Polyamine Transport and Metabolism in Mouse Mammary Gland

GENERAL PROPERTIES AND HORMONAL REGULATION

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Mouse mammary gland has been shown to possess a transport system for spermidine, spermine, and putrescine. The uptake system for spermidine, as studied in detail on mammary explants in culture is a time-dependent, energy-requiring process which can be stimulated by insulin and prolactin. The stimulatory effect of insulin involves both enhancement of $V_{\text{max}}$ for spermidine influx and prevention of efflux of the polyamine, whereas prolactin, in the presence of insulin, elicits a greater increase in $V_{\text{max}}$ for spermidine. Studies are also reported on the effects of temperature, concentration, and various inhibitors on this system. The accumulated spermidine exists virtually in an unchanged form with little metabolic conversion to either spermine or putrescine or to its conjugated form. In contrast, spermine and putrescine, both of which are also taken up by mammary explants, undergo metabolic conversion to spermidine.

The polyamines spermidine, spermine, and putrescine occur widely among mammalian cells, and in high concentrations during cell growth and development (1-5). In recent years a body of data has been accumulated that implies a fundamental role for the polyamines in various cellular processes (1-5).

In mammary epithelium, the concentration of spermidine increases markedly during lactation (6). A similar increase has been found during the hormonal induction of lactogenesis in vitro, which is effected by cultivation of mouse mammary explants with insulin, glucocorticoid, and prolactin (7, 8). Recent studies on the role of spermidine in this in vitro system (7-10) have indicated that the polyamine may serve a vital function in milk protein synthesis. This view is supported by the observation (8) that addition of spermidine together with insulin and prolactin elicits a marked stimulation of milk protein synthesis. In the present studies, we have extended our previous observation (8) by studying the uptake and metabolism of exogenous spermidine in mammary cells. The data demonstrate the existence of a transport system for spermidine in mammary cells. Some features of this transport system and subsequent metabolism of the polyamines are described.

EXPERIMENTAL PROCEDURES

Materials

Materials were purchased as follows: [3-3H]spermidine trihydrochloride (specific activity 423 mCi/mmol), [3H]spermine tetrahydrochloride (specific activity 1070 mCi/mmol), [2,3-3H]putrescine (specific activity 125 mCi/mmol), and [1,4-14C]putrescine (specific activity 19.5 mCi/mmol) from New England Nuclear; all unlabeled polyamines except cadaverine from Sigma Chemical Co.; cadaverine, actinomycin D, cycloheximide, and crystalline bovine serum albumin from Calbiochem; collagenase (type I) from Worthington; dinitrophenol from Eastman Kodak; NCS tissue solubilizer from Amer sham/Searle; Medium 199 from Grand Island Biological Co.; hydrocortisone from ICN Pharmaceuticals, Inc. Crystalline pork zinc insulin was a gift from Eli Lilly. Bovine prolactin was obtained from the National Institutes of Health.

Each radioactive polyamine was routinely checked for purity and whenever necessary was purified by high voltage electrophoresis prior to use.

Methods

Organ Culture The abdominal mammary glands of C57 H/HeN mice in the middle of their first pregnancy were used throughout the experiments. Tissue explants, each weighing about 1 mg, were cultured in Medium 199 containing penicillin G (50 μg/ml) as described previously (11) with addition of appropriate hormones and other chemicals. Each hormone was used at a concentration of 5 μg/ml of medium. All procedures were done under sterile conditions.

Isolation of Epithelial Cell Fraction—Mammary epithelial cells were prepared by treating the tissue explants with collagenase as described previously (11). Uptake of Radioactive Polyamines—Tissue explants in Medium 199 were incubated with labeled polyamines as indicated in the appropriate experiments. At various times thereafter, explants were analyzed for radioactivity in the following manner. Explants were weighed and placed for washing on a Whatman GF/C disc filter paper which had been immersed for at least 15 min in Medium 199 containing 40 mM corresponding unlabeled polyamine to prevent absorption of the radioactive polyamines. Explants were washed on a Millipore filter apparatus with 15 ml of Medium 199 and 5 ml of phosphate-buffered...
0.15 M NaCl (pH 7.2) using suction. Each washing solution also contained the appropriate unlabeled polyamine at 40 mM. This washing procedure removed approximately 10% of the radioactivity associated with explants which presumably represented nonspecific adsorption of the polyamines to the cells. Further washing did not remove any more radioactivity from the explants. The washed explants were transferred into a scintillation vial, digested with 1 ml of NCS tissue solubilizer, and assayed for the radioactivity in a toluene-based scintillation fluid with a liquid scintillation spectrometer.

