Polyamine metabolism and function

PEGG, ANTHONY E., AND PETER P. McCANN. Polyamine metabolism and function. Am. J. Physiol. 243 (Cell Physiol. 12): C212-C221, 1982.—Polyamines are ubiquitous organic cations of low molecular weight. The content of these amines is closely regulated by the cell according to the state of growth. The reactions responsible for the biosynthesis and interconversion of the polyamines and their precursor putrescine are described and the means by which polyamine content can be varied in response to exogenous stimuli are discussed. The role of polyamines in the cell cycle, cell division, tissue growth, and differentiation is considered. Recent studies using highly specific inhibitors of polyamine biosynthesis such as α-difluoromethylornithine to prevent accumulation of polyamines have indicated that the synthesis of polyamines is intimately associated with these processes. Such inhibitors have great potential for investigation of the cellular role of polyamines.

cell cycle; tissue growth; differentiation

VIRTUALLY ALL CELLS of higher eukaryotes contain significant amounts of the polyamines, spermidine and spermine, and their precursor, putrescine. Although the physiological function of these amines is still not well understood at the molecular level, recent studies have shown that their concentration is highly regulated and that normal cellular growth and differentiation require polyamines. This review outlines the metabolic reactions responsible for the maintenance of polyamine content, describes some compounds that have been used to interfere with this process, and indicates some of the areas in which polyamines appear to be of particular importance. Brief mention of possible pharmacologic uses for drugs interfering with polyamine metabolism is made. There is no intention to be comprehensive in coverage, and it should be stressed that this review does not attempt to answer the question of the “physiological role of polyamines.” These cations may have many roles and only in certain experimental circumstances is a particular requirement for a parameter under study made apparent.

Polyamine Metabolism

Biosynthesis. The pathway for mammalian polyamine biosynthesis is shown in Fig. 1. The structures for polyamines and some of the inhibitors described later in the text are indicated in Fig. 2. Polyamine levels vary significantly from one cell type to another, but, as typical examples, the content of polyamines and related nucleosides in control and regenerating rat liver and in cultured transformed cells are given in Table 1. Detailed description of the metabolic reactions responsible for polyamine synthesis are given in recent reviews (21, 44, 64, 70). Many microorganisms and higher plants are able to produce putrescine from agmatine produced by decarboxylation of arginine, but all mammalian cells and many lower eukaryotes lack arginine decarboxylase. In these species, therefore, the only route to putrescine is via the enzyme, ornithine decarboxylase. Ornithine is available for these reactions from the plasma (44) and can also be formed within the cell by the action of arginase. It is possible that arginase, which is much more widely distributed than other enzymes of the urea cycle, is present in extrahepatic tissues to ensure the availability of ornithine for polyamine production. Arginase can, therefore, be thought of as an initial step in the polyamine biosynthetic pathway.

Ornithine decarboxylase is a pyridoxal phosphate-dependent enzyme. It is present in very small amounts in quiescent cells, and its activity can be increased manyfold within a few hours of exposure to trophic stimuli (21, 30). Such stimuli include hormones, drugs, tissue regeneration, and growth factors. Even after such stimulation, ornithine decarboxylase is only a very small fraction of the total cellular protein ranging from 0.01% of the cytosolic protein in androgen-stimulated mouse kidneys to 0.00012% in thioacetamide-stimulated rat liver (39, 52). There is evidence for a macromolecular inhibitor of ornithine decarboxylase that may regulate the activity of the enzyme under some circumstances (21, 30, 44).

To convert putrescine into spermidine, an aminopropyl group must be added. This aminopropyl moiety is derived from methionine, which is first converted into S-adenosylmethionine and is then decarboxylated. The resulting decarboxylated S-adenosylmethionine is used as an aminopropyl donor in an analogous manner to the use of S-adenosylmethionine itself as a methyl donor. Once it has been decarboxylated, S-adenosylmethionine is committed
FIG. 1. Pathway of polyamine synthesis and interconversion in mammalian cells. Enzymes involved are 1) arginase, 2) S-adenosylmethionine decarboxylase, 3) ornithine decarboxylase, 4) spermidine synthase, 5) spermine synthase, 6) spermidine N1-acetyltransferase, 7) polyamine oxidase.

FIG. 2. Structure of polyamines, related compounds, and inhibitors of polyamine synthesis.

ted to polyamine production as no other reactions utilizing decarboxylated S-adenosylmethionine at any physiologically significant rate are known. Therefore, the production of decarboxylated S-adenosylmethionine is kept low (Table 1) and constitutes the rate-limiting factor in spermidine formation. Mammalian S-adenosylmethionine decarboxylase is activated by putrescine and repressed by spermidine, linking the supply of decarboxylated S-adenosylmethionine to the need for spermidine and the availability of the other substrate (putrescine) for spermidine synthesis. S-adenosylmethionine decarboxylase (which has an enzyme-bound pyruvate as cofactor) is also present in mammalian tissues in very small amounts equal to 0.015% of the soluble protein in ventral prostate and to 0.0007% in liver (38). Its activity is also regulated by many hormones and other growth-promoting stimuli.

