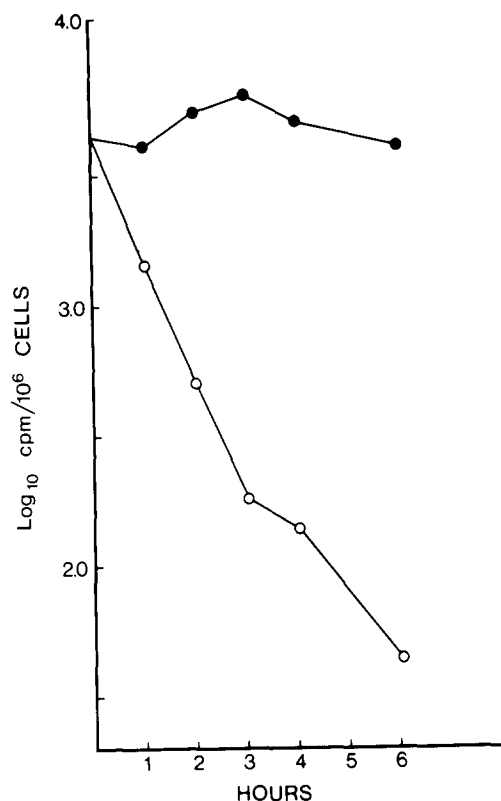


FIGURE 3 Effect of removal of serum on the rate of putrescine transport. Cells were incubated overnight in fresh medium containing 10% serum; half of them were then rinsed twice with MEM at 37°C and incubated further without serum. At different time intervals, 15-min pulses of [ $^{14}\text{C}$ ]putrescine ( $10^{-7}$  M) were given and the radioactivity in the TCA-soluble fraction was determined. O, 0% serum. ●, 10% serum.

transport in 6 h varied from one-eighth to one-hundredth.

For comparison, the effect of serum on the rate of transport of uridine, deoxyglucose, leucine, and thymidine was determined. Fig. 4 demonstrates that in human fibroblasts the addition of serum resulted in a smaller increase in the rate of transport of these substances than in the rate of putrescine transport.

**INSULIN:** Insulin, which acts as a growth factor in low serum medium (19), raised the rate of putrescine transport, but only about 2.5-fold, as Fig. 5 illustrates.

**CELL DENSITY:** Fig. 6 shows that in semiconfluent, exponentially growing cultures ( $4 \times 10^4$  cells/cm<sup>2</sup>) the rate of putrescine transport per  $10^6$  cells was about 15 times higher than in densely populated, more stationary cultures ( $8 \times 10^5$  cells/cm<sup>2</sup>).

**TRYPSIN:** Trypsin is known to stimulate cell proliferation in mouse (2) and chicken fibroblasts (17). This was found to be true also with human fibroblasts. In an attempt to rule out the effect of serum the experiments were carried out in serum-less medium. Before adding trypsin, the cultures, seeded 3 days earlier, were rinsed three times and starved of serum for 12 h. Table II demonstrates that trypsin stimulated DNA synthesis and cell division in serum-less medium, as measured by incorporation of [ $^3\text{H}$ ]thymidine into the acid-insoluble fraction and by counting the percentage of mitoses. After 24 h of incubation with 0.3  $\mu\text{g}/\text{ml}$  of trypsin, DNA synthesis had increased 15-fold as compared to the control, and after 36 h of incubation the number of mitoses had increased about 10-fold. Fig. 7 demonstrates that trypsin also increased the rate of putrescine transport. In the presence of 0.3  $\mu\text{g}/\text{ml}$  trypsin, the rate of putrescine transport had increased ninefold in 12 h.

#### *Effect of cAMP, Dibutyryl cAMP, and Prostaglandin E<sub>1</sub> on the Rate of Putrescine Transport*

Cyclic AMP has been found to inhibit uridine transport in mouse fibroblasts (11). To test whether this was true also with putrescine transport in human fibroblasts, cAMP, dibutyryl cAMP and prostaglandin E<sub>1</sub> were added separately to cultures together with serum and the rate of putrescine transport was tested 4 h later. Table III shows that these drugs did not inhibit the

