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Current Status of the Polyamine Research Field

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Abstract

This chapter provides an overview of the polyamine field and introduces the 32 other chapters that make up this volume. These chapters provide a wide range of methods, advice, and background relevant to studies of the function of polyamines, the regulation of their content, their role in disease, and the therapeutic potential of drugs targeting polyamine content and function. The methodology provided in this new volume will enable laboratories already working in this area to expand their experimental techniques and facilitate the entry of additional workers into this rapidly expanding field.

Keywords

Putrescine; Spermidine; Spermine; Hypusine; Antizyme; Polyamine transport

1. Structure of Physiological Polyamines

Mammalian tissues contain putrescine (1,4-diaminobutane), which is strictly speaking a diamine, spermidine, and spermine (1), while a wider spectrum of natural polyamines exists in other organisms (Fig. 1). Plants have these three polyamines and also thermospermine (2, 3) and agmatine. Some fungi and some, but not all, bacteria contain only putrescine and spermidine since they lack a spermine synthase. Specific organisms also contain other diamines such as 1,3-diaminopropane and cadaverine (1,5-diaminopentane) and *sym*-norspermidine (caldine). Thermophilic bacteria and archea are a rich source of polyamines including these compounds and a wide variety of other polyamines. Since thermophiles were the first source for isolation of polyamines such as thermospermine, caldine, and thermine (Fig. 1) this explains their trivial names, but these polyamines are not unique to organisms living at high temperature. Similarly, spermidine and spermine are so named because the first report of crystals in seminal plasma by Leeuwenhoek was subsequently shown to be due to the precipitation of insoluble spermine phosphate, but these polyamines are present in all tissues and many extracellular fluids of mammals (4).

2. Synthesis of Polyamines

Diamines such as putrescine, cadaverine, and 1,3-diaminopropane are formed by the decarboxylation of the relevant amino acid (L-ornithine, L-lysine and L-1,4-diaminobutyric acid respectively); reactions catalyzed by pyridoxal phosphate-dependent decarboxylases (Fig. 2a) (5). An alternative pathway to putrescine occurs in plants and microorganisms in which L-arginine is decarboxylated forming agmatine (6). This can then be converted to putrescine either by the reaction of agmatinase releasing urea, as in *E. coli,* or by the combined actions of agmatine deiminase releasing ammonia forming *N*-carbamoylputrescine and *N*-carbamoylputrescine amidase as in plants.

There are two pathways for the addition of aminopropyl groups to form the higher polyamines. By far the most extensively studied is that first demonstrated by the Tabors (7), which occurs in mammals, plants, fungi, and many bacteria including *E. coli*, where decarboxylated *S*-adenosylmethionine (dcAdoMet) is used as the aminopropyl donor. This

nucleoside is produced by the action of the pyruvoyl-dependent *S*-adenosylmethionine decarboxylase (AdoMetDC) (8, 9). Enzymes termed aminopropyl-transferases (10–12) use dcAdoMet and an amine acceptor to form the higher polyamines (Fig. 3). Thus, putrescine is used as a substrate by the aminopropyltransferase, termed spermidine synthase, to form spermidine. A distinct aminopropyltransferase, spermine synthase, then uses a second dcAdoMet molecule to add the aminopropyl group to the N^8 of spermidine forming spermine. In addition to a spermine synthase, plants contain a thermospermine synthase that attacks the N^1 end of spermidine producing thermospermine. The aminopropyltransferases mentioned above in mammals and plants, and in some bacteria and fungi, are quite specific for the amine acceptor (13). Other organisms including the thermophiles have less specific aminopropyltransferases that can make a variety of polyamines including thermine. Some contain an enzyme that uses agmatine to produce aminopropylagmatine (Fig. 3), which is then converted into spermidine and urea by an agmatinase-like enzyme allowing for the production of spermidine without a putrescine intermediate. It is likely that some thermophiles and some other organisms such as diatoms where very long chain polyamines are involved in biomineralization of the shell contain additional aminopropyltransferases supporting their synthesis but these enzymes have not been characterized.

The polyamine biosynthetic pathway dependent on dc-AdoMet described above is very well understood and methods for the assay of these enzymes including L-ornithine decarboxylase (ODC), AdoMetDC, and aminopropyltransferases were provided in Volume 79 of this series published in 1998.

There is, however, a second pathway for the synthesis of polyamines containing aminopropyl moieties in which L-aspartic-β-semialdehyde is used to provide the aminopropyl group (14, 15) (Fig. 4). The condensation of this molecule with putrescine forms carboxyspermidine, which is then decarboxylated to form spermidine. Similar reactions using 1,3-diamino-propane form *sym*-norspermidine and the same enzymes (termed carboxynorspermidine synthase and carboxynorspermidine decarboxylase) are able to bring about both syntheses (Fig. 4). This pathway is used in many microbes.

Finally, there is also a pathway for the synthesis of *sym*-homospermidine, which involves the use of NAD and putrescine with either a second molecule of putrescine or spermidine to generate the product via condensation with 4-aminobutyralehyde intermediate (16, 17) (Fig. 5a). In plants, the homospermidine synthase, which is responsible for the latter reaction, is derived from deoxyhypusine synthase (18), a critical enzyme for a posttranslational modification, which involves polyamines and is described below.

The second chapter of this volume describes genomic methods for identifying key polyamine biosynthetic genes and thus determination of the pathways used in particular organisms. This is a very important area since polyamines are essential for normal growth and their biosynthetic pathways provide targets for drug development. Organisms such as *Vibrio cholerae* that use the L-aspartic–β-semialdehyde condensation pathway may be sensitive to potential therapeutics that are innocuous to host organisms that do not depend on these reactions.

Polyamines with quaternary ammonium centers such as tetrakis(3-aminopropyl)ammonium (Fig. 1) or tertiary N atoms such as mitsubishine (see Chapter 5) respectively are found in acute thermophiles and are needed for growth at extreme temperatures (19, 20). The biosynthetic reactions leading to these polyamines have not yet been elucidated.