The transfer of the aminopropyl group from decarboxylated S-adenosylmethionine to putrescine is catalyzed by spermidine synthase. Another aminopropyl group is needed to convert spermidine into spermine, and this also comes from decarboxylated S-adenosylmethionine in a reaction catalyzed by a second aminopropyltransferase termed spermine synthase. Despite the similarity between these reactions, spermidine synthase and spermine synthase are discrete enzymes each specific for its own particular substrate (42). The aminopropyltransferases are present in many cells in amounts much greater than the decarboxylases and are thought to be regulated by the availability of their substrates, particularly decar-
These acetyl derivatives are extremely good substrates for the enzyme polyamine oxidase, which cleaves at the internal nitrogen to yield N-acetylpropionaldehyde and putrescine or spermidine depending on the substrate (7, 54). Although polyamine oxidase can be shown to work on the polyamines themselves in vitro, this reaction requires unphysiological levels of aldehyde activators, whereas the acetyl derivatives are very rapidly oxidized under physiological conditions (54). Therefore, it appears probable that acetylation is the limiting step in this interconversion, and it has been shown that spermidine-N1-acetyltransferase is rapidly induced and increased manyfold after exposure to toxic agents that enhance the conversion of spermidine into putrescine and spermine into spermidine (39). Only very small amounts of the acetylated derivatives are present in rat tissues even at the peak of induction because of the much greater activity of polyamine oxidase. In the mouse, larger amounts of N1-acetyl spermidine and N1-acetyl spermine can be found because mouse tissues have twenty times lower levels of polyamine oxidase (47, 54). However, even in this species, the acetylated derivatives are all degraded within a few hours (47).

The stimuli leading to the largest stimulation of polyamine interconversion are exposures to toxic agents, but the process is also induced by fasting and by exposure to excess spermidine itself (39, 54) and may be of physiological importance in preventing the level of spermidine and spermine from exceeding certain limits.

Degradation of polyamines. Instead of conversion into spermidine, putrescine can be oxidized by diamine oxidase, yielding \( \gamma \)-aminobutyraldehyde, which can be further oxidized to \( \gamma \)-aminobutyrate (GABA), or the \( \Delta^1 \) pyrroline (the spontaneously cyclized form of \( \gamma \)-aminobutyraldehyde) may be converted into 2-pyrrolidone and 5-hydroxy 2-pyrrolidone (54, 64). Putrescine can also be acetylated by a microsomal enzyme and the monoacetyl-pu- trescine oxidized by monoamine oxidase. This pathway can also give rise to GABA and is more important in GABA formation from putrescine in those tissues like brain that have little diamine oxidase (53).

Acetylation of putrescine, spermine, and spermidine can also be brought about by a nuclear enzyme that, with spermine as substrate, forms predominantly the N1-acetylspermidine derivative. The only known metabolic fate for this conjugate is deacetylation by an enzyme that can also degrade monoacetylpurines, but not N1-acetyl spermidine (54). Acetylated polyamines are found in blood and urine, but in quite small amounts (54), and the importance of acetylation in permitting polyamine excretion is unclear.

No other intracellular metabolic reactions for polyamines are firmly established, but transglutaminases can incorporate polyamines into proteins, and protein or peptide-linked polyamines have been reported but have not been fully characterized. Extracellular oxidation of polyamines by serum oxidases yields aldehyde derivatives from the primary amino moieties. These aldehydes are unstable and can liberate putrescine and acrolein but can also be taken up into the cell and further metabolized, producing putreanine, \( N^2 \)-carboxyethyl-spermidine, and possibly spermid acid (54). These derivatives have been found in small amounts in normal tissue and urine but may be produced on a large scale only in experiments.

### Table 1. Content of polyamines and related substances in control and regenerating rat liver and in SV3T3 cells

<table>
<thead>
<tr>
<th>Substance</th>
<th>Liver, nmol/g</th>
<th>Regenerating Liver, nmol/g</th>
<th>SV3T3 Cells, fmol/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine</td>
<td>10</td>
<td>42</td>
<td>0.26</td>
</tr>
<tr>
<td>Spermidine</td>
<td>1,380</td>
<td>2,510</td>
<td>3.18</td>
</tr>
<tr>
<td>Spermine</td>
<td>980</td>
<td>2,000</td>
<td>1.17</td>
</tr>
<tr>
<td>S-adenosylmethionine</td>
<td>68</td>
<td>74</td>
<td>0.09</td>
</tr>
<tr>
<td>Deacetylated S-adenosylmethionine</td>
<td>1.5</td>
<td>2.4</td>
<td>0.0008</td>
</tr>
<tr>
<td>5'-methylthioadenosine</td>
<td>1.2*</td>
<td>1.4*</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Regenerating liver was 32 h after partial hepatectomy. Cells were exposed to 5 mM N-acetylpropionaldehyde (DFMO) for 3 days (see Refs. 40, 46). *These values may be overestimates due to degradation of S-adenosylmethionine to 5'-methylthioadenosine during sample preparation.