High Voltage Electrophoresis—Quantitative as well as qualitative analyses of the radioactive polyamines and possible metabolites in the cells were made by high voltage electrophoresis as described previously (8). In brief, explants were washed as described above, homogenized in 2 ml of 0.01 N HCl, and then 2 ml of 10% trichloroacetic acid were added. After centrifugation of the homogenate, the supernatant was extracted with alkaline butanol and processed further to prepare a sample for electrophoresis by the method of Pegg et al. (12). During the entire procedure, recovery of radioactivity was monitored to assess possible loss of the polyamines or some metabolites which may not have been extracted by this procedure. In electrophoresis, standards were included to locate the positions of spermidine, spermine, putrescine, cadaverine, and ornithine, which were subsequently identified by staining with acid ninhydrin. Each spot was cut out, eluted with 1 ml of solution containing water/ethanol/acetic acid (1/4/5 by volume) and 0.2% cadmium acetate. The amount of the radioactivity was determined in Bray’s (13) scintillation fluid with a liquid scintillation spectrometer.

RESULTS

Hormonal Effect on Polyamine Uptake—Fig. 1A shows the time course of spermidine uptake by mammary explants in various culture conditions. It can be seen that the uptake of spermidine in the absence of added hormones rapidly increased, with time to reach a plateau level at about 24 hours. In the presence of insulin, the uptake of spermidine was increased by about 50% over the no hormone control. Addition of prolactin with insulin elicited an even greater increase, although prolactin by itself was not effective. Hydrocortisone alone or in any combination with insulin and/or prolactin caused no stimulation of the polyamine uptake during a 2-day culture (not shown). Similar results were obtained with mammary epithelial cell fractions derived from the cultured explants except that the net amount of radioactivity was about half that observed in the corresponding explants. Similar results were obtained by experiments with 14C-labeled spermidine.

As shown in Fig. 1, B and C, the uptake of putrescine and spermine, other naturally occurring polyamines, also occurred in mammary explants. In contrast to spermidine, however, the uptake of these polyamines continued to increase for 48 hours in the presence of insulin and prolactin.

Metabolic Fate of Exogenous Polyamines—The data in Table I show that over 90% of the [3H]spermidine taken up by mammary explants during a 48-hour incubation was recovered as spermidine while less than 9% was converted to spermine. The percentage of conversion of spermidine to spermine was essentially the same in explants cultured with various combinations of hormones. Similar results were obtained in other experiments with explants cultured for up to 24 hours.

It is unlikely that spermidine is converted into other major metabolites and conjugated forms since (a) essentially all the radioactivity in the tissue homogenate was recovered as spermidine and spermine, (b) no radioactivity was present in the acid-insoluble fraction which normally contains the conjugated polyamines (14), and (c) the only radioactive compound recovered from the culture medium was spermidine.

In contrast to the metabolic stability of spermidine, both added spermine and putrescine underwent metabolic conversion in cultured mammary explants. After a 48-hour incubation, about 30% of the spermine and approximately 70% of the putrescine in the tissues were converted to spermine. The extent of the conversion of each polyamine was similar regardless of hormonal milieu. Analogous results were obtained with higher concentrations of added spermine and putrescine (0.2 ~ 1 mM), which indicate that such conversions may occur at physiological levels of these polyamines.

Characterization of Spermidine Uptake Process—As shown in Fig. 2, the accumulation of spermidine in cultured mammary explants is dependent on the incubation temperature. At 4°C, the concentration of radioactivity in explants was about the same as that in the culture medium, indicating that active

Fig. 1. Uptake of polyamines by mouse mammary explants in vitro. Mammary explants from midpregnant mice were cultured in the absence of added hormones (O—O) or in the presence of insulin (■—■) or prolactin (△—△) as described under “Experimental Procedures.” At the beginning of the culture, (A) [3H]spermidine (1 μCi/ml) or (B) [3H]spermine (2 μCi/ml) or (C) [14C]putrescine (0.01 μCi/ml) was added to each dish, and the radioactivity in explants was determined at the indicated times as described under “Experimental Procedures.” Each point represents the average of five to six separate experiments. Standard error is within the range of 7 to 9% for each point.