3. Functions of Polyamines

Polyamines have a multitude of functions affecting growth and development, and these pleiotropic effects complicate efforts to understand the physiological and pathophysiological effects of perturbing polyamine content. Recent studies have identified a number of key areas in which polyamine effects are initiated (21–23). These include regulation of gene transcription, multiple effects on posttranscriptional regulation, control of the activity of ion channels as well as modulation of protein kinase activities, the cell cycle, membrane structure/function, and nucleic acid structure and stability.

A critical new concept increasing understanding of the role of polyamines in maintaining optimal growth rates and cell viability has been provided by studies showing that there is a bacterial “polyamine modulon” that consists of a set of genes whose expression is increased by polyamines as a result of increased translation (24, 25). Polyamines stimulate translation in a number of ways including alteration of mRNA structure allowing initiation of protein synthesis encoded by genes that lack Shine–Dalgarno sequences or have them placed at nonoptimal positions. The proteins whose synthesis is directly stimulated by polyamines also include transcription factors and kinases that can in turn enhance gene expression of other proteins. The polyamine modulon concept has been extended to yeast (26) and is likely to also apply to mammalian cells (27). Methods for identifying members of the polyamine modulon are described in Chapter 3.

In eukaryotes, another factor is also involved in the role of polyamines in stimulating gene expression. This is the protein eIF5A, which is only active after a posttranslational modification to form hypusine (Fig. 5b). This reaction has an absolute requirement for spermidine as a precursor. The functions of eIF5A have been the subject of considerable debate and it may have multiple functions, but recent studies indicate that it is a translation elongation factor (28–30). Many other sites of posttranscriptional gene regulation, such as mRNA transport, and turnover are also influenced by polyamines either via eIF-5A or other proteins. These include RNA-binding proteins such as the HuR family(31–33). The HuR proteins are highly regulated by polyamines and methods for their study are described in Chapter 4.

The novel polyamines present in thermophiles are essential for growth at higher temperatures and have effects on nucleic acid stability and structure, and in protein synthesis (19, 20). Methods for the synthesis and the study of the function of these polyamines are described in Chapter 5.

A new and critically important area in polyamine research was revealed when it was observed that the steep voltage-dependence of the inwardly rectifying potassium (Kir) channels is caused by the binding of polyamines (34) and that polyamines profoundly affect the activities of NMDA receptors (35). Subsequent studies have shown that a wide variety of ion channels are affected by polyamines including the Kir potassium channels, which control membrane potential and potassium homeostasis in many cell types, glutamate receptors that mediate excitatory synaptic transmission in the mammalian brain, as well as other channels affecting intracellular calcium signaling, Na^+ transport, and some connexin-linked gap junctions (22, 23). Chapter 6 describes methodology characterizing polyamine interactions with both prokaryotic and eukaryotic Kir channels.

4. Use of Transgenics to Investigate Polyamine Function

The ability to generate transgenic rodents that have reductions or increases in polyamine content due to alteration in the activities of key enzymes has now provided important tools

to evaluate polyamine function in mammals (36–38). Various aspects of these studies are covered in Chapters 7–9.

There is much evidence linking elevated polyamine content to tumor growth and development. Consequently a number of animal models have been developed in which polyamine content is disturbed and studies are carried out on their susceptibility to tumor induction by chemicals, oncogene activation, UV radiation, and other stimuli. Remarkably, even the small reduction in ODC activity brought about by the *Odc* heterozygosity in *ODC*$^{(+/-)}$ transgenic mice reduced lymphoma and skin tumor development (39, 40). Similarly, moderately increased levels of antizyme (AZ), which as described below is a negative regulator of ODC, dramatically lowered skin carcinogenesis in response to two-stage carcinogenesis protocols (37, 41). The use of these and other models for studying the role of polyamines in neoplasia is described in Chapter 7.

These studies correlate well with a number of investigations using human patients that show that interference with polyamine synthesis by drugs such as DFMO (an irreversible inactivator of ODC) has a profound cancer preventative effect (42). Thus, clinical trials examining the reoccurrence of colonic polyps have shown that prolonged treatment with this drug combined with sulindac caused a 70% reduction of recurrence of all adenomas, and over a 90% reduction in advanced and/or multiple adenomas (43). Treatment with DFMO also reduced the development of non-melanoma skin cancer in subjects with a previous history of this disease (44). Some encouraging results in a Phase IIb clinical trial suggest that DFMO may be useful for chemoprevention of prostate cancer (45). At present, all of the clinical data supporting the use of the polyamine pathway for cancer chemoprevention are derived from studies with DFMO, but animal studies suggest that polyamine analogs described below and the targeting of drugs to other enzymes in the pathway, such as spermidine synthase (which like ODC is regulated by the oncogene *c-myc*), may also be successful (46).

Remarkable phenotypic changes occur in rodents in which the activity of the catabolic enzyme spermidine/spermine-N^1-acetyltransferase (SSAT) (Fig. 6) is increased. This contrasts to the relatively minimal effects in untreated mice with inactivation of the *Sat1* gene. The lack of significant changes in these mice is consistent with the concept that normally SSAT levels are very low and that physiological and pathophysiological effects of SSAT occur in response to its induction (47, 48). Multiple lines of transgenic mice and transgenic rats overexpressing SSAT have been described (47, 49). These mice also show a wide variety of other defects including hair loss, female infertility, weight loss, CNS effects, altered carbohydrate and lipid metabolism, a tendency to develop pancreatitis and altered responses to chemical carcinogens. These effects are related not only to the alterations in polyamine content, but also to the effects on levels of acetyl-CoA, ATP, and oxidative damage associated with the increased activity of the SSAT/APAO pathway (Fig. 4) described below, and the increased metabolic flux through the polyamine pathway due to the futile cycle set up by compensatory increases in polyamine synthesis in response to constitutively elevated SSAT levels (48). Chapter 7 describes methods to study pancreatitis and altered lipid and carbohydrate metabolism in rodents with elevated expression of SSAT.

