The Effect of Inhibitors of Ornithine Decarboxylase on DNA Synthesis in Mouse Mammary Gland in Culture

THE IMPORTANCE OF OSMOLARITY OF THE MEDIUM AND OF THE INITIAL INTRACELLULAR LEVEL OF PUTRESCINE

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In cultured mammary explants from both virgin and midpregnant mice which contained a low and a high initial level of putrescine, respectively, addition of an ornithine decarboxylase inhibitor, DL-α-hydrazino-δ-aminovaleric acid at the onset of culture prevented an early, "osmolarity-sensitive" rise in the putrescine level that occurred prior to induction of DNA synthesis by insulin. In the presence of the drug, hormonal stimulation of DNA synthesis occurred normally in virgin mouse mammary explants cultured in isotonic medium and in midpregnant mouse mammary explants cultured in both isotonic and hypotonic media. However, in virgin mouse mammary explants cultured in hypotonic medium, the drug caused varying degrees of inhibition of DNA synthesis which were inversely related to the osmolarity of the medium. The inhibitory effect of the drug on DNA synthesis was also dependent on the time of its addition, being most effective within the first 2 h of culture, but virtually ineffective at 12 h. Inhibition of DNA synthesis by the drug was reversible by addition of putrescine (0.5 to 1 μM), which was most effective within 1 h of culture and thereafter became less effective. The polyamines, spermidine, spermine, and cadaverine were ineffective in this respect. Similar results were obtained with another ornithine decarboxylase inhibitor, α-methylornithine. In contrast to the effect on DNA synthesis, DL-α-hydrazino-δ-aminovaleric acid inhibited neither protein synthesis nor the uptake of amino acids and glucose and caused a much smaller degree of inhibition of RNA synthesis. These results suggest that an osmolarity-sensitive increase in putrescine is important for hormonal induction of DNA synthesis in mammary cells containing a low, initial level of putrescine which are cultured in hypotonic medium.

The diamine putrescine and the polyamines spermidine and spermine are aliphatic polyamines which are synthesized by mammalian cells and are considered to play essential roles in cell growth and development (1-5). The biosynthesis and function of spermidine in the hormonally induced development of mouse mammary epithelium have been studied in an organ culture system. Biosynthesis of spermidine may be a pivotal regulatory step in hormonal induction of both cell proliferation and milk protein synthesis, the two major events in the terminal development of mammary epithelium (6-16). The hormonal stimulation of spermidine biosynthesis in cultured mammary explants is mediated by increases in the activity of various enzymes in the polyamine biosynthetic pathway (7-9, 15). The activity of ornithine decarboxylase, which catalyzes the formation of putrescine from ornithine, increases in a biphasic manner (9). The first peak of activity occurs within a few hours of culture and is caused largely by incubation of tissue explants in a hormone-free culture medium (9). This early rise in ornithine decarboxylase activity is sensitive to the osmolarity of the culture medium and appears to represent an adaptive response of mammary cells in culture (17). Thus, putrescine may have a function in the development of mammary cells in vitro which is different from its role as a precursor in the biosynthesis of spermidine.

Our previous observation (17) that an "osmolarity-sensitive" increase in ornithine decarboxylase activity with a subsequent rise in putrescine level invariably precedes induction of mammary cell proliferation by insulin in vitro led us to investigate the possible involvement of putrescine in the hormonal stimulation of DNA synthesis in cultured mammary explants. We have employed various culture conditions in which the intracellular level of putrescine was varied by alterations in the osmolarity of culture medium (17), use of DL-α-hydrazino-δ-aminovaleric acid, as an inhibitor of ornithine decarboxylase (17), and use of mammmary explants derived from virgin and midpregnant mice, which contained initially a low and a high level of putrescine, respectively (12). The data suggest that an early rise in the level of putrescine itself is important for hormonal stimulation of DNA synthesis in virgin mouse mammary explants cultured in hypotonic medium but such a requirement is not apparent in the other culture conditions.