1 In all the subsequent experiments, the results were confirmed by the use of appropriate 14C- or 3H-labeled polyamine.
spermidine by mouse mammary explants in vitro. Mammary explants were cultured with [3H]spermidine (1 μCi/ml) for 6 hours at indicated temperatures in the absence of added hormones (O---O) or in the presence of insulin (O---O) or in the presence of insulin and prolactin (A---A). Each point represents the mean of three to four separate experiments. Standard error is within the range of 2 to 5% for each point.

Mammary explants were cultured for 24 hours in the absence of added hormones (O---O) or in the presence of insulin (O---O) or in the presence of insulin and prolactin (A---A). Each point represents the mean of three separate experiments. Standard error is within the range of 4 to 6% for each value. Each value in parentheses refers to the percentage of the radioactivity recovered relative to the total isotope content in the final extract.

### Table I

**Metabolism of polyamines in mouse mammary explants in culture**

Mammary explants were cultured for 48 hours in medium containing the indicated combination of hormones and either [3H]spermidine (1 μCi/ml) or [3H]spermine (2 μCi/ml) or [14C]putrescine (1 μCi/ml). At the end of culture, 40 to 60 mg of explants in each group were processed to examine the metabolic fate of exogenous polyamines as described under “Experimental Procedures.” Each value represents the mean of three to four separate experiments. Standard error is within the range of 4 to 6% for each value. Each value in parentheses refers to the percentage of the radioactivity recovered relative to the total isotope content in the final extract.

<table>
<thead>
<tr>
<th>Polyamine added and culture condition</th>
<th>Spermidine</th>
<th>Spermine</th>
<th>Putrescine</th>
<th>Unknown fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermidine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No hormone</td>
<td>7,920 (90.0)</td>
<td>680 (8.7)</td>
<td>15 (0.2)</td>
<td>60 (0.8)</td>
</tr>
<tr>
<td>Insulin</td>
<td>21,770 (92.7)</td>
<td>1,570 (6.7)</td>
<td>24 (0.1)</td>
<td>130 (0.5)</td>
</tr>
<tr>
<td>Prolactin</td>
<td>11,970 (82.5)</td>
<td>830 (6.4)</td>
<td>40 (0.3)</td>
<td>80 (0.7)</td>
</tr>
<tr>
<td>Insulin + prolactin</td>
<td>20,770 (94.9)</td>
<td>1,150 (4.5)</td>
<td>40 (0.2)</td>
<td>110 (0.4)</td>
</tr>
<tr>
<td>Spermine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No hormone</td>
<td>2,620 (30.5)</td>
<td>5,400 (63.0)</td>
<td>44 (0.5)</td>
<td>510 (5.9)</td>
</tr>
<tr>
<td>Insulin</td>
<td>5,185 (21.1)</td>
<td>18,510 (75.3)</td>
<td>25 (0.1)</td>
<td>840 (3.4)</td>
</tr>
<tr>
<td>Prolactin</td>
<td>1,800 (23.9)</td>
<td>5,560 (73.8)</td>
<td>7 (0.1)</td>
<td>160 (2.1)</td>
</tr>
<tr>
<td>Insulin + prolactin</td>
<td>8,590 (31.1)</td>
<td>18,850 (68.3)</td>
<td>28 (0.1)</td>
<td>110 (0.4)</td>
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<tr>
<td>Putrescine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No hormone</td>
<td>1,070 (70.5)</td>
<td>140 (8.4)</td>
<td>300 (19.9)</td>
<td>2 (0.1)</td>
</tr>
<tr>
<td>Insulin</td>
<td>1,390 (65.0)</td>
<td>140 (6.7)</td>
<td>600 (28.2)</td>
<td>2 (0.1)</td>
</tr>
<tr>
<td>Prolactin</td>
<td>960 (67.2)</td>
<td>140 (8.8)</td>
<td>520 (22.4)</td>
<td>9 (0.6)</td>
</tr>
<tr>
<td>Insulin + prolactin</td>
<td>5,110 (74.5)</td>
<td>240 (8.1)</td>
<td>710 (17.0)</td>
<td>12 (0.2)</td>
</tr>
</tbody>
</table>

*Fig. 2 (left).* Effect of incubation temperature on the uptake of spermidine by mouse mammary explants in vitro. Mammary explants were cultured with [3H]spermidine (1 μCi/ml) for 6 hours at indicated temperatures in the absence of added hormones (O---O) or in the presence of insulin (O---O) or in the presence of insulin and prolactin (A---A). Each point represents the mean of three separate experiments. Standard error is within the range of 2 to 3% for each point.