The critical importance of polyamines for mammalian development is shown by studies with mouse knockouts of the *ODC* or *AdoMetDC* genes, which are lethal at very early embryonic stages. Spermidine synthase knockouts would almost certainly also be lethal since spermidine is essential for viability in yeast (50). In contrast, mice lacking spermine synthase do survive on an appropriate strain background and can be used to evaluate the specific functions of spermine (51). Chapter 9 describes the phenotypic changes in these

mice and their use to evaluate the role of spermine in growth, behavior, fertility, hearing, and cardiac function.

Transgenic approaches have also been used to evaluate the functions of polyamines in plants. It is noteworthy that although many plants contain both ODC and arginine decarboxylase, the former is not required in all species since there is no gene for ODC in *Arabidopsis thaliana* (52). Genes for AdoMetDC and arginine decarboxylase are essential for normal plant development as is the gene for thermospermine synthase (2, 3, 13, 53, 54).

5. Polyamine Catabolism

The enzymatic reactions forming polyamines are effectively irreversible. Polyamines are interconverted and removed by a combination of acetylation, oxidation, and excretion to vacuoles or extracellular fluids. Putrescine can be attacked by diamine oxidase and other copper-dependent enzymes that oxidize the terminal amino groups of spermidine and spermine are well known (55–57). These enzymes are present extracellularly in mammals. The products of their reaction, which can rearrange to form acrolein, are highly toxic (58, 59) and this reaction is the basis of many publications that erroneously claim that polyamines exert direct toxic effects on the basis of observations that addition of polyamines to cell cultures causes cell death. The observed toxicity in such systems is due to the formation of toxic metabolites by enzymes in the serum used to support cell culture. Although elevated levels of intracellular polyamines can lead to apoptosis or increased formation of reactive oxygen species, the polyamine transport systems are highly regulated to avoid uptake of excess polyamines.

Interconversion of polyamines in mammals is brought about by two FAD-dependent oxidases (Fig. 7). Spermine oxidase (SMO), first described in 2001, is specific for spermine acting on it to produce spermine, H_2O_2, and 3-aminopropanal (48, 60). It exists as multiple forms due to splicing variations and is highly inducible by polyamine analogs and other stimuli. The other oxidase, N^1-acetylpolyamine oxidase (APAO), is generally a constitutively expressed enzyme that has minimal activity on free polyamines, but is highly active on N^1-acetylspermidine (forming putrescine) and N^1-acetylspermine (forming spermidine). With both substrates, H_2O_2 and *N*-acetyl-3-aminopropanal are produced (48, 61). The substrates for APAO are produced by SSAT (Fig. 6), which is very highly regulated and inducible by polyamines (48). Therefore, the SSAT/APAO pathway acts to prevent the overaccumulation of polyamines. This occurs at the expense of the generation of reactive oxygen species (ROS) and potentially toxic aldehydes. Such damage may be limited by the subcellular localization of APAO, which is a peroxisomal enzyme, whereas similar production of ROS by SMO may be more damaging due to its nuclear and cytoplasmic localization. Assay of SMO is described in Chapter 10.

Plants also use polyamine oxidation to interconvert polyamines H_2O_2 that may and regulate polyamine content and also to form act as a signaling molecule (54, 62–64). Methods for the investigation of these enzymes are described in Chapter 11.

6. Formation and Function of Hypusine

One essential function of polyamines in eukaryotes, as stated above, is to serve as a precursor of hypusine [N^{ε}-(4-amino-2-hydroxybutyl)lysine] (Fig. 5b). Hypusine production is a posttranslational modification of the protein eIF5A. This modification is essential for the activity of eIF5A, which as described above is needed for protein synthesis at the elongation step and probably also for other functions essential for growth and for viral replication (29, 50, 65–67). Drugs preventing the hypusination of eIF5A inhibit the expression of HIV-1 genes (68). The ability to serve as the source of hypusine can only be fulfilled by

spermidine, which is used as a substrate by the enzyme deoxyhypusine synthase (66). This enzyme attaches the aminobutyl group of spermidine to a specific lysine residue in the protein eIF5A forming deoxyhypusine (Fig. 5b). This protein is then further modified by deoxyhypusine hydroxylase (69) to produce the complete hypusinated eIF5A. Assays for these enzymes are described in Chapters 12 and 13.

7. Regulation of Polyamine Content

In mammals, polyamine content is controlled primarily by alterations in the activity of the key enzymes ODC, AdoMetDC, and SSAT, along with alterations in efflux and uptake (22, 70, 71). Changes in ODC and SSAT activity are brought about by alterations in the content of enzyme protein and this level is controlled at the levels of transcription, mRNA stability, translation, and protein turnover (47, 48, 71–73). AdoMetDC is formed as a proenzyme that undergoes an autocatalytic cleavage to form its pyruvoyl cofactor and its two subunits (8, 22, 71). AdoMetDC activity is also varied by alterations in the level of the processed protein and, additionally, by activation of the protein by putrescine in mammals, yeast, and some other organisms (8, 22, 74) and by binding of an activating protein (75–77) in trypanosomes. This protein, which was discovered in 2007, has been named prozyme. Methods to identify and assay this important regulatory molecule in these parasites that are responsible for major human diseases including African sleeping sickness, Chagas disease, and Leishmaniasis are described in Chapter 14. DFMO is highly effective as a therapeutic agent for some forms of African sleeping sickness, but other forms and other parasitic diseases are less susceptible to DFMO and AdoMetDC may be a critical target for their treatment (78, 79).