The other product of the aminopropyltransferase reactions is 5'-methylthioadenosine. Although this nucleoside is produced in stoichiometric amounts with the polyamines, its concentration in the cell (Table 1) is very low due to rapid degradation by a phosphorylase producing adenine and 5'-methylthioribose-1-phosphate (71). The adenine is then converted to 5'-AMP by action of adenine phosphoribosyltransferase and the 5'-methylthioribose-1-phosphate is converted back to methionine in a reaction that conserves the methylthio group and all but the C-1 of the carbon atoms of this sugar. Therefore, all of the S-adenosylmethionine molecule not used for polyamine production is effectively salvaged. 5'-Methylthioadenosine phosphorylase is a widely distributed enzyme that is present in all normal tissues examined in amounts sufficient to maintain 5'-methylthioadenosine at very low levels (71). Certain tumor cell lines including some derived from humans have lost this enzyme, and these appear to excrete 5'-methylthioadenosine to reduce the intracellular content of this nucleoside. It cannot be ruled out that 5'-methylthioadenosine (which has a number of effects on cellular physiology when applied exogenously) rather than the polyamines is the critical product of the pathway in Fig. 1, but the rapid degradation of more than 99% of this product argues against this possibility.

**Interconversion.** The spermidine synthase and spermine synthase reactions are effectively irreversible, but it has been known for many years that conversion of spermine into spermidine and spermidine into putrescine can occur in vivo. This interconversion takes place by the action of two enzymes, spermidine N1-acetyltransferase and polyamine oxidase. The former enzyme uses acetyl CoA to convert spermidine into N1-acetyl spermidine and will also acetylate spermine forming N1-acetyl spermine (39). These acetylated derivatives are extremely good substrates for the enzyme polyamine oxidase, which cleaves at the internal nitrogen to yield N-acetylpropionaldehyde and putrescine or spermidine depending on the substrate (7, 54). Although polyamine oxidase can be shown to work on the polyamines themselves in vitro, this reaction requires unphysiological levels of aldehyde activators, whereas the acetyl derivatives are very rapidly oxidized under physiological conditions (54). Therefore, it appears probable that acetylation is the limiting step in this interconversion, and it has been shown that spermidine-N1-acetyltransferase is rapidly induced and increased manyfold after exposure to toxic agents that enhance the conversion of spermidine into putrescine and spermine into spermidine (39). Only very small amounts of the acetylated derivatives are present in rat tissues even at the peak of induction because of the much greater activity of polyamine oxidase. In the mouse, larger amounts of N1-acetyl spermidine and N1-acetyl spermine can be found because mouse tissues have twenty times lower levels of polyamine oxidase (47, 54). However, even in this species, the acetylated derivatives are all degraded within a few hours (47).

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with pharmacologic doses of polyamines inasmuch as the extracellular polyamine content is normally low.

Transport of polyamines. An active transport system distinct from those for amino acids is present in all cells tested and can take up putrescine or the polyamines. This system is stimulated by polyamine-depleted cells and can maintain cellular polyamine levels when only very low extracellular concentrations are provided. The role of the transport system under normal circumstances is unclear because extracellular polyamine concentrations are normally small and intracellular synthesis is used to provide polyamines. The transport system can also take up other basic substances including some drugs such as methylglyoxal bis(guanylhydrzone) (MGBG, see below), and stimulation of its activity by polyamine depletion may provide a mechanism to enhance their uptake (20). Some cell mutants have been isolated that lack the transport system and these are resistant to MGBG (28).

Efflux of polyamines from some cultured cells occurs when cell growth is restricted by inhibitors, confluence, or lack of growth factors. Only spermidine is excreted under these conditions, although the content of spermine in the cell also declines, and it appears to be converted to spermidine prior to excretion. Such efflux did not occur in transformed fibroblasts (6) or in cells infected with herpes viruses (69), but at present the mechanism and regulation of the release is not understood.

Inhibition of polyamine synthesis. Many substances inhibiting enzymes in the polyamine biosynthetic pathway (particularly ornithine decarboxylase) have been described (15, 21, 44, 55, 57). Ornithine decarboxylase is inhibited competitively by α-methylornithine and in a complex manner by α-hydrazino-δ-aminopropionic acid (HAVA; also known as α-hydrazinoornithine). Some interesting experiments were carried out with these compounds but more recently enzyme-activated irreversible inhibitors have been synthesized (57). Compounds of this type such as α-difluoromethylornithine (DFMO) (Fig. 2), α-ethylnornithine, and 5-hexylyl-1,4-diamine have very high specificity and significantly reduce putrescine synthesis in vivo. Labeled DFMO can also be used to titrate the number of active ornithine decarboxylase molecules, to localize the enzyme in the cell, and to label it both in vitro and in vivo since it forms a stable covalent bond with ornithine decarboxylase and no other protein (41, 51).

S-adenosylmethionine decarboxylase is strongly inhibited by MGBG (Fig. 2), and this drug can be used to inhibit polyamine synthesis in vivo. However, it is not specific, also inhibiting diamine oxidase and has a structure with considerable resemblance to polyamines and is taken up by the same transport system (15, 21, 70). Therefore, it is difficult to prove that the effects of MGBG are due to polyamine depletion as reversal of its action by spermidine could be due to displacement of MGBG from intracellular sites or interference with drug transport.