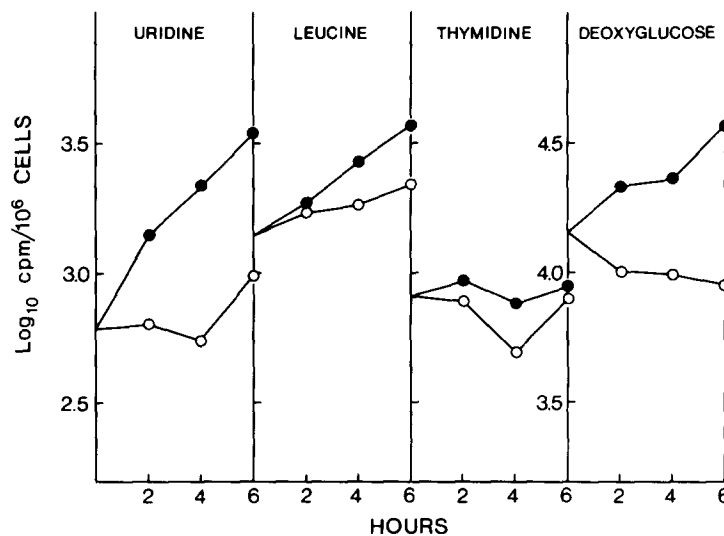


FIGURE 4 Effect of serum on the rate of transport of deoxyglucose, leucine, uridine, and thymidine. Cells were incubated without serum for 18 h. Then 10% serum was added to half of the cultures, and 10-min pulses of [<sup>3</sup>H]deoxyglucose ( $6 \times 10^{-8}$  M), [<sup>3</sup>H]leucine ( $6 \times 10^{-8}$  M), [<sup>3</sup>H]uridine (1  $\mu$ Ci/ml), and [<sup>3</sup>H]thymidine (1  $\mu$ Ci/ml) were given at different time intervals and the radioactivity in the TCA-soluble fraction of the cells was determined. O, 0% serum. ●, 10% serum.

serum-induced acceleration of putrescine transport.

#### *Rate of Putrescine Transport in Normal and Transformed Human Fibroblasts*

The two cell lines that were compared were human fetal lung fibroblasts WI38 and the same cell line transformed by SV40, designated as WI38/VA13. The latter cell line behaves like a transformed cell line in that it has an altered morphology, is able to grow on soft agar, and shows three times faster deoxyglucose transport than the parent cell line. In addition the transformed cells contained the SV40 T antigen.

When the two cell lines were grown under similar conditions, there was little difference in the rate of putrescine transport per  $10^6$  cells. Nor did the rates at which putrescine transport was altered after addition or removal of serum differ greatly. The transformed cell line, however, had a tendency to respond a little more rapidly to these changes than did the parent cell line.

### DISCUSSION

#### *Nature of Transport*

Cells exposed to [<sup>14</sup>C]putrescine retained 85% of the radioactivity after washing with a 10,000-fold

excess of unlabeled putrescine. This indicates that there was no extensive exchange between putrescine molecules in the cell and outside the cell, and that therefore a true uptake of [<sup>14</sup>C]putrescine had taken place. However, during prolonged incubation at 37°C some radioactivity was lost in the medium. If cells were washed after [<sup>14</sup>C]putrescine pulse and further incubated for 24 h, about one-third of the radioactivity originally present in the cells was recovered in the medium (unpublished results).

In rapidly growing cultures the concentration of radioactivity after a [<sup>14</sup>C]putrescine pulse was about 85 times higher in the cells than in the medium, and over 95% of this radioactivity was as unchanged putrescine. It is not known whether a true concentration gradient across the membrane was present because some of the [<sup>14</sup>C]putrescine may exist as a complex with polyacids, and it is not possible to measure the free ionized putrescine in the cell. However, the fact that butanol treatment removed 70% of the [<sup>14</sup>C]putrescine from the cells but did not affect the morphology of human fibroblasts grown on a solid surface is compatible with the idea that butanol treatment may disrupt only the permeability barriers in the cells. If this is true, [<sup>14</sup>C]putrescine released from the cells during butanol treatment should represent the free ionized