It has been known for 40 years that ODC protein has a very rapid rate of turnover (80). More recent studies have shown that a key part of the regulation of ODC content is due to two proteins that influence the proteasomal degradation of ODC termed antizyme (AZ) and antizyme inhibitor (AZIn) (71, 72, 81, 82). AZ was first described and named in 1976 as an inducible protein inhibitor of ODC (83). It does act as an inhibitor by binding to the ODC monomer. ODC functions as a homodimer with two active sites formed at the dimer interface. Association between the two subunits is quite weak and the dimers are in rapid equilibrium with inactive monomers. Therefore, binding of AZ causes loss of ODC activity, but more importantly it targets the protein for degradation by the 26S proteasome. AZ acts in a catalytic manner in this reaction since most of the AZ is released and can cause further ODC degradation (72, 84). AZ is not restricted to mammals having been found in yeast and many other species (85, 86).

AZ synthesis is increased in response to high polyamine levels predominantly via increasing a +1 frameshifting mechanism, which is needed to allow read-through of a stop codon that prevents AZ synthesis (87, 88). Polyamines stimulate this frame-shifting, hence increasing the level of AZ and providing a regulatory feedback to control polyamine levels. Procedures for quantifying, assay, and characterization of AZ, which has at least four isoforms, some of which may be phosphorylated (89), are described in Chapter 15. AZ-3 is expressed specifically in germ line cells during spermatogenesis and is needed for haploid germ cell differentiation (90).

A second component of the system regulating ODC protein stability is AZIn (22, 73, 91). This protein, which has a structure similar to that of ODC itself, but does not dimerize or have any activity (92), binds to AZ more tightly than ODC. It can therefore displace ODC from the ODC:AZ complex or prevent this complex from forming, thus preventing ODC degradation. There are two *AZIn* genes (91, 93) and inactivation of the *AZIn-1* gene is lethal (94). Methods for the study of AZIn are covered in Chapter 16.

The ODC gene promoter region contains sequences including E-boxes, a cAMP response element, CAAT and LSF motifs, AP-1 and AP-2 sites, GC-rich Sp1 binding sites, and a TATA box. These binding sites allow for regulation of the synthesis of ODC mRNA by many factors including oncogenes such as c-*Myc*, hormones, tumor promoters, and other growth factors (21, 22, 36, 65, 95, 96). There are also many studies showing that translational regulation of ODC synthesis via the long 5′UTR of its mRNA affects ODC production. Both cap-dependent and internal ribosome entry site (IRES)-mediated translation have been reported with the initiation factor eIF4E playing an important regulatory role (97, 98). Early studies also suggested that the 3′UTR of the ODC mRNA might exert regulatory activity (99) and this possibility has now been confirmed with studies showing that mRNA content is also regulated at the level of mRNA stability via interactions of key proteins with this region. Chapter 17 describes methods for studying the posttranscriptional regulation of ODC.

8. Polyamine Transport

The polyamines exist predominantly in a charged form at physiological pH and are excluded by cell membranes. The existence of transport systems underlying both uptake and efflux has been known for many years and several chapters outlining methods to determine polyamine transport into and out of the cell were included in Volume 79 of this series. At that time, almost nothing was known about the mechanism of this transport and the components of the transporters. Recently, great progress has been made in the characterization and study of these systems. This work has been led by a series of elegant studies in which the components of the carrier-mediated systems for both uptake and efflux of polyamines in bacteria have been fully characterized using proteins from*E. coli* (100, 101). Detailed studies of these transport systems in yeast have also been published (101–103). Chapter 18 describes methods for investigating these transporters in detail.

Specific polyamine transporters have also been isolated from protozoan parasites (104, 105) and methods for investigation of these proteins are covered in Chapter 19. Some protozoa such as *T. cruzi*, which causes Chagas disease, lack enzymes capable of putrescine synthesis and uptake is therefore essential for their ability to possess adequate polyamines to maintain viability. Even in those parasites possessing a complete polyamine synthesis system, a full understanding of the uptake systems and methods for their inhibition or down-regulation may be needed to maintain the therapeutic activity of drugs targeting the polyamine pathway. Some progress has been made on the synthesis of inhibitors of polyamine transport using amino acid-spermidine conjugates (106–108). Lipophilic polyamine analogs where a C16-lipophilic substituent is attached to a Lys-spermine conjugate have been shown to have considerable promise, but reports so far have been limited to studies of inhibition of transport into cancer cells (108).

Many attempts to isolate similar carrier-mediated systems from higher organisms have not yet been successful, although recently a transporter protein CATP-5, a P5B-type ATPase associated with the plasma membrane that is involved in polyamine transport, has been identified in *C. elegans* (109). It should be noted that mammalian polyamine transport system can transport a number of related compounds including paraquat (110, 111), antiproliferative agents such as MGBG (112–114) and mepacrine (115), and synthetic drugs conjugated with a polyamine (115–118). The latter property may be valuable to deliver drugs to tumor cells, although obvious problems such as maintaining the tumor-killing properties of the drug or making a version cleavable inside the cell, as well as imparting tumor selectivity, since the uptake systems are not unique to tumor cells, must be overcome. An additional difficulty when reactive drugs are attached to polyamines is that these drugs may inactivate the carrier and thus rapidly inactivate the uptake process.

The polyamine transport system in mammals is also responsible for the uptake of many polyamine analogs, which, as described below, are under investigation and in some cases detailed clinical trials as antitumor agents. AZ (Chapter 15) has a dual function in maintaining polyamine homeostasis (72). It not only reduces putrescine synthesis by its effects reducing ODC activity, but also inhibits polyamine transport. The molecular mechanism for this inhibition is not yet understood. Many of the polyamine analogs also induce AZ synthesis and thus limit their own accumulation (119, 120). Finding an analog that lacks this property but is still actively transported is an important goal, although providing selectivity towards tumors is also a key limiting issue.