*Fig. 3 (right).* Apparent $K_m$ value and $V_{max}$ for spermidine uptake by mouse mammary explants in vitro. Mammary explants were cultured for 24 hours in the absence of added hormones (O---O) or in the presence of insulin (O---O) or insulin and prolactin (A---A), and then exposed to various concentrations of unlabeled spermidine with constant amounts of [3H]spermidine (1 μCi/ml) for 3 hours. The radioactivity in explants was determined as described under “Experimental Procedures.” Data are expressed by double reciprocal plot of spermidine concentration, micromolar (abscissa), and the amount of spermidine taken up by the explants, nanomoles/mg of tissue/hour (ordinate). Each point represents the mean of two separate experiments.

The extent of spermidine uptake by mammary explants also depends on the concentration of the polyamine in the medium (not shown). Thus the amount of spermidine taken up by the cells increased with increasing concentrations of added spermidine until it reached a maximum at a concentration of 12 μM in the medium. It was also found that the stimulatory effects of insulin and prolactin on the uptake of spermidine became smaller as the concentration of spermidine in the medium was increased. The apparent $K_m$ value for the uptake process was approximately $4.76 \times 10^{-9}$ μM (Fig. 3). The addition of insulin and/or prolactin did not alter the apparent $K_m$ value, but the $V_{max}$ value was increased 2.86 x $10^{-4}$ μmol/mg of tissue/hour to $4.76 \times 10^{-4}$ μmol/mg of tissue/hour by insulin and to $7.24 \times 10^{-4}$ μmol/mg of tissue/hour by the combination of insulin and prolactin. The uptake of spermidine was inhibited in a competitive manner by other naturally occurring polyamines such as spermine, putrescine, and cadaverine. For example, the uptake of 2.3 μM spermidine was inhibited by 84% with 0.2 mM spermine, 30% with 0.2 mM putrescine, and 30% with 0.2 mM cadaverine. The uptake, however, was not inhibited by basic amino acids such as lysine and arginine, each at 2.3 mM.

*Influx of Spermidine—* Fig. 4 shows the change in the net rate of spermidine uptake by mammary explants cultured with various combinations of insulin and prolactin. It can be seen that the rate of uptake in the absence of added hormones uptake did not occur. With higher incubation temperatures, however, an increasing amount of spermidine was taken up into the cells. The data in Fig. 2 also show that the stimulatory effect of insulin was most evident at 37°. In another series of experiments, it was found that mammary explants incubated in various media such as distilled water, 0.15 M saline, and Hank's salts solution did not accumulate spermidine over the level in the medium.

The extent of spermidine uptake by mammary explants also depends on the concentration of the polyamine in the medium (not shown). Thus the amount of spermidine taken up by the cells increased with increasing concentrations of added spermidine until it reached a maximum at a concentration of 12 μM in the medium. It was also found that the stimulatory effects of insulin and prolactin on the uptake of spermidine became smaller as the concentration of spermidine in the medium was increased. The apparent $K_m$ value for the uptake process was approximately $4.76 \times 10^{-9}$ μM (Fig. 3). The addition of insulin and/or prolactin did not alter the apparent $K_m$ value, but the $V_{max}$ value was increased 2.86 x $10^{-4}$ μmol/mg of tissue/hour to $4.76 \times 10^{-4}$ μmol/mg of tissue/hour by insulin and to $7.24 \times 10^{-4}$ μmol/mg of tissue/hour by the combination of insulin and prolactin. The uptake of spermidine was inhibited in a competitive manner by other naturally occurring polyamines such as spermine, putrescine, and cadaverine. For example, the uptake of 2.3 μM spermidine was inhibited by 84% with 0.2 mM spermine, 30% with 0.2 mM putrescine, and 30% with 0.2 mM cadaverine. The uptake, however, was not inhibited by basic amino acids such as lysine and arginine, each at 2.3 mM.