MATERIALS AND METHODS

Materials were purchased as follows: [methyl-3H]thymidine (specific activity 58.7 Ci/mmol), [3H]-Juridine (specific activity 7.3 Ci/mmol), 3H-labeled L-amino acid mixture (specific activity 1 mCi/ml) and Protosol® from New England Nuclear Corp., calf thymus DNA from Worthington Biochemicals; putrescine, spermidine, spermine, and cadaverine from the Sigma Co.; α-methylornithine, and thymidine from Calbiochem; Medium 199 (Hanks' salts) from Grand Island Biological Co.; and tolube-base scintillation fluids from Research Products International Corp., Ill. Crystalline porcine zinc insulin was a gift from Eli Lilly and Co. Medium 199 without NaCl and KCl was prepared by the Media Preparation Unit, National Institutes of Health. HAVA1 synthesized by the method of Sawaya et al. (19)

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was kindly supplied by Dr. H. Nishimura of Dainippon Pharmaceutical Company, Limited, Osaka, Japan.

Three- to four-month old C3H/HeN virgin female mice and mice in the 10th to 13th day of pregnancy were obtained from the Animal Breeding Facility, National Institutes of Health, Md. The animals were killed by cervical dislocation, and the abdominal mammary glands were removed for organ culture studies. The tissue explants were prepared at 25°C and cultured as described previously (20). Medium 199 with varying osmolality was prepared by mixing regular Medium 199 and NaCl- and KCl-free Medium 199 in various ratios (17). Culture medium was changed every 48 h. Insulin was used at a concentration of 5 μg/ml of medium.

The extent of DNA synthesis was determined by allowing explants to incorporate [3H]thymidine (1 μCi/ml) into acid-insoluble materials for the indicated periods. At the end of the labeling period, explants were collected, weighed, and processed for determination of the amount of radioactivity as described previously (21). The final tissue residue was dissolved in 0.5 ml of Protosol(R) and assayed for radioactivity in a toluene-based scintillation fluid with a liquid scintillation spectrometer. Autoradiographic and mitotic index studies (22) previously established that the incorporation of [3H]thymidine into acid-insoluble material reflected DNA synthesis in mammary epithelium. The concentration method was chosen over short pulse labeling in assessing the overall pattern of DNA synthesis because the latter method required extensive time point determinations in order to identify the peak time for DNA synthesis, which normally proceeds for 2 to 4 days depending on the developmental stage of mammary explants used (21). The amount of [3H]thymidine, namely, 1 μCi/ml, was sufficient for 3-day labeling experiments, as judged by preliminary data which showed that the replenishment of culture medium at every 24 h gave identical results. RNA synthesis and protein synthesis were measured by labeling explants with [3H]uridine or [3H]-labeled amino acid mixture, respectively, for the indicated period and determining the radioactivity incorporated into RNA and protein, respectively, as described previously (23). DNA content in epithelial cell-enriched fractions (23) was measured by the method of Giles and Myers (24) using calf thymus DNA as a standard.

The content of putrescine, spermidine, and spermine was determined by an amino acid analyzer as described previously (15).

**RESULTS**

Mammary glands of virgin mice contained a low level of putrescine, 4 to 6 pmol/mg of tissue, whereas the tissue in midpregnant mice contained an elevated level of putrescine, 20 to 30 pmol/mg of tissue (Table I). When mammary explants derived from mice in the two physiological stages were placed in culture, the activity of ornithine decarboxylase increased within 1 h, irrespective of the initial level of putrescine, and both the extent and the duration of the increase were found to be inversely related to the osmolarity of the culture medium and augmented further by insulin (17). At 0.53 isotonicity, the cellular content of putrescine increased more than 10-fold over the initial level in both virgin and midpregnant mouse mammary explants cultured with insulin for 12 h.

Addition of HAVA, an inhibitor of ornithine decarboxylase, at the onset of culture inhibited the large increase in the putrescine level observed in virgin mouse mammary explants cultured in hypotonic medium. The intracellular concentration of spermidine and spermine, however, did not change appreciably in culture, regardless of the presence of HAVA. HAVA completely inhibited the stimulatory effect of insulin on DNA synthesis (Table I). In contrast, in similar experiments with mammary explants from midpregnant mice, HAVA did not inhibit hormonal stimulation of DNA synthesis, despite its inhibitory action on putrescine accumulation. In addition, DNA synthesis was at low levels in mammary explants cultured in hypotonic medium containing no insulin even though the intracellular level of putrescine was increased moderately. Thus, the increase in putrescine accumulation alone was not sufficient for augmentation of DNA synthesis in mammary explants.