Many attempts to isolate mammalian carrier-mediated uptake systems similar to those characterized from bacteria, fungi, and protozoal parasites have not yet been successful. However, endocytic pathways for polyamine transport have been described and fluorescent polyamine derivatives have been localized in discrete vesicles (121). Polyamine transport can follow a dynamin-dependent and clathrin-independent endocytic uptake pathway.

Cell surface heparin sulfate proteoglycans have been implicated in polyamine transport (122–124), and uptake of polyamines was blocked by a single chain variable fragment antiheparin sulfate antibody (125). Such uptake mechanisms are covered in Chapter 20. A caveolin-regulated system that transports polyamines in colon cancer cells has been described (126). Phosphorylation of caveolin-1 at Tyr^{14} is stimulated by the oncogene *k-Ras* and increases the activity of this system (126).

There is also compelling evidence for a polyamine efflux system which excretes excess putrescine and the acetylpolyamines formed by SSAT (127–129). Important recent studies have identified SLC3A2, previously known as a glycosylated heavy chain of a cationic amino acid transporter and its partner y+ LAT light chain, as a part of a putrescine and acetylpolyamine efflux system. This complex supports coupled arginine uptake and putrescine efflux, indicating that there is a putrescine/arginine exchange reaction (130). Methods for the study of these mammalian transport systems are included in Chapter 21. Interestingly, SSAT was colocalized with SLC3A2, so this efflux system may be closely linked to polyamine acetylation. Expression of SLC3A2 was negatively regulated by *k-Ras* (130). Thus, this oncogene can increase polyamine content by affecting both influx and efflux.

Since polyamines are ubiquitous components of plant and animal cells and are produced by intestinal microorganisms, there is substantial dietary exposure to polyamines (131). Uptake from this source is a significant potential problem leading to resistance to drugs that block endogenous polyamine synthesis which are used for cancer chemotherapy or chemoprevention (124, 132). Diets low in polyamines and supplemented with antibiotics to reduce synthesis by intestinal organisms have been designed to overcome this problem (133–135). On the other hand, enhanced use of dietary polyamines may be helpful in some conditions in which pathology is associated with depleted polyamines (136, 137). Detailed descriptions of the polyamine contents of various foodstuffs are available to aid in the design of diets with appropriate polyamine content (131, 138). Chapter 22 details simple and reliable methods for precisely measuring dietary polyamines from a number of food sources. Understanding the variety of dietary sources and their actual content of polyamines will be critical as chemotherapeutic and chemopreventive strategies using polyamine-like molecules move forward.

9. Deranged Polyamine Metabolism and Human Disease

There is an increasing realization that polyamines and/or their metabolites may have critical roles in human disease. Conditions causing reduced polyamine content, increased polyamine

oxidation, or overproduction of certain polyamines are associated causatively in a number of conditions. In some cases, it is not entirely clear how the changes seen are produced or how they are linked to the conditions, but they provide an important diagnostic test. For example, urinary excretion of N^1, N^{12}-diacetylspermine has now been shown to be a valuable marker for a variety of cancers (139). The possible value of polyamine measurements in cancer diagnosis and monitoring of therapy was first proposed by Russell and colleagues (140, 141) who showed that increased extracellular polyamines were associated with neoplasia, but was not generally useful. The rationale/explanation for such increases was that the rapid turnover of cancerous cells led to release of increased polyamines that were found in blood and urine. However, many other conditions where there was increased cell proliferation/turnover, tissue damage, pregnancy, and dietary considerations can all influence such polyamine levels leading to a far more variation than is acceptable for such tests. More recent studies in which the focus has been on N^1, N^{12}-diacetylspermine have provided a more reliable assay (139). This product can be formed by SSAT as shown in Fig. 6 and is readily excreted from cells (142), but it is not clear how the N^1, N^{12}-diacetylspermine found in urine of cancer patients originates. Another advantage of N^1, N^{12}-diacetyl-spermine is that antibodies to this polyamine can be produced that have adequate specificity for analytical use (143), whereas it is difficult to produce antibodies for a free polyamines, such as spermine, which have sufficient selectivity over closely related molecules such as spermidine. Chapter 23 gives full details of this method and its diagnostic applicability.

Polyamine oxidation and the subsequent production of H_2O_2 (Fig. 7) leading to cell death by apoptosis may be an important tissue-remodeling event during development (144, 145). However, inadequately regulated oxidative damage caused by the production of H_2O_2 and/or aldehydes produced by the APAO and the SMO reactions may be involved in the initiation of cancer and the etiology of several other pathologies (48, 60). High levels of SMO may be particularly damaging since this enzyme is not located in peroxisomes as is APAO.

Recent work clearly implicates a significant role for ROS generated via the SSAT/APAO, and possibly SMO, pathway in ischemia reperfusion injury (IRI) (48, 146–148). SSAT was one of the genes that was significantly up-regulated in a mouse model of IRI (149) and plasmid-derived induction of SSAT in kidney epithelial cells (which also leads to SMO induction) showed ROS production leading to injury that could be prevented by catalase. H_2O_2-mediated DNA damage leads to decreased proliferation and repair capability in injured kidneys (148). Confirmation that SSAT plays an important role in kidney IRI has been provided by studies using the *SSAT* knockout mouse, which was less sensitive to both liver and kidney IRI (150). These results clearly have important implications in the treatment and prevention of IRI, and methods to study the role of ROS in IRI, which have more general potential for study of tissue damage related to increased polyamine catabolism, are described in Chapter 24.

Further studies in human renal failure (151, 152) and cerebral stroke (153, 154) have also focused on the role of the polyamine oxidation. These studies have shown that the metabolite, acrolein, may be of particular importance (154–157). The acute toxicity of acrolein, which can be formed from spermine by extracellular Cu-dependent oxidases present in serum, has been known for many years (58, 59, 158), but only recently this metabolite been demonstrated to be an important potential cause and biomarker for ischemic disease. Methods for these assays are given in Chapter 25.