Spermidine synthase is quite sensitive to dicyclohexylammonium sulfate for unknown reasons (15). It is also strongly inhibited by the transition-state analog, S-adenosyl-1,8-diaminooctane (AdoDATO), and this drug can be used to reduce spermidine synthase activity in vivo (13). At present, there are no selective inhibitors for spermine synthase, although 5'-methylthiothreoborcidin (a deaza derivative of 5'-methylthioadenosine, which is not degraded by the phosphorylase) does inhibit spermine synthase more strongly than spermidine synthase (15, 70). There are no specific inhibitors available for the acetolysyl/oxidase pathway.

The effect of potent ornithine decarboxylase inhibitors such as DFMO on cellular polyamines is to decrease the content of putrescine and spermidine to virtually undetectable values, but there is little change in spermine (see Table 1). The small amount of putrescine that is produced even in the presence of the inhibitor is very efficiently converted into spermine. Also, since cell growth decreases and the degradation of spermine is very slow, the content of spermine falls very little when expressed as content per cell. The combination of DFMO with AdoDATO reduced the spermine content of SV3T3 cells by 80% presumably by preventing the conversion of the residual putrescine into spermidine (43). Such cells, in which putrescine and spermidine were reduced by more than 95% and spermine by 80%, represent the greatest reduction in cellular polyamine levels yet achieved with inhibitors and grew very slowly unless spermidine was added.

One factor contributing to the maintenance of spermine in DFMO-treated cells is the enormous build-up of decarboxylated S-adenosylmethionine in such cells (see Table 1 and Ref. 40). This occurs because of the absence of putrescine and spermidine to act as aminopropyl acceptors and because S-adenosylmethionine decarboxylase increases in response to the decline in spermidine. The very large increase in decarboxylated S-adenosylmethionine could play a role in the effects of DFMO. For example, the recycling of adenine and methionine would be prevented, and interference with methyltransferase reactions could occur. Reversal of the effects by putrescine does not rule out this possibility as addition of putrescine rapidly reduces the accumulation of decarboxylated S-adenosylmethionine.

Turnover of enzymes involved in polyamine biosynthesis and interconversion. Three of the enzymes described above, namely, ornithine decarboxylase, spermidine N1-acetyltransferase, and S-adenosylmethionine decarboxylase, have very rapid rates of turnover. Ornithine decarboxylase has been shown to have a half life (t1/2) of less than 1 h in many different cells (21, 30, 44). Arguments have been raised that the fall in enzyme activity after inhibition of protein synthesis by cycloheximide by which many of these measurements have been made is an invalid method for demonstrating enzyme turnover. However, the very short t1/2 for ornithine decarboxylase has been confirmed by use of antibodies and recently by following the loss of the DFMO-labeled enzyme in vivo (51). The t1/2 of S-adenosylmethionine decarboxylase has also been confirmed to be of the order of 1 h by immunochemical techniques (38); although this has not yet been accomplished for spermidine-N1-acetyltransferase, the very rapid loss of this activity when the induced enzyme declines even in the absence of cycloheximide provides strong support that its t1/2 is also about 1 h (39).

The short half-lives of these three enzymes that appear to be the key regulators of putrescine and polyamine
content permit the rapid change in their activity in response to stimuli, and this is observed in many situations. Changes in the total activity of 10- to 100-fold can occur within a few hours, and for the decarboxylases it has been confirmed that these changes are parallel to changes in enzyme protein. Such a system is uncommon in eukaryotes in which regulatory fluctuations in enzyme activities are more usually accomplished by posttranslational modifications or by allosteric effectors.

One consequence of the short half life of ornithine and S-adenosylmethionine decarboxylase is that stabilization of the protein is able to bring about a considerable increase in the total enzyme present. Such stabilization occurs with competitive inhibitors such as α-methylornithine and MGBG and can play a major part in overcoming the effects of the inhibitors (30, 38). This problem is obviated by use of irreversible inhibitors such as DFMO, but even with such compounds the rapid rate of synthesis of new enzyme makes it essential to maintain the drug concentration at a high level since full activity can be restored rapidly once it drops. A constitutively high level of enzyme may also confer resistance to such inhibitors, and one way in which this might be brought about would be via a change in the degradation rate. A mutant clone of HTC cells having a much longer half-life for ornithine decarboxylase and resistant to inhibitors of this enzyme has been isolated by Mamont and colleagues (26).

As the aminopropyltransferases do not turn over rapidly, the use of inhibitors of these enzymes would not be subject to these problems, and this advantage may more than compensate for the higher activity of these enzymes, particularly since their substrate concentration of decarboxylated S-adenosylmethionine is normally low. However, at present, no irreversible inhibitors are available.