The effect of HAVA on DNA synthesis in virgin mouse mammary explants was dependent on the toxicity of the culture medium (Table II). The degree of inhibition of DNA synthesis by the drug increased as the toxicity of culture medium was reduced such that the inhibition was essentially zero at isotonic medium and increased up to about 90% at 0.50 isotonicity. The mammary explants in hypotonic medium retained responsiveness to insulin (Table II).

The extent of accumulation of putrescine in mammary cells increased progressively as the osmolality of culture medium was reduced and HAVA effectively inhibited such increases, regardless of the osmolality of the medium (Table III).

**Table I**

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Putrescine pmol/mg tissue</th>
<th>Spermidine pmol/mg tissue</th>
<th>Spermine pmol/mg tissue</th>
<th>DNA synthesis cpm/mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Explants from virgin mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncultured control</td>
<td>3.8</td>
<td>64.7</td>
<td>48.6</td>
<td></td>
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<tr>
<td>No hormone</td>
<td>31.6</td>
<td>75.4</td>
<td>26.9</td>
<td>109</td>
</tr>
<tr>
<td>Insulin</td>
<td>55.6</td>
<td>79.8</td>
<td>28.8</td>
<td>1,194</td>
</tr>
<tr>
<td>Insulin + HAVA (1 mM)</td>
<td>9.1</td>
<td>71.5</td>
<td>28.3</td>
<td>101</td>
</tr>
<tr>
<td>Explants from midpregnant mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncultured control</td>
<td>30</td>
<td>399</td>
<td>114</td>
<td></td>
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<tr>
<td>No hormone</td>
<td>326</td>
<td>409</td>
<td>108</td>
<td>358</td>
</tr>
<tr>
<td>Insulin</td>
<td>356</td>
<td>499</td>
<td>108</td>
<td>810</td>
</tr>
<tr>
<td>Insulin + HAVA (1 mM)</td>
<td>32</td>
<td>401</td>
<td>78</td>
<td>820</td>
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</table>

**Table II**

<table>
<thead>
<tr>
<th>Culture system</th>
<th>DNA synthesis with isotonicity of 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/mg tissue</td>
</tr>
<tr>
<td>No hormone</td>
<td>1,083</td>
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<tr>
<td>Insulin</td>
<td>2,542</td>
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<tr>
<td>Insulin + HAVA (1 mM)</td>
<td>780</td>
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**Table III**

<table>
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<tr>
<th>Culture system</th>
<th>Content of putrescine with isotonicity of 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>No hormone</td>
<td>4.5 pmol/mg tissue</td>
</tr>
<tr>
<td>Insulin</td>
<td>15.3</td>
</tr>
<tr>
<td>Insulin + HAVA (1 mM)</td>
<td>5.8</td>
</tr>
</tbody>
</table>
results suggest that the "osmolarity-dependent" inhibitory effect of HAVA on DNA synthesis may not be due to altered uptake of HAVA by cultured explants in various hypotonic cultures.

In experiments not presented here, the extent of inhibition of DNA synthesis by HAVA in virgin mouse mammary explants in a given hypotonic medium was somewhat variable among different preparations of tissue explants. No greater inhibition was produced by increasing the concentration of HAVA from 1 mM to 4 mM. These results may be due to the known variation in the initial level of putrescine of tissue explants (12).

If the inhibitory effect of HAVA on DNA synthesis was the result of an intracellular putrescine deficiency caused by blocking ornithine decarboxylase activity, the effect should be reversed by exogenous addition of putrescine. When the mammary explants were cultured in hypotonic medium with insulin, DNA synthesis began to increase after a lag period of 1 day and thereafter remained elevated during a 5-day period.