SMO is likely to provide an important mechanistic link between infection, inflammation, and carcinogenesis (48). It is induced in ulcerative colitis leading to increased oxidative stress and altered immune responses (159). Infection of gut macrophages with *Helicobacter*

pylori, which causes gastric ulcers and stomach cancer, induced arginase II and ODC increasing polyamine content and also induced SMO producing ROS including H_2O_2 leading to DNA damage and apoptosis (160–163). Thus, *H. pylori* infection may escape the immune system by killing the immune cells responsible for eradicating the infection. Furthermore, the increased SMO expression in the gastric epithelial cells could result in ROS production leading to mutagenic DNA damage and initiation of neoplastic transformation (163). Chapter 26 describes procedures for these investigations.

As mentioned above, animal models with increased SSAT activity have suggested that this enzyme may be involved in a wide variety of other conditions including pancreatitis, blockage of regenerative tissue growth, obesity, diabetes, and carcinogenesis. Such SSAT overexpression leads to a variety of biochemical alterations that may cause pathogenesis. These include: the loss of polyamines and the reduction in acetyl-CoA and ATP due to the futile cycle set up by increased degradation causing increased synthesis of polyamines and the formation of ROS (47, 49, 164–168). Human genetic studies also suggest that the SSAT/APAO and SMO pathways may be involved in heritable disease. One form of keratosis follicularis spinulosa decalvans (KFSD), a rare X-linked inherited disease affecting primarily the skin and eyes, may be associated with an increased gene copy of the *Sat1* gene encoding SSAT (169). KFSD is characterized by keratosis and hair loss and elevated SSAT in mouse models does cause such changes (49, 170). Cells from patients with the neurodegenerative lysosomal storage disease Niemann–Pick type C are more sensitive to the toxic effects of aldehydes formed by the polyamine catabolic pathways (171). Low SSAT activity may be related to behavioral changes particularly a propensity to suicide (172–174). Decreased *Sat1* gene expression due to reduced levels of SSAT mRNA that may be imparted by the C allele of the Sat1 342A/C gene polymorphism has been correlated to depression and a propensity to suicide. Microarray analyses indicating a link between decreased SSAT expression and suicide were supported by studies with reverse transcription-polymerase chain reaction; immunohistochemistry and Western blotting for SSAT have been used to show that SSAT content was lower in the brains of persons committing suicide than in normal controls. Alterations in polyamine levels in such depressive patients have also been reported (175) and methods to examine this and relate it to disease are described in Chapter 27.

The mechanism by which SSAT is linked to behavioral effects is not understood, but may be linked to polyamine-mediated alterations in ion channels including NMDA receptors, AMPA receptors, K^+ channels, and Ca^2 in the brain. Kainate-induced seizures increase SSAT activity (176) and transgenic overexpression of SSAT protects from kainate (177), and epilepsy-like seizure activity induction by pentylenetetrazol (177). These SSAT transgenic mice also showed a reduced activity and spatial learning impairment (36, 178). The critical role of polyamines in cognitive function is shown most clearly by the finding that an inherited defect in spermine synthase causes Snyder–Robinson syndrome (SRS) (179). SRS is an X-linked recessive disease that causes mild-to-moderate mental retardation as well as hypotonia, cerebellar circuitry dysfunction, thin habitus and kyphoscoliosis. The first gene alteration shown to cause this condition was a splice variant of the *SMS* gene that resulted in an inactive truncated protein. Subsequent studies showed that similar phenotypes were associated with point mutations in the coding region that produce virtually complete loss of spermine synthase activity (13, 180, 181). The analysis of human samples to determine SRS is covered in Chapter 28.

10. Polyamine Analogs and Derivatives as Research Tools and Therapeutics

A wide variety of polyamines have been produced by synthetic chemists and these have been used for a variety of experimental and therapeutic experiments. One interesting series of compounds first described by the laboratories of Coward and of Ganem has methyl substituents on the α carbon atom or both the terminal carbon atoms of spermidine or spermine (182–186). Such substitution does not interfere with the uptake or many physiological functions of the polyamines, but renders them resistant to some oxidative catabolism and as substrates for acetylation and the generation of ROS (187–189). They can therefore be used to investigate polyamine function and to replace polyamines in conditions of polyamine deficiency (188, 190). More recent studies, in which (S)- and (R)- isomers of α-methylspermidine and (S,S)-, (R,S)-, and (R,R)-diastereomers of α,α′-(dimethyl)spermine have been used, have confirmed and extended these important findings (67, 191–196). Such studies have shown the maintenance of nucleic acid structure (196, 197), the stereospecificity of oxidation reactions (192, 194), the ability of derivatives that can support hypusine synthesis to support growth (67, 188, 190), and the role of polyamines in other growth processes including hair synthesis (198), liver regeneration, and adipocyte differentiation (199). Of particular interest is the ability to overcome acute liver damage and pancreatitis related to polyamine depletion by provision of α-methylspermidine or α,α′-(dimethyl)spermine (168, 193, 195, 200, 201). The synthesis of such derivatives is covered by Chapter 29. Other active polyamine analogs have also been produced that contain unsaturated moieties leading to structural changes that can be used to investigate polyamine function (185, 186, 190, 202). *N*-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene [the *cis* isomer of the alkene analog of spermidine] was a good substrate for spermine synthase, but that the *trans* isomer and the alkyne analog [*N*-(3-aminopropyl)-1,4-diaminobut-2-yne] were not substrates. Only the *cis* isomer was able to act as a precursor of hypusine and support sustained growth in cells treated with an inhibitor of polyamine synthesis (190).