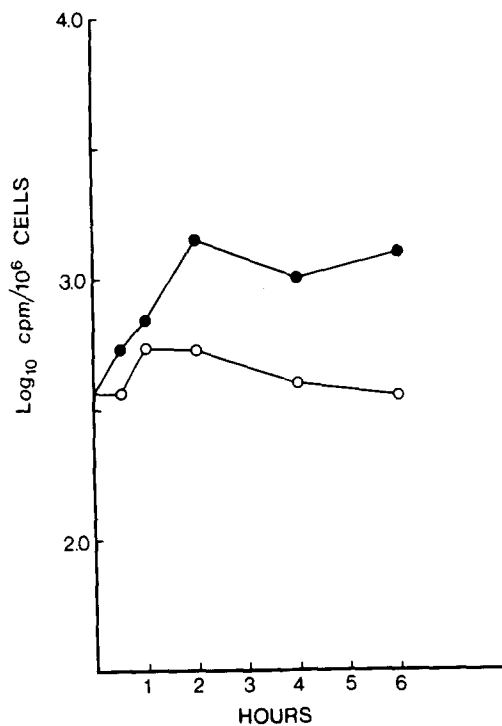


FIGURE 5 Effect of insulin on the rate of putrescine transport. Growth medium containing 10% serum was replaced with serum-less medium without rinsing the cells, and incubated for 18 h. Then insulin was added to half of the cultures, 15-min pulses of [<sup>14</sup>C]putrescine ( $10^{-7}$  M) were given at different intervals, and the radioactivity in the TCA-soluble fraction of the cells was determined. O, control. ●, 10  $\mu$ g/ml insulin.

putrescine, and a true concentration gradient across the membrane would be present.

#### *K<sub>m</sub> and V<sub>max</sub>*

When the rate of putrescine transport was determined at different putrescine concentrations, a Lineweaver-Burk plot of the data gave a straight line. The calculated  $K_m$  was approximately the same for exponentially growing and for starved cultures. The apparent  $V_{max}$  was, however, higher for the former than for the latter cultures. The results are compatible with one transport mechanism for putrescine in human fibroblasts.

#### *Inhibition of Putrescine Transport*

Ornithine, arginine, and other amino acids did not significantly inhibit putrescine transport, nor did divalent cations have an inhibitory effect. This lack of competition shows that putrescine is not

transported by the same mechanism as amino acids or divalent cations. The polyamines cadaverine, spermidine, and spermine inhibited putrescine transport, and this inhibition was more effective as their molecular weights increased. It remains to be elucidated whether inhibition of putrescine transport by other polyamines is due to competition, to some unspecific effect on the cell membrane, or possibly to feedback inhibition, bearing in mind that putrescine is a precursor of spermidine and spermine.

#### *Effect of cAMP*

Cyclic AMP, dibutyryl cAMP, and prostaglandin E<sub>1</sub> did not retard the serum-induced acceleration of putrescine transport, although human fibroblasts, like mouse fibroblasts (3) but unlike chicken fibroblasts (9), have been reported to respond to the administration of cAMP by inhibition of DNA synthesis (4). In this respect, putres-

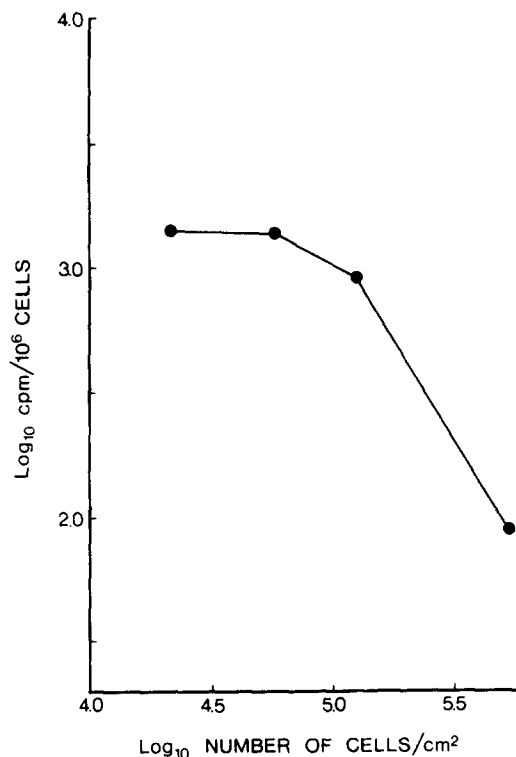


FIGURE 6 Effect of cell density on the rate of putrescine transport. Cultures of different cell densities were incubated overnight with fresh medium containing 10% serum. Then 15-min pulses of [<sup>14</sup>C]putrescine ( $10^{-7}$  M) were given and the radioactivity in the TCA-soluble fraction of the cells was determined.