Addition of HAVA at the onset of culture inhibited the hormonal stimulation of DNA synthesis by more than 70% throughout the culture, but the concomitant presence of putrescine virtually abolished the inhibition of DNA synthesis by HAVA (Fig. 1). Addition of putrescine to hormone-free or insulin-containing medium did not cause stimulation of DNA synthesis (data not shown).

The effective concentration of exogenous putrescine needed to reverse the inhibitory effect of HAVA on DNA synthesis was 5 x 10^-4 to 10^-3 M (Table IV). When putrescine was added at 10^-3 M to the medium containing insulin and HAVA, the intracellular level of putrescine increased to 34.0 pmol/mg of tissue in 17 h, essentially the same level as that found in cultures without hormone (see Table I). In contrast, addition of spermidine or spermine or cadaverine or ornithine (not shown) in concentrations ranging from 1 μM to 1 mM, did not reverse the inhibitory effect of HAVA on DNA synthesis. As mentioned above, the addition of putrescine alone (1 to 10 μM) did not stimulate DNA synthesis, but often produced a small inhibition of insulin-stimulated DNA synthesis (see also Table VII).

The data in Table V show that another inhibitor of ornithine decarboxylase, α-methylornithine (25) also inhibited the induction of DNA synthesis in virgin mammary explants cultured in hypotonic medium. The inhibition of DNA synthesis was overcome by the concomitant addition of putrescine, but not by spermidine, ornithine, spermine, or cadaverine at 1 μM to 1 mM (data not shown). α-Methylornithine, like HAVA, did not inhibit insulin-induced DNA synthesis either in virgin mouse mammary explants cultured in isotonic medium or midpregnant mouse mammary explants cultured either in isotonic or hypotonic medium (data not shown).

To determine the critical period of the requirement of putrescine for DNA synthesis, the time of addition of HAVA and putrescine was altered. The inhibitory effect of HAVA on DNA synthesis was most effective when added at 0 h and decreased progressively as the addition of the drug was delayed (Table VI). Similarly, the "reversal" effect of putrescine was largest when added within 1 h and thereafter decreased progressively.

Several lines of evidence indicate that the inhibitory effect of HAVA on DNA synthesis was not due to the inhibition of [3H]thymidine uptake: (a) determination of the DNA content

**Table IV**

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>DNA synthesis (cpm/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No hormone</td>
<td>177</td>
</tr>
<tr>
<td>Insulin</td>
<td>843</td>
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<tr>
<td>Insulin + HAVA</td>
<td>400</td>
</tr>
<tr>
<td>Insulin + HAVA + putrescine (0.5 μM)</td>
<td>705</td>
</tr>
<tr>
<td>Insulin + HAVA + putrescine (1 μM)</td>
<td>921</td>
</tr>
<tr>
<td>Insulin + HAVA + putrescine (10 μM)</td>
<td>689</td>
</tr>
<tr>
<td>Insulin + HAVA + spermine (1 mM)</td>
<td>423</td>
</tr>
<tr>
<td>Insulin + HAVA + spermine (1 mM)</td>
<td>323</td>
</tr>
<tr>
<td>Insulin + HAVA + cadaverine (1 mM)</td>
<td>405</td>
</tr>
</tbody>
</table>

**Table V**

<table>
<thead>
<tr>
<th>Culture system</th>
<th>DNA synthesis (cpm/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No hormone</td>
<td>576</td>
</tr>
<tr>
<td>Insulin</td>
<td>1,677</td>
</tr>
<tr>
<td>Insulin + α-methylornithine</td>
<td>1,092</td>
</tr>
<tr>
<td>Insulin + α-methylornithine + putrescine (1 μM)</td>
<td>1,605</td>
</tr>
<tr>
<td>Insulin + α-methylornithine + spermidine (10 μM)</td>
<td>1,055</td>
</tr>
<tr>
<td>Insulin + α-methylornithine + ornithine (2 mM)</td>
<td>1,000</td>
</tr>
</tbody>
</table>