Other very useful polyamine derivatives involve substitution at N-groups. Such derivatives of both the terminal and internal N atoms have been used to provide analogs that are transported by the polyamine transport system and thus can be used to study this system (121, 203–206). Fluorescent derivatives have been particularly useful in this regard (121, 204, 206). They have been used to study transport and to provide useful assay substrates for polyamine metabolic enzymes as described in Chapter 30, and also to study the binding of polyamines to receptors and ion channels. Polyamines with N-substitutions are also potentially useful as delivery vehicles for drugs. They can incorporate additions that are known antitumor agents (118, 204, 207–209).

Many synthetic polyamine analogs are profoundly antiproliferative and/or cause apoptosis in cancer cells and some are at various stages of development as antitumor agents (210–216). Addition of terminal alkyl groups renders the analogs more resistant to metabolism and large numbers of analogs with both symmetrical terminal substituents and unsymmetrical terminal substituents have been made by the laboratories of Samejima, Bergeron, Woster, Frydman, and others (211, 217–223). Other internal modifications leading to conformational restriction of bis(ethyl) polyamines have provided particularly promising derivatives that are currently in clinical trials (224, 225).

The original hypothesis underlying the production of these N-alkyl substituted analogs was related to the homeostatic regulation of polyamine content and the importance of polyamines for neoplastic growth. Thus, it was hypothesized that if analogs could be developed, which did not themselves act in the roles of polyamine essential for cell growth, but were able to down-regulate polyamine synthesis and upregulate degradation/excretion,

these would prevent tumor growth. Remarkably, many polyamine analogs such as N^1, N^{11}-bis(ethyl)norspermine and N^1, N^{12}-bis(ethyl)spermine do indeed seem to have these properties being strong inducers of antizyme and of SSAT, thus leading to a drastic fall in polyamine content and apoptosis (119, 226–228). Induction of high levels of SSAT also may exert antitumor effects via ROS production through the activated SSAT/APAO pathways (60, 229, 230) or a reduction in acetyl-CoA and malonyl-CoA, which would decrease fatty acid synthesis (231, 232).

However, many analogs that are also effective against human tumor cells in xenografts and cell cultures do not cause as large an increase in SSAT (213, 214, 224, 233–236). They may act by directly inducing SMO, which is highly responsive to many analogs, and thus reduce spermine and increase ROS (48, 60, 229), by metabolism generating toxic metabolites via SMO or the SSAT/APAO pathways (60, 237) or by additional mechanisms such as blocking binding of polyamines to key sites for normal effects on growth, interfering with polyamine-mediated cell signaling (238) or by acting on the cytoskeleton (230). It should also be noted that the potential of polyamine analogs for therapeutic use is not limited to cancer therapy. A variety of analogs have been shown to have antiparasitic activity (216, 239–242).

Recently, a novel use of polyamine analogs for therapy has been developed based on the observations that some of these compounds are potent and specific inactivators of enzymes that play key roles in chromatin modification (215, 238, 243). Alteration of chromatin structure to reverse epigenetic changes leading to neoplastic growth is a major current focus of the development of novel therapeutics. The synthesis of polyaminohydroxamic acid and polyaminobenzamide derivatives that selectively inactivate histone deacetylase isoforms is described in Chapter 31.

11. Chemical Properties and Analysis of Polyamines

It is important to realize that the properties of polyamines are not simply due to their total charge but that the spatial distribution of this charge and the abilities of cellular macromolecules to interact, not only via electrostatic interactions, but also by hydrophobic interactions with the aliphatic moieties, are critical to understanding their effects. The many crystal structures of proteins and nucleic acids complexed with polyamines that are now available show clearly the importance of both types of interaction.

Early studies with polyamine analogs containing fluorine substituents also demonstrate the importance of the charged nitrogen atoms in the ability of these analogs to fulfill polyamine functions (244–247), but with some exceptions (196) this aspect of the potential effects of synthetic substitutions of the polyamine backbone is not often explored. Other considerations of the charge distribution in the naturally occurring polyamines are of great importance and Chapter 32 provides a comprehensive discussion of this topic and the measurement of the pK_a values of individual polyamines.

It is a critical part of experiments on polyamine metabolism and function to measure the content of polyamines. Many methods are available for this assay and, depending on the polyamines to be assayed and the equipment available, an appropriate method can be selected. Acid extraction followed by deproteinization provides a suitable starting material. Very early methods involved separation by thin layer chromatography of either the amines followed by detection with reagents such as ninhydrin (248) or of fluorescent derivatives formed by dansylation (249, 250) or use of an amino acid analyzer with modified elution to release the more basic polyamines (251). These have largely been replaced by HPLC separation, which can be carried out with either postcolumn derivatization using *o*-phthalaldehyde (252) or by precolumn derivatization to form dansyl or benzoyl- or other readily quantifiable derivatives (253, 254). These methods are covered in Volume 79 of this

series. One advantage of the precolumn dansylation procedure is that polyamine analogs with terminal alkyl or acetyl substituents can still be detected. Extension of HPLC analysis to separation of the wider variety of polyamines first characterized in thermophiles is covered in Chapter 5 of the present volume.

More recent analytical methods have employed gas chromatography/mass spectrometry (255–257). These assays, detailed procedures for which are described in Chapter 33 and for human samples in Chapter 27, are the method of choice with respect to specificity and sensitivity as long as the required equipment, marker compounds, and expertise are available and the extensive development of these assays has been a major advance.

In summary, it is hoped that the detailed methods provided in this new volume will facilitate the work in the many laboratories currently interested in polyamine function and metabolism and will foster a new generation of researchers interested in this exciting and challenging field. As we have entered a new era of polyamine research with our better understanding of their molecular functions, many challenges lie ahead. Our goal is to make meeting those challenges somewhat easier.