TABLE II  
Effect of Trypsin on DNA Synthesis and on Cell Division in Serum-less Medium

| Concentration of trypsin | DNA synthesis                               |          | Mitoses Mit/no cells |      |
|--------------------------|---|----------|----------------------|------|
|                          | cpm/10 <sup>6</sup> cells × 10 <sup>2</sup> | increase |                      |      |
| μg/ml                    |   | %        |                      | %    |
| —                        | 22  |          | 2/11,224             | 0.02 |
| 0.3                      | 351   | 1495     | 23/11,819            | 0.19 |
| 0.1                      | 197   | 795      | 14/13,356            | 0.10 |
| 0.03                     | 133   | 509      |                      |      |

Cells were grown for 3 days in medium containing 10% serum. They were then rinsed three times, incubated for 12 h in serumless medium, and different concentrations of trypsin were added. After further incubation for 24 h, the cells were exposed to [<sup>3</sup>H]thymidine (2 μCi/ml) for 1 h and the radioactivity in the acid-insoluble material was determined. The percentage of mitoses was counted under the microscope after a 36-h incubation with trypsin.

cine transport resembles phosphate transport which seems to be independent of the intracellular concentration of cAMP (1), and differs from uridine transport which in mouse fibroblasts is inhibited by cAMP (11).

#### Effect of Factors Affecting Cellular Growth

Stimulation of cell proliferation by the addition of serum was followed by an early and extensive rise in the rate of putrescine transport. Subsequent to the removal of serum, the rate of putrescine transport decreased rapidly. This type of response to the addition and removal of serum was not confined to human fibroblasts but was exhibited also by chicken fibroblasts (Pohjanpelto, unpublished results).

Because transformed mouse fibroblasts showed a slower retardation of uridine transport after removal of serum than did their untransformed parent cells (7), the rates of putrescine transport in SV40-transformed human fibroblasts and their untransformed parent cells were compared. No significant difference in the rate of putrescine transport between these two cell lines was observed after the addition or the removal of serum. There is, however, preliminary evidence that chicken fibroblasts transformed by Rous sarcoma virus may behave differently. The rate of putrescine transport decreased after the removal of serum more slowly and to a lesser degree in Rous sarcoma virus-transformed than in normal chicken fibroblasts (Pohjanpelto and Vaheri, unpublished results).

Insulin, which acts as a growth factor in low

serum medium (19), increased also the rate of putrescine transport, and trypsin, known to stimulate DNA synthesis and cell proliferation in mouse and chicken fibroblasts (2, 17), was found to increase both cell proliferation and the rate of

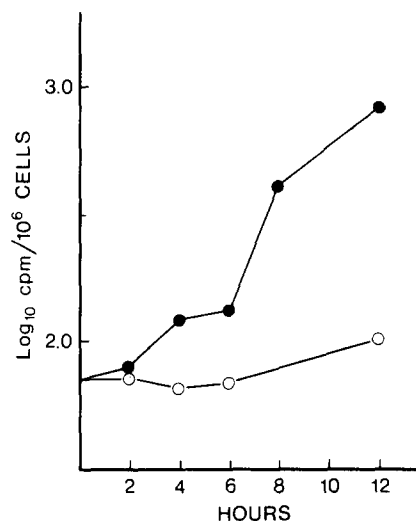


FIGURE 7 Effect of trypsin on the rate of putrescine transport. Cultures grown for 3 days in medium containing 10% serum were rinsed three times and incubated for 12 h in serum-less medium. Trypsin was then added, 15-min pulses of [<sup>14</sup>C]putrescine (10<sup>-7</sup> M) were given at different time intervals, and the radioactivity in the TCA-soluble fraction of the cells was determined. O, control. ●, trypsin 0.3 μg/ml.

TABLE III  
Effect of cAMP, Dibutyryl cAMP, and Prostaglandin E<sub>1</sub> on the Rate of Putrescine Transport

| Compound added to the medium         | cpm/10 <sup>6</sup> cells × 10 <sup>2</sup> |
|--------------------------------------|---|
| No additions                         | 23  |
| cAMP 3.14 mmol                       | 24  |
| cAMP 1.00 mmol                       | 27  |
| cAMP 0.31 mmol                       | 25  |
| Db-cAMP 2.00 mmol                    | 23  |
| Db-cAMP 1.00 mmol                    | 28  |
| Db-cAMP 0.50 mmol                    | 25  |
| Db-cAMP 0.25 mmol                    | 21  |
| Prostaglandin E <sub>1</sub> 5 μg/ml | 19  |
| Control without serum                | 1   |

Cells were starved overnight without serum. Then serum was added to a final concentration of 10% together with the compound to be tested. After 4 h of incubation at 37°C, 15 min-pulses of [<sup>14</sup>C]putrescine (10<sup>-7</sup> M) were given and the radioactivity in the TCA-soluble fraction of the cells was determined.



putrescine transport in human fibroblasts, even if serum was omitted. Moreover, sparsely populated, exponentially growing cultures had a higher rate of putrescine transport than densely populated, more stationary cultures. Because trypsin was able to accelerate putrescine transport in the absence of serum and since there are indications that the serum-dependent and the density-dependent regulation of cell proliferation may, at least in mouse fibroblasts, operate by different mechanisms (13), it is concluded that an increase in the rate of putrescine transport is not determined merely by serum, or by insulin-like factors in the serum, but may generally accompany initiation of cell proliferation.