*Influx of Spermidine—* Fig. 4 shows the change in the net rate of spermidine uptake by mammary explants cultured with various combinations of insulin and prolactin. It can be seen that the rate of uptake in the absence of added hormones
increased for the first 6 hours and thereafter remained virtually constant. In the presence of insulin, the rate of uptake increased by about 40% in the first 3 hours over the no hormone control and then continued to increase more slowly with time up to 48 hours. In contrast, the stimulation of the uptake by prolactin in the presence of insulin had an approximately 9-hour lag period, after which time the rate of uptake was increased, reaching the maximum of about 200% above the no hormone control at 48 hours. Prolactin alone, however, again did not stimulate the rate of uptake, and hydrocortisone by itself or in combination with insulin or prolactin showed no stimulatory effect.

The effects of several types of metabolic inhibitors on the net rate of uptake of spermidine are shown in Fig. 5. Addition of dinitrophenol to a culture medium resulted in a rapid decrease in the uptake of spermidine and also prevented the stimulatory effects of both insulin and prolactin. In the presence of actinomycin D, the rate of uptake was virtually unaffected for the first 19 hours, but thereafter it declined rapidly, irrespective of the culture conditions. Cycloheximide, on the other hand, stimulated the rate of spermidine uptake in the first 14 hours by almost 100% in all the culture systems, although longer exposure of explants to the drug resulted in a marked inhibition of the uptake. Puromycin, another inhibitor of protein synthesis, also produced a stimulation of the spermidine uptake in the first 14 hours. Cytosine arabinoside, an inhibitor of DNA synthesis, was found to have no effect on the rate of spermidine uptake in any culture system.

**Efflux of Spermidine**—Studies on the effect of hormones on the efflux of spermidine from mammary explants indicate that insulin prevents the release of the polyamine from the tissue (Fig. 6A). Thus, when explants which had been allowed to accumulate labeled spermidine in the presence of insulin and prolactin for 24 hours were transferred to a non-insulin-containing medium, more than half of the polyamine in the cells was released into the medium in 24 hours. Neither prolactin nor hydrocortisone alone was effective in preventing the efflux of the polyamine from the cells. The effect of withdrawal of insulin was reversible in the sense that re-addition of insulin prevented the release of the polyamine and allowed the cells to accumulate the exogenously added spermidine (not shown). Electrophoretic analysis of radioactive materials released into the culture medium showed that almost all of the radioactivity (>99%) was in the form of spermidine.

**Fig. 5.** Effect of various inhibitors on the rate of spermidine uptake by mouse mammary explants *in vitro*. Mammary explants were cultured in the presence of (A) 1 mM dinitrophenol, or (B) 5 µg of actinomycin D/ml, or (C) 1 µg of cycloheximide/ml. These concentrations of the drugs were chosen on the basis of our previous studies whereby possible side effects of the drugs were shown to be minimal (15). Other details were described in the legend to Fig. 4. O—O, no hormone; — —, insulin; A—A, insulin and prolactin. Dotted and solid lines refer to the presence and absence of the inhibitor, respectively. Each point represents the mean of two to four separate experiments. Standard error is within the range of 4 to 9% for each point.

**Fig. 6.** Effect of hormones and various inhibitors on the efflux of spermidine from mouse mammary explants *in vitro*. Mammary explants were first incubated with [3H]spermidine (1 µCi/ml) for 24 hours in the presence of insulin and prolactin. At the end of the first incubation, explants were washed thoroughly with Medium 199 containing the indicated combination of hormones used in the second incubation, and then incubated in medium containing no hormones (O—O), or insulin (— —) or insulin plus prolactin (A—A). (A) 1 mM dinitrophenol, (B) 5 µg of actinomycin D/ml; and (C) 1 µg of cycloheximide/ml were added at the beginning of the second incubation. Dotted lines and solid lines refer to the presence and the absence of the inhibitor used. The washing and the second incubation medium contained nonisotopic spermidine (2.4 µM) in place of [3H]spermidine. At the indicated times after the second incubation, the amount of radioactive spermidine in the explants was determined as described under "Experimental Procedures." The data are expressed as relative amount (%) of spermidine remaining in the explants where 100% refers to the amount present in the explants at the beginning of the second culture. Each point represents the mean of two separate experiments for dinitrophenol and actinomycin D, and three separate experiments for cycloheximide. Standard error is within the range of 8 to 11%.
similar transport system for the polyamines in mammalian cells. Earlier studies on bacteria and other lower organisms, however, have clearly shown the existence of active transport systems for spermidine, spermine, and putrescine (18-21). The present studies demonstrate a transport system for spermidine by mammalian cells. The stimulatory effect of prolactin on the accumulation of exogenous spermidine in mammary cells is shown to be mediated at the level of both entry and exit of spermidine. The present study shows that insulin also enhances the uptake of spermidine, although the efflux of the polyamine has been reported to be diminished by the hormone (8). Previous studies (8) also showed that spermidine was effective at a concentration as low as 4 x 10^{-8} M. According to the present study, such a concentration of spermidine in the medium should amount to about 400 nmol of the polyamine/g of tissue, which is very similar to that found in the cells that are actively engaged in milk protein synthesis under the influence of insulin, glucocorticoid, and prolactin (7, 8). Thus it can be concluded that mammary cells can attain a sufficiently high level of spermidine needed for the formation of milk protein by transporting it from the external medium under the influence of insulin and prolactin.