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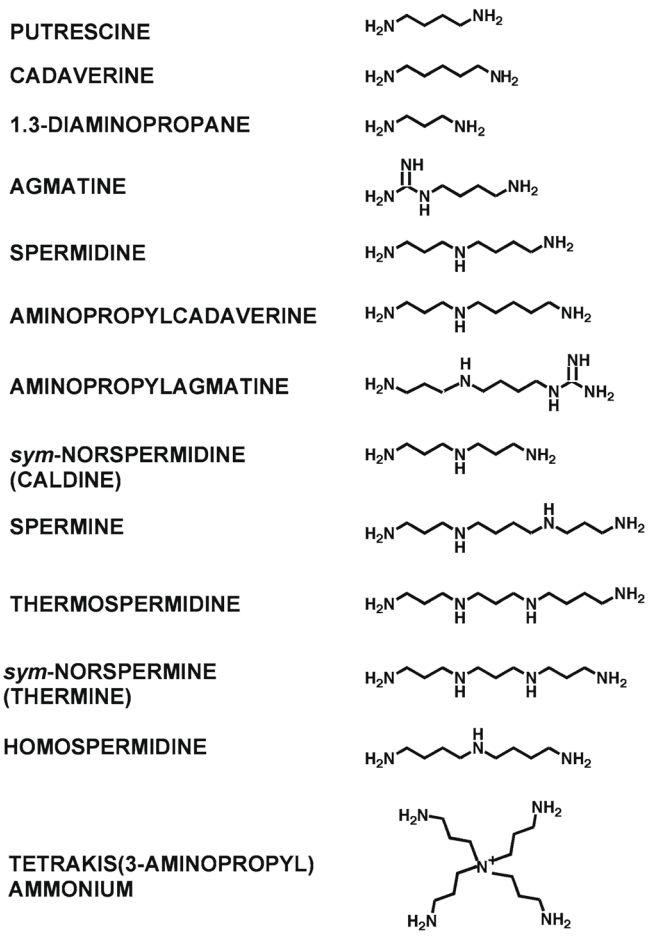

Fig 1.

Structures of some naturally occurring polyamines. It should be noted that at physiological pH values the nitrogen atoms in these structures (and in subsequent figures) would be predominantly protonated (see Chapter 32). Additional polyamines found in thermophiles are described in Chapter 5.

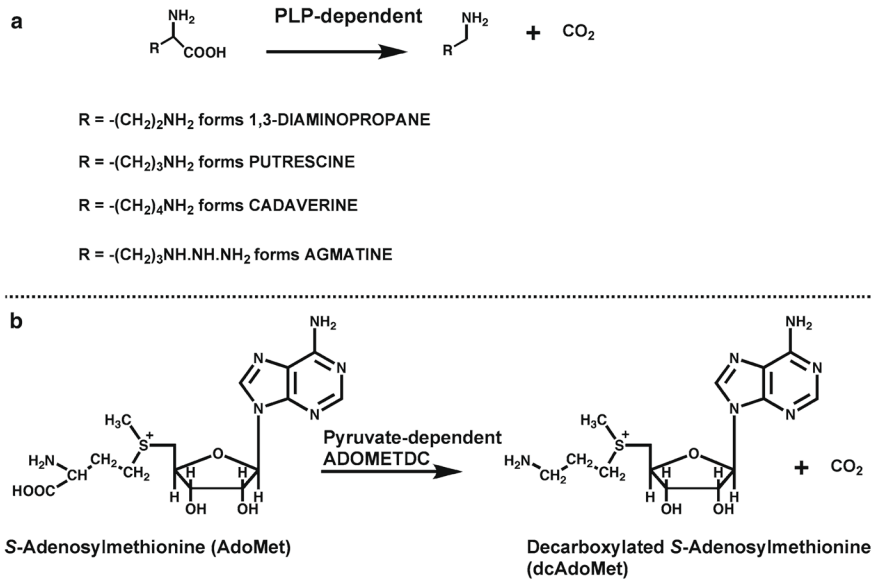

Fig 2.

Decarboxylases involved in polyamine synthesis. (**a**) Pyridoxal phosphate (PLP)-dependent decarboxylases [L-1,4-diaminobutyric acid decarboxylase, L-ornithine decarboxylase (ODC), L-lysine decarboxylase and L-arginine decarboxylase]. (**b**) *S*-adenosylmethionine decarboxylase, which contains a covalently bound pyruvate group essential for activity.

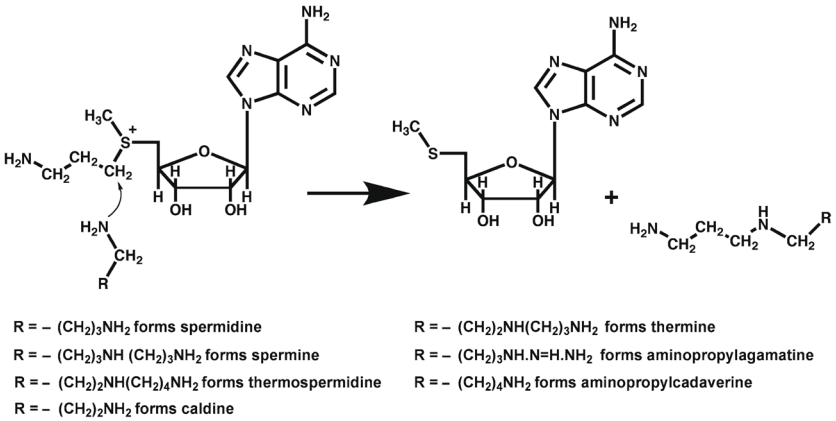

Fig 3.

Aminopropyltransferases involved in polyamine synthesis. The reaction catalyzed by these enzymes involves the attack by the unprotonated terminal N of the amine substrate on the methylene C atom adjacent to the sulfonium center of the dcAdoMet aminopropyl donor. The deprotonation of the attacking N, which is facilitated by residues in the enzyme, and the positive charge on the sulfonium S of dcAdoMet allow the reaction to proceed forming the polyamine product. The reactions shown have all been demonstrated by purified aminopropyltransferases. Other members of this family of enzymes may occur to bring about the synthesis of longer chain polyamines.