**Fig. 1.** Time course of DNA synthesis in virgin mouse mammary explants in hypotonic culture medium. Mammary explants were cultured for 5 days in 0.67 isotonic medium containing [3H]thymidine (1 μCi/ml) and the following additions: insulin (C), or insulin plus HAVA (1 mM) (C), or insulin plus HAVA (1 mM) plus putrescine (0.5 μM) (A), or no addition (O). At the indicated time points, incorporation of [3H]thymidine into DNA in cultured explants was determined as under "Materials and Methods." Each point represents an average of two to three determinations. Standard error for each point was less than 10%.
of mammary epithelial cells at the end of 3 days of culture showed that HAVA produced over 90% inhibition of the increase in DNA which was effected by insulin, and that the inhibition was reversed by the addition of putrescine at 0 h (Table VII). Putrescine, added to the insulin-containing medium, caused a small inhibition in the increase in DNA content; (b) use of [3H]thymidine with 100-fold lower specific activity, added at 10 μCi/ml, to expand the endogenous pool size, gave the same pattern of inhibition (data not shown); (c) [3H]thymidine in the acid-soluble pool was essentially unaltered by the drug, i.e., 750 dpm/mg of tissue in the presence of insulin and 720 dpm/mg of tissue in the presence of insulin and HAVA (1 mM) at the end of a 3-day culture. Attempts to determine the intracellular pool of dTTP were unsuccessful because the amount of dTTP was too small to measure accurately even with use of 200 mg of tissue explants (whole gland equivalent).

In order to assess whether the inhibitory effect of HAVA is specific for DNA synthesis, we examined the effect of the drug on RNA synthesis and protein synthesis in the explants cultured with insulin in hypotonic medium (Table VIII). HAVA produced 15 to 20% inhibition of RNA synthesis and no inhibition of protein synthesis at 12, 24, and 48 h in culture. In an experiment not shown, HAVA also failed to inhibit the uptake of either glucose or a nonmetabolizable amino acid, α-aminobutyric acid, as determined by the methods previously described (20).

### Table VI

<table>
<thead>
<tr>
<th>Culture System</th>
<th>DNA synthesis (cpm/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No hormone</td>
<td>247</td>
</tr>
<tr>
<td>Insulin</td>
<td>1,544</td>
</tr>
<tr>
<td>Insulin + HAVA (0 h)</td>
<td>297</td>
</tr>
<tr>
<td>Insulin + HAVA (3 h)</td>
<td>791</td>
</tr>
<tr>
<td>Insulin + HAVA (6 h)</td>
<td>946</td>
</tr>
<tr>
<td>Insulin + HAVA (12 h)</td>
<td>1,435</td>
</tr>
<tr>
<td>Insulin + HAVA (0 h) + putrescine (0 h)</td>
<td>1,462</td>
</tr>
<tr>
<td>Insulin + HAVA (0 h) + putrescine (1 h)</td>
<td>1,055</td>
</tr>
<tr>
<td>Insulin + HAVA (0 h) + putrescine (2 h)</td>
<td>1,055</td>
</tr>
<tr>
<td>Insulin + HAVA (0 h) + putrescine (3 h)</td>
<td>706</td>
</tr>
<tr>
<td>Insulin + HAVA (0 h) + putrescine (12 h)</td>
<td>548</td>
</tr>
</tbody>
</table>

### Table VII

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>DNA synthesis (μg/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No hormone</td>
<td>0.2216 0.3876</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.9922 0.8165</td>
</tr>
<tr>
<td>Insulin + putrescine (10 μM)</td>
<td>0.4442 0.7055</td>
</tr>
<tr>
<td>Insulin + HAVA (1 mM)</td>
<td>0.2548 0.5000</td>
</tr>
<tr>
<td>Insulin + HAVA + putrescine (10 μM)</td>
<td>0.4426 0.6933</td>
</tr>
</tbody>
</table>

### Discussion

In this paper we have shown that an ornithine decarboxylase inhibitor, HAVA, prevented an early, osmolarity-sensitive increase in putrescine accumulation and also inhibited subsequent induction of DNA synthesis by insulin in mammary explants from virgin mice cultivated in hyperosmotic medium. The present data suggest that putrescine per se may have an essential role in hormonal induction of DNA synthesis in mouse mammary explants containing a low, initial level of putrescine, which are placed in hyperosmotic culture medium. Alternatively, however, the ornithine decarboxylase inhibitors may directly interfere with some process (es) necessary for DNA synthesis, which could be overcome by putrescine.