The inhibitory effect of dinitrophenol was again manifested rapidly while the effects of the other two drugs became apparent about 12 hours after their addition.

Spermidine Uptake in Vivo—It was of interest to determine whether the uptake of spermidine by mammary cells as demonstrated in vitro occurs also in vivo. The results presented in Table II show that when spermidine was administered intravenously, the mammary gland of midpregnant mice did not accumulate spermidine, the level of which was about three times higher than that in serum, at 48 hours after injection. In contrast, neither mammary nor muscle tissue of virgin mice, nor muscle of pregnant mice demonstrated the ability to concentrate spermidine over the serum level. Electrophoretic analysis of radioactive materials in these tissues and sera showed that essentially all of the material was spermidine. In another experiment, the serum concentration of spermidine in mature virgin and midpregnant mice was determined since the apparent inability of mature virgin mouse mammary gland to accumulate spermidine could have resulted from the difference in the endogenous serum level of spermidine. However, the results showed that the serum concentration of spermidine in mature virgin mice, 1 - 5 mg/ml, was even lower than that in midpregnant mice, 13 - 19 mg/ml. Thus it appears that the apparent inability of jack mouse mammary gland to concentrate spermidine may be due to its insulin insensitivity, as suggested by the previous observations that the mammary cells at this stage of development are unresponsive to insulin (16, 17).

**DISCUSSION**

In spite of much work on the polyamines in various mammalian systems, there have been few studies on the uptake of the polyamines by mammalian cells. Earlier studies on bacteria and other lower organisms, however, have clearly shown the existence of active transport systems for spermidine, spermine, and putrescine (18-21). The present studies demonstrate a similar transport system for the polyamines in mammary cells in vitro and in vivo. The uptake of spermidine, as studied in detail on cultured mammary cells, is a time-dependent process which requires active metabolic processes: the uptake is dependent on temperature, is sensitive to metabolic inhibitors such as dinitrophenol, and also is inhibited by prolonged exposure to actinomycin D and cycloheximide. The extent of spermidine uptake depends on a concentration with an apparent $K_a$ value of $4.76 \times 10^{-8}$ M. The present data, however, do not allow us to conclude that the uptake of spermidine occurs against a real concentration gradient since some of the polyamine binds to cellular polyacids such as DNA and RNA. In addition, the uptake of spermidine is inhibited by other polyamines such as putrescine, cadaverine, and spermine but not by various basic amino acids, which suggests that the transport system for spermidine is different from that for the basic amino acids, but may be shared by the other polyamines. Some of these features of the polyamine transport system in mammary cells are analogous to those described for bacteria by Tabor and Tabor (18).

The accumulation of exogenous spermidine in mammary cells is augmented in the presence of both insulin and prolactin, hormones which are required for the development of mammary epithelium in vitro. However, glucocorticoid, another hormone necessary for lactogenesis in vitro, showed no stimulatory effect on the transport of the polyamine. It should be noted that the requirement of glucocorticoid for mammary development was previously shown to be replaced by exogenous spermidine and that the combination of insulin, prolactin, and spermidine elicited a marked stimulation of milk protein synthesis in mammary explants (7, 8). Previous studies (8) also showed that spermidine was effective at a concentration as low as $4 \times 10^{-8}$ M. According to the present study, such a concentration of spermidine in the medium should amount to about 400 nmol of the polyamine/g of tissue, which is very similar to that found in the cells that are actively engaged in milk protein synthesis under the influence of insulin, glucocorticoid, and prolactin (7, 8). Thus it can be concluded that mammary cells can attain a sufficiently high level of spermidine needed for the formation of milk protein by transporting it from the external medium under the influence of insulin and prolactin.