Polyamine Biosynthesis and the Cell Cycle

Polyamines and new polyamine biosynthesis have been associated for some time with the traverse of cells through the cell cycle. There is evidence for this apart from a number of early and even recent papers dealing with the phenomenon of induction of ornithine decarboxylase during cell proliferation (for reviews see Refs. 15, 30, 44). A few early reports suggested that, in some cells, exogenous polyamines were needed for a maximal growth rate suggesting that polyamines may be needed for cell growth. Recent studies have provided convincing evidence for this. Heby, Marton, and their co-workers (18) were able to demonstrate that cellular putrescine, spermidine, and spermine increased progressively as the cells traversed the cell cycle from G1 to mitosis. The increase was seen first for putrescine, then for spermidine, and finally for spermine, which reflects the order of the biosynthesis of the polyamines. They extended their work to show that the spermidine content of cells in culture showed a direct linear correlation with the specific growth rate, indicating to them that spermidine accumulation is an event primarily associated with the process of cell replication (18). They were also able to conclude that putrescine and spermidine participated in the regulation of the cellular growth rate; a high content seemed to augment, and a low content seemed to restrain the actual growth rate (15, 16, 18). Heby and Andersson (16) have recently attempted to dissect results obtained by flow cytometric analysis correlating the cell cycle with changes observed in the rate of polyamine biosynthesis. Using Chinese hamster ovary (CHO) fibroblasts, they found that synthesis of polyamines was initiated in mid G1 and that polyamines started to accumulate toward the end of the G1 phase. The rate of synthesis peaked as the cells started to synthesize DNA (S phase), and the highest polyamine content was observed at the beginning of the S phase. The levels of all three polyamines (and ornithine decarboxylase activity) decreased significantly during mid-S, but toward the end of the S phase they increased again. Prior to cell division the polyamine biosynthetic activity and the concentration of the polyamines reached a second maximum close to the levels achieved in late G1 to early S. After mitosis the cellular polyamine content decreased by dilution to values half of those previously seen. Thus the biphasic peaks of polyamine, i.e., putrescine and spermidine, synthesis that occur in late G1-early S and in S-G2 may be part of the cell's preparation for DNA synthesis and division. Very similar ideas were deduced from the results of McCann et al. (94) using rat hepatoma tumor (HTC) cells whereby biphasic increases of putrescine were noted in each generation period that occurred just before and after DNA synthesis.

Sunkara et al. (61) working with human cervical carcinoma (HeLa) cells found that although polyamine levels fluctuated throughout the cell cycle in a fashion similar to those reported by earlier investigators, the actual measured rates of polyamine synthesis during these periods did not necessarily correspond with the absolute levels. When diamine oxidase, one of two degradative enzymes involved in metabolism of the polyamines, was measured, it was found to be high during mitosis and late G1-early S phase whereas there is active DNA synthesis and when there is also a “valley” between two maximal levels of putrescine. Ornithine levels were also measured and found to fluctuate in a similar fashion, arguing that overall catabolism of polyamines and regulation of ornithine pools play an important role in the regulation of the intracellular concentrations of polyamines during the cell cycle. Other studies have also indicated there are large increases in ornithine pools at the beginning of the cell division cycle (33).

Specific Inhibition of Polyamine Biosynthesis and Effects on Cell Cycle and Cell Division

Cellular effects of inhibition. The correlative studies of looking at polyamine levels during the cell cycle could never definitively answer the question of the true relationship between the availability of polyamines and the cell growth and division processes. With the discovery and synthesis of inhibitors of the biosynthetic pathways much more conclusive experiments could be done. Several groups utilized MGBG, the competitive inhibitor of S-adenosylmethionine decarboxylase, to block the synthesis of spermidine in activated lymphocytes. They were able to see a dramatic effect on DNA synthesis that was related to the block in spermidine synthesis. Based on their data and the fact that the block on DNA synthesis...
was totally reversible by addition of spermidine, Morris and colleagues (11) postulated that the increased levels of spermidine and spermine generally seen in rapidly proliferating eukaryotic systems are necessary for enhanced rates of DNA replication.

One of the most dramatic findings that truly linked polyamines, DNA synthesis, and ultimately cell division involved the use of the competitive inhibitor of ornithine decarboxylase, a-methylornithine, in HTC cells (25). In this way, Mamont, McCann, and their co-workers (25) clearly and unambiguously demonstrated that in situ inhibition of ornithine decarboxylase in ITC cells induced to proliferate causes a rapid fall in the levels of putrescine, which is followed by a striking decrease of spermidine. In parallel with the depletion of spermidine, DNA synthesis and cell proliferation were inhibited. Addition of polyamines resulted in an immediate resumption of cell proliferation. A final important conclusion was the fact that such inhibition of ODC and depletion of putrescine and spermidine had essentially no irreversibly deleterious effects on the cells (25).

Subsequent experiments were carried out using the irreversible inhibitor of ornithine decarboxylase, DFMO, confirmed these findings. Exposure to DFMO restricted growth in HTC cells, L1210 mouse leukemia cells, and MA 100 human prostate adenoma cells with the effects again completely reversible by the addition of exogenous polyamines (27). This study also pointed out the in situ superiority of DFMO over a-methylornithine as the latter compound had no effect on the MA 100 cells (27). One of the most striking findings with 1DFMO was the effect on the survival of human small-cell lung carcinoma in culture. After the onset of decreased proliferation caused by polyamine depletion, there was a pronounced cell loss directly related to viability. These data suggest that such cells are among the most sensitive to the effects of polyamine depletion, a fact that may prove useful in the therapy of this human tumor (24, 57).