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## REFERENCES

- ASUA, L. J., E. ROZENGURT, and R. DULBECCO. 1974. Kinetics of early changes in phosphate and uridine transport and cyclic AMP levels stimulated by serum in density-inhibited 3T3 cells. *Proc. Natl. Acad. Sci. U. S. A.* **71**:96-98.
- BURGER, M. M. 1970. Proteolytic enzymes initiating cell division and escape from contact inhibition of growth. *Nature (Lond.)* **227**:170-171.
- BURGER, M. M., B. M. BOMBIK, B. BRECKENRIDGE, and J. R. SHEPPARD. 1972. Growth control and cyclic alterations of cyclic AMP in the cell cycle. *Nat. New Biol.* **239**:161-163.
- FROELICH, J. E., and M. RACHMELER. 1972. Effect of adenosine 3'-5'-cyclic monophosphate on cell proliferation. *J. Cell Biol.* **55**:19-31.
- HAM, R. G. 1964. Putrescine and related amines as growth factors for a mammalian cell line. *Biochem. Biophys. Res. Commun.* **14**:34-38.
- HATANAKA, M., C. AUGL, and R. V. GILDEN. 1970. Evidence for a functional change in the plasma membrane of murine sarcoma virus-infected mouse embryo cells. *J. Biol. Chem.* **245**:714-717.
- HERSHKO, A., P. MAMONT, R. SHIELDS, and G. TOMKINS. 1971. Pleiotypic response. *Nat. New Biol.* **232**:206-211.
- HOGAN, B. L. M. 1971. Effect of growth conditions on the ornithine decarboxylase activity of rat hepatoma cells. *Biochim. Biophys. Res. Commun.* **45**:301-307.
- HOVI, T., and A. VAHERI. 1973. Cyclic AMP and cyclic GMP enhance growth of chick embryo fibroblasts. *Nat. New Biol.* **245**:174-177.
- KAY, J. E., and V. J. LINDSAY. 1973. Polyamine synthesis during lymphocyte activation. *Exp. Cell Res.* **77**:428-436.
- KRAM, R., P. MAMONT, and G. M. TOMKINS. 1973. Pleiotypic control by adenosine 3'-5'-cyclic monophosphate: A model for growth control in animal cells. *Proc. Natl. Acad. Sci. U. S. A.* **70**:1432-1436.
- MACPHERSON, I. 1969. Agar suspension culture for quantitation of transformed cells. In *Fundamental Techniques in Virology*. K. Habel and N. P. Salzman, editors. Academic Press Inc., New York. 214-219.
- OEY, J., A. VOGEL, and R. POLLACK. 1974. Intracellular cyclic AMP concentration responds specifically to growth regulation by serum. *Proc. Natl. Acad. Sci. U. S. A.* **71**:694-698.
- POHJANPELTO, P. 1973. Relationship between putrescine and the proliferation of human fibroblasts in vitro. *Exp. Cell Res.* **80**:137-142.
- POHJANPELTO, P., and A. RAINA. 1972. Identification of a growth factor produced by human fibroblasts in vitro as putrescine. *Nat. New Biol.* **235**:247-249.
- RAINA, A., and S. S. COHEN. 1966. Polyamines and RNA synthesis in polyauxotrophic strain of *E. coli*. *Proc. Natl. Acad. Sci. U. S. A.* **55**:1587-1593.
- SEFTON, B. M., and H. RUBIN. 1970. Release from density dependent growth inhibition by proteolytic enzymes. *Nature (Lond.)* **227**:843-845.
- TABOR, H., and C. WHITE TABOR. 1972. Biosynthesis and metabolism of 1,4-diaminobutane, spermidine, spermine and related amines. *Adv. Enzymol.* **36**:203-268.
- TEMIN, H. 1967. Studies on carcinogenesis by avian sarcoma viruses LV. Differential multiplication of uninfected and of converted cells in response to insulin. *J. Cell. Physiol.* **69**:337-384.
- WHITE TABOR, C., and H. TABOR. 1966. Transport systems for 1,4-diaminobutane, spermidine and spermine in *E. coli*. *J. Biol. Chem.* **241**:3714-3723.