**Table II**

<table>
<thead>
<tr>
<th>Time after injection (hr)</th>
<th>Midpregnant</th>
<th>Mature virgin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mammary gland</td>
<td>Muscle</td>
</tr>
<tr>
<td></td>
<td>cmg/mg tissue</td>
<td>cmg/µl</td>
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<tr>
<td>6</td>
<td>177 ± 14</td>
<td>74 ± 1</td>
</tr>
<tr>
<td>24</td>
<td>286 ± 37</td>
<td>129 ± 33</td>
</tr>
<tr>
<td>48</td>
<td>526 ± 53</td>
<td>191 ± 9</td>
</tr>
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</table>

The stimulatory effect of insulin on the accumulation of exogenous spermidine in mammary cells is shown to be mediated at the level of both entry and exit of spermidine. The hormone stimulates rapidly the rate of entry of spermidine by increasing $V_{max}$. Studies with actinomycin D and cycloheximide suggest that the effect of insulin does not require new RNA and protein synthesis for the first 12 hours or so, although at later times the hormonal effect is abolished by these drugs. The present study shows that insulin also enhances the accumulation of spermidine by preventing the efflux of the polyamine from the cells. This observation may have a significant implication for the regulation of the cellular level of the polyamine, although the efflux of the polyamine has received little attention in the past. It remains to be determined whether this effect of insulin on the polyamine transport is elicited by interaction of the hormone on the cell membrane, as suggested by a number of studies on the mechanism of action of insulin in the transport of various small molecules such as amino acids and glucose (22-24).

The present data also show that in the presence of insulin prolactin stimulates the accumulation of exogenous spermidine in cultured mammary cells. This effect of prolactin is

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* R. Kano, and T. Oka, unpublished observation.
exerted mainly through an increase in $V_{\text{max}}$ for spermidine. In contrast to insulin, however, the stimulatory effect of prolactin is manifested only after a 9- to 12-hour lag period, and is inhibited by actinomycin D. These results suggest that the effect of prolactin may be mediated by a different mechanism from that of insulin. Previous studies on the effect of prolactin in lower invertebrates have shown (25) that the hormone stimulates the transport of the small ions such as Ca$^{2+}$ and Na$^{+}$. Since spermidine is also a naturally occurring cation, it is possible that this effect of prolactin on the polyamine transport in mammary cells may have some evolutionary counterpart in lower invertebrates.

Studies on the metabolism of spermidine taken up by mammary cells indicate that over 90% of the polyamine remains virtually unchanged, although the existence of a very labile derivative cannot be completely excluded. This is in contrast to both putrescine and spermine, which undergo considerable metabolic conversion to spermidine. Conversion of putrescine to spermidine is probably effected, at least in part, by a small increase in the activity of S-adenosylmethionine decarboxylase, which occurs during the culture of mammary cells (8). On the other hand, the mechanism of spermidine formation from spermine is not known. Several investigators have also reported (26, 27) that spermine is converted to spermidine in various rat tissues and Ehrlich ascites cells in culture.

It has been shown repeatedly (1-5) that the intracellular concentrations of the polyamines increase markedly during cell growth and development. Because such increases are often accompanied by enhanced activities of spermidine biosynthetic enzymes, it has been customary to ascribe an increase in the polyamine largely to its enhanced biosynthesis. Although polyamine biosynthesis is undoubtedly an important mechanism for the regulation of the polyamine level in the cells, the present findings that the polyamines are taken up and released by mammary cells, and that spermine and putrescine undergo metabolic conversion to spermidine, may suggest another possible means of modulating the cellular polyamine level. In fact, earlier studies by Holm and Emanuelsson (28) have presented some evidence indicating that the concentration of the polyamine in tetrahymena may be regulated primarily by the uptake of the polyamine from external medium. In the mammalian system, Pohjanpelto and Raina (29) have shown that cultured fibroblasts excrete putrescine which acts as a growth-promoting agent. Furthermore, it has been shown recently (30, 31) that regression of the hormone-dependent rat mammary tumor by ovariectomy or of the rat hepatoma by treatment with fluorodeoxyuridine resulted in an elevation of the spermidine level in serum with a concomitant decrease in the concentration of the polyamine in the tumor. It would be of interest to determine how much the uptake, release, and metabolic conversion of the polyamines may contribute to the regulation of the cellular polyamine level during tissue growth and development, as well as various pathophysiological states, since there is growing evidence for the critical role of the polyamines in these cellular processes (1-5).

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