Inhibition of polyamine metabolism may also prove to be a means for restricting replication of some viruses in host cells. DNA viruses, including vaccinia and human cytomegalovirus, cause an induction of ornithine decarboxylase that may be necessary for viral replication in the host. With the use of MGBG, a methylornithine, and later DFMO (68), it was shown that polyamine biosynthesis is an absolute requirement for the replication of these viruses. Another factor to be considered is that the host cells themselves normally have large complements of polyamines that may or may not be available for virus-directed DNA synthesis. Tuomi et al. (67), for example, have shown that DFMO restricts replication of herpes simplex virus and Semliki forest virus, but only in pretreated host cells, suggesting that these viruses utilize the polyamine complements of their hosts.

Effects on cell cycle. The availability of such compounds as MGBG and DFMO have allowed for a more precise investigation of the effects of polyamine depletion on specific events in the cell cycle. Seyfried and Morris (56) concluded that using MGBG and DFMO alone or together specifically inhibited DNA replication in activated lymphocytes without altering the number of cells in S phase. Later, Harada and Morris (14) using a-methylornithine in CHO cells found that the longer doubling time of inhibitor-treated cells was a consequence of increases in the lengths of the G1 and S phases. Expansion of both were approximately double and proportional to the doubling time, whereas the lengths of G2 and mitosis were unchanged. Heby and Andersson (16) using either MGBG or a-methylornithine caused cell cycle perturbations resulting in accumulation of Ehrlich ascites cells in S and into G2. On the other hand, Rupniak and Paul (50) found that inhibition of polyamine biosynthesis with MGBG led to growth arrest of rat embryo fibroblasts in G1. Sunkara and his co-workers (60) may have resolved part of this seeming conflict when, using MGBG and later DFMO (59), they reported that “normal” fibroblasts (human PA2 cells and mouse 3T3 cells) were preferentially arrested in early G1 phase, whereas a majority of various “transformed” (CHO, HeLa, SV3T3) cells were blocked in S phase. When they examined the transformed cells for prematurely condensed chromosomes they found a significant decrease in the number of DNA replication sites, suggesting that polyamines may have an important role in DNA chain initiation. Their subsequent work with HeLa and CHO cells using MGBG and a-methylornithine suggests that spermidine may play a role in the structure and function of microfilaments during cytokinesis and division (62). All of these data confirm the fact that polyamines are important in the traverse of the cell cycle and that a restriction of polyamines will significantly affect new DNA synthesis and ultimately cell division.

In vivo effects of polyamine inhibitors. Putrescine biosynthesis was inhibited in vivo in mouse sarcoma-180 by a-hydrazinoornithine (HAVA) a potent but not completely specific competitive inhibitor of ODC (22). Neoplastic growth of the tumor was suppressed by HAVA and could be reversed by putrescine (22). With the availability of 1DFMO, Danzin and his colleagues (55) were able to demonstrate that in vivo administration of this inhibitor would lower polyamine levels in various rat tissues, i.e., ventral prostate, testis, thymus. They were thus able to selectively slow weight gain of the testosterone-induced regeneration of rat prostate (9). DFMO also suppressed the normal age-dependent hypertrophic and hyperplastic growth of rat prostate (57) and would also selectively slow the weight gain of young rats over a period of days (55). Subsequent studies clearly indicated the efficacy of 1DFMO in several in vivo tumor models including L1210 leukemia in mice, although the most dramatic effects were on the growth of EMT-6 mammary sarcoma (48). Administration of DFMO in the drinking water (3%) of mice 5 days after subcutaneous inoculation of EMT-6 cells resulted in an 80% inhibition of tumor weight gain by day 27 compared with controls. The acute oral median lethal dose (LD50) for DFMO was also determined to be 5,000 mg/kg, indicating DFMO treatment was essentially nontoxic.

DFMO has also been extremely useful in elucidating the importance of polyamine biosynthesis for the in vivo replication of several protozoan parasites. Dacchi (1) had suggested that polyamine biosynthesis was a critical target for the development of drugs against trypanosomatid flagellates. However, it was the dramatic discovery that DFMO administered in the drinking water of mice infected with Trypanosoma brucei brucei would completely cure the mice within 3 days that led to a general...
replication and polyamine biosynthesis (2). Subsequent work has established that 1) this effect can be completely antagonized by administration of polyamines back to infected animals during DFMO treatment, and 2) that DFMO does indeed inhibit \( T. b. brucei \) ornithine decarboxylase and deplete putrescine and spermidine in the parasite infecting the host animal (31). Other experiments have demonstrated that ornithine decarboxylase inhibition by DFMO will block replication of other African trypanosomes in vivo including \( T. b. rhodesiense \), a cause of human sleeping sickness (32). The parasite \( E. tenella \), an organism that causes coccidiosis in chickens, is also completely restricted from dividing in vivo by DFMO, which cures the infection (32). In vitro replication of the malaria parasite, \( P. falciparum \), is also restricted by DFMO, which again was shown to act by a specific inhibition of DNA synthesis (32). In each of the above examples, the specificity of the DFMO effect was proven by the fact that polyamines, administered simultaneously with the inhibitor, would reverse the inhibition and allow cell division to proceed.

Liver regeneration after partial hepatectomy is accompanied by a substantial increase in polyamine content (21) (see Table 1). Prevention of this increase by application of inhibitors retards liver growth (21, 46), but only part of the increase in polyamine content was essential for the stimulation of DNA synthesis (46). In contrast, thyroxin-induced cardiac hypertrophy, which is normally accompanied by an increased content of polyamines, was not blocked by DFMO or other inhibitors of ornithine decarboxylase even though these completely prevented the increase in polyamines (37). However, Bartolome et al. (4) report that DFMO attenuated isoproterenol-induced cardiac hypertrophy, suggesting that there may be fundamental differences in the requirement for polyamines in the mechanisms by which thyroid hormones and sympathomimetics elicit increased cardiac growth.