Mammary explants from midpregnant mice were also sensitive to the inhibitory action of HAVA on putrescine accumulation, but responded normally to insulin in DNA synthesis regardless of the osmolarity of the medium. These explants differ from mammary tissues of virgin mice in that mammary cells are already in a proliferative phase (12, 21) and contain a high, initial level of putrescine. Thus, both the initial cellular level of putrescine and the developmental state of cells are important factors for the efficacy of HAVA in inhibition of DNA synthesis. Accordingly, the requirement for putrescine may not be apparent in those instances when the cellular levels of putrescine are initially high enough for subsequent DNA synthesis even though the drug inhibited an increase in putrescine. A similar view was presented in earlier studies on the role of spermidine in cell proliferation (12, 26-28). Such considerations may be important in cases where inhibitors of polyamine biosynthesis are found to be ineffective in preventing DNA synthesis (29-31).

The precise mode of involvement of putrescine in mammary DNA synthesis in hypotonic culture remains to be elucidated. Since putrescine itself was ineffective in stimulating DNA synthesis in mammary explants, direct involvement of putrescine as a mitogenic factor is unlikely. The participation of putrescine in protein synthesis and RNA synthesis is unlikely (Table VIII). However, these results need to be evaluated further because the assay methods used were relatively crude. Qualitative or small quantitative changes in macromolecular synthesis might not be detected. On the other hand, time course studies suggest that the diamine may be required for events occurring within a few hours of culture. A rapid os-
molarity-sensitive increase in ornithine decarboxylase and in the accumulation of putrescine may represent an adaptive response of mammary cells in culture. Mammary cells synthesize and accumulate more putrescine in hypotonic medium than in isotonic medium. Based on these findings, together with earlier observations (32) that alteration in the osmolarity of medium causes perturbation of the normal balance of intracellular cations in cultured cells, we suggest that mammary cells in hypotonic culture synthesize increasing amounts of putrescine, a cation, as a means of modulating the altered intracellular ionic environment.

HAVA is a potent inhibitor of ornithine decarboxylase with a \( K \) value of \( 5 \times 10^{-7} \) M (18). HAVA selectively inhibits putrescine biosynthesis without altering the concentration of polyamines and therefore is a useful tool in assessing the involvement of putrescine itself in cell proliferation in various mammalian systems. An increase in accumulation of putrescine per se may be causally related to the subsequent proliferative changes in isoproterenol-stimulated mouse parotid glands (19), sarcoma 180 (33), regenerating liver (34), and growth of epidermis in vivo (35). Our present results also support an essential role of putrescine itself in cell proliferation.

The importance of polyamines for cell growth is indicated by earlier work on polyamine-deficient bacterial and yeast mutants (36-38). Enhanced synthesis of putrescine and subsequent accumulation of spermidine occur prior to cell multiplication (1-5). Previously, other inhibitors of ornithine decarboxylase such as \( \alpha \)-methylornithine (25, 39), diaminopropane (40), and \( \beta \)-difluoromethylornithine (26, 27) have also been used to examine the causal relationship of putrescine biosynthesis to cell proliferation. These studies have also suggested an important role of putrescine in the regulation of cell proliferation, but the mode of involvement of putrescine was considered to be through its precursor role in the biosynthesis of spermidine, which has been implicated as an essential growth regulator in mammalian cells (39-44). Our previous studies with an inhibitor of S-adenosyl-L-methionine decarboxylase, methylglyoxal bis(guanylylhydrazone), have also suggested that spermidine is a necessary agent for insulin-stimulated cell proliferation in mammary explants from virgin mice (12). While some caution should be exercised in interpreting the results of experiments with such inhibitors, it appears from these results that putrescine may have at least a dual role in DNA synthesis in cultured mammary cells, one being of its own, and the other as a precursor of spermidine biosynthesis.

In summary, the results of the present studies may give a new insight into the role of putrescine in the proliferation of mammary cells in culture, and these, together with previous findings (6-17), reemphasize the importance of biosynthesis of putrescine and spermidine in hormonal regulation of the development of mammary epithelium in vitro.

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