Tumor promoters such as phorbol esters are known to enhance ornithine decarboxylase activity and increase cell putrescine content in mouse skin. This can be blocked by application of DFMO, and such blockage greatly reduces the incidence of polycyclic hydrocarbon-initiated papillomas (66).

Potential of inhibitor combinations. Combining polyamine antimetabolites together or with other types of inhibitors has had unexpected results that can be defined as synergistic. Morris et al. (35) used MGBG and \( \alpha \)-methylornithine together in activated lymphocytes and found accentuation of inhibition of DNA synthesis with no effect on total protein or RNA synthesis. However, Järne et al. (20) demonstrated the potential of using DFMO together with MGBG. As mentioned above, MGBG is a “spermidinelike” analogue that shares a common cellular transport system with the natural polyamines. They showed that DFMO-pretreated and polyamine-depleted cells tended to more effectively retain higher levels of MGBG than untreated cells, this in turn leads to an accumulation of the drug and to rapid cell death. This exciting finding was reproducible in the same Ehrlich ascites cells grown in the peritoneal cavity of mice and has already led to therapeutic use of DFMO and MGBG together in human leukemia (20). Initial pilot studies with a number of patients have shown that a short “priming” with DFMO followed by a low dose of MGBG greatly increased the concentration of MGBG in the circulating leukemia blast cells. The combined regimen also produced a true therapeutic synergism (20). It is not completely clear at this point if part of the synergistic effect is due to combined inhibition of polyamine biosynthesis per se, but in light of the considerable amount of data indicating such effects in animal tumor models it seems likely that this may be true.

Other drugs have been tried in combination with DFMO, for example, cytotoxic drugs such as cytosine arabinoside (araC) and other drugs that have been used in anticancer chemotherapy. Combination of DFMO with araC, an S phase-specific drug, resulted in a synergistic killing of HeLa cells in culture (59). It was suggested that use of DFMO and araC in “nontransformed” cells, i.e., W1-38 and 3T3 cells, caused an arrest in G1 by polyamine depletion that, in fact, protected these cells from the cytotoxic effects of araC acting later in the traverse in S phase (59). Thus, in effect, use of a polyamine inhibitor in vivo such as DFMO might selectively potentiate effects of a cytotoxic drug in a tumor and help protect normal cells undergoing replication, an intriguing idea. Marton and colleagues (29) have recently shown that DFMO, used as single agent, has little or no effect on murine glioma 26 and rat 9L gliosarcoma intracerebral tumors. However, in both brain tumor animal models, pretreatment with DFMO before a single dose of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) significantly enhanced the effect of BCNU without increasing toxicity. DFMO, in combination with either vindesine or Adriamycin, produced significant increase of survival time of mice inoculated with L1210 leukemia cells and synergistically inhibited growth of a solid hepatoma (HTC) in rats in EMT-6 mammary tumors in mice (3).

One of the most interesting and potentially most useful combinations of a polyamine antimetabolite and a cytotoxic drug is that of bleomycin and DFMO used against trypanosome infections. Bleomycin, a potent antitumor antibiotic, cures infections of \( T. b. brucei \) and, most interestingly, such bleomycin cures are reversed by administration of spermidine and spermine. Recently, Bach and his collaborators (31) found that normally subeffective doses of bleomycin and DFMO acted together in a synergistic fashion to cure \( T. b. brucei \) infections. These amounts of drug represented \( \frac{1}{4} \) and \( \frac{1}{12} \) the amount of the individual curative dosages of DFMO and bleomycin, respectively. One of the most important subsequent results of this work was that A. B. Clarkson, Jr. and his colleagues (31) found that the DFMO-bleomycin combination somehow crosses the blood-brain barrier to cure sequestered acute \( T. b. brucei \) infections in the brain and the central nervous system. Neither drug alone, even at very high concentrations, has this effect against brain-involved infections that are today the major problem of the human disease as it exists in Africa.

The reason why a compound such as DFMO and so many different cytotoxic drugs are apparently effective in combination may lie with the ultimate biological and
biochemical actions of the polyamines in a cell and particularly in the nucleus. These drugs have effects directly at the DNA level, and as spermidine and spermine are known to be associated with DNA, it may well be that depletion of the normal component of polyamines makes such cells more susceptible to such DNA-acting cytotoxic drugs.

Finally, an approach of much potential value would involve treatments with combinations of strictly specific inhibitors of polyamine biosynthesis, something which MGBG is not. The synthesis of S-adenosyl-1,8-diamino-3-thiooctane (AdoDATO), an effective transition-state analog inhibitor of spermidine synthase, which for the first time effectively lowers spermine levels (43), will allow investigators to more precisely define the cellular effects of polyamine depletion.

Genetic Approaches

Another way to investigate the role or importance of polyamines in cell growth and division is the genetic approach. Growth-retarded polyamine deficient mutants have been isolated from microorganisms for several years, but achievement of the same sort of auxotrophs in mammalian cells is considerably more complex. Scheffler and his colleagues (23, 58) and Pohjanpelto et al. (45) independently have finally described true polyamine auxotrophs in mammalian cells. Landy-Otsuka and Scheffler (23) first reported an ODC phenotype in a temperature-sensitive line of CHO cells. More recently Steglich and Scheffler (58) have described an ODC mutant of CHO cells that is auxotrophic for putrescine and requires at least $10^{-5}$ M putrescine in the medium to maintain a normal growth rate.

Pohjanpelto et al. (45) have isolated a CHO cell mutant dependent on putrescine (5 x $10^{-5}$ M) or other polyamines. They showed that although putrescine and spermidine were not detectable in these cells after 2 days of putrescine starvation, cellular replication and DNA synthesis were not strongly affected until after 3-4 days. This agrees with results in which DFMO does not markedly cause inhibition of DNA synthesis and cell division until after polyamine depletion, i.e., the second generation period. Analogous to growth-arrested DFMO-treated cells, these putrescine-starved auxotrophs are still viable and will start to grow and divide after addition of polyamines to the medium (45). They also lack 90% of their actin filament bundles and show essentially no assembly of microtubules. Similar effects have been observed in CHO cells using MGBG to decrease spermidine levels (62). Thus depletion of polyamines may, in part, restrict cell division because the integrity of the microfilaments and microtubules depends on polyamines, a major clue in understanding the various cellular roles of the polyamines.

Polyamines and Differentiation

There is convincing evidence that polyamines play a role in the differentiation of mammalian cells and that interference with polyamine biosynthesis can influence the state of differentiation. An elegant series of studies by Oka and colleagues (36) have shown that spermidine is needed for milk protein synthesis by the cultured mouse mammary gland. Synthesis of casein and $\alpha$-lactalbumin is blocked by inhibition of spermidine production, and the requirement for glucocorticoids in this production of milk proteins can be replaced completely for $\alpha$-lactalbumin and partially for casein by provision of exogenous spermidine. An increased synthesis of putrescine and spermidine is brought about by the combination of insulin, cortisol, and prolactin, which in the mouse mammary gland are all needed for milk production. In other species, the requirement for glucocorticoid or increased spermidine content is not so clear, but spermidine is still required for normal development. The increased synthesis of polyamines, particularly putrescine, is needed for mammary cell growth, but the requirement for spermidine for milk protein production appears to be distinct from the need for polyamines for growth. Similarly, not only growth and nucleic acid synthesis but the secretory functions of the rat ventral prostate are inhibited by interference with polyamine synthesis by DFMO (9).

The prevention of polyamine synthesis by DFMO has striking inhibitory effects on embryonic development in a wide variety of species. Contragestational effects were observed in mice, rats, and rabbits when DFMO was present during early embryonic development (12). The critical time period in the mouse was over the period around days 5-8 of gestation, which corresponds to a time of normally very high ornithine decarboxylase activity in deciduomal extracts. Development of the chick embryo was blocked by DFMO at the primitive streak phase (gastrulation), which corresponds well with mammalian results (17). A similar block at gastrulation was seen in the invertebrate, Ophryotrocha labronica, and an inhibition of formation of nucleoli was seen suggesting that polyamine synthesis may be essential for ribosomal gene expression that occurs at this stage (17). Evidence that DFMO-modulated reduction of putrescine content inhibits the maturation of Xenopus oocytes under the influence of progesterone or human chorionic gonadotropin is consistent with this finding and shows that putrescine is needed for amphibian oocyte development (63).

The differentiation of Friend erythroleukemic cells in culture to erythroid cells under the influence of dimethylsulfoxide and other agents has been reported to be prevented by inhibition of polyamine synthesis and is accompanied by an increase in ornithine decarboxylase activity (13). Unfortunately, these experiments were not carried out with the most potent inhibitors of ornithine decarboxylase, and the reversal of the inhibitory effects of MGBG by the very high levels of polyamines that were used is not satisfactory evidence for specificity. Enhanced putrescine and spermidine levels were produced in response to agents inducing terminal differentiation in human HL-60 promyelocytic cells suggesting a role for polyamines in this process (19). However, the differentiation was not affected by exposure to DFMO or MGBG. The differentiation of neuroblastoma cells is accompanied by marked changes in polyamine metabolism including reduced inducibility of ornithine decarboxylase, increased conversion of putrescine to GABA, and a decreased covalent binding of a putrescine metabolite to an 18,000 M, protein (8), but the significance of these
changes in the differentiative process have not yet been established.

Polyamines have been implicated in the complex process of bone differentiation. Rath and Reddi (49) found that polyamine synthesis was enhanced during osteogenesis using a matrix-induced endochondral bone differentiation model system. Suzuki and colleagues (65) have established a matrix-induced endochondral bone differentiation model system. Suzuki and colleagues (65) have established.

Overall, it is frequently difficult to separate effects on cellular proliferation from those on differentiation as many differentiative processes require cell division. The requirement for polyamine synthesis for cell growth is well established and differentiation can clearly be affected in this way. It appears likely that a most direct involvement of polyamines in differentiation may also occur in some systems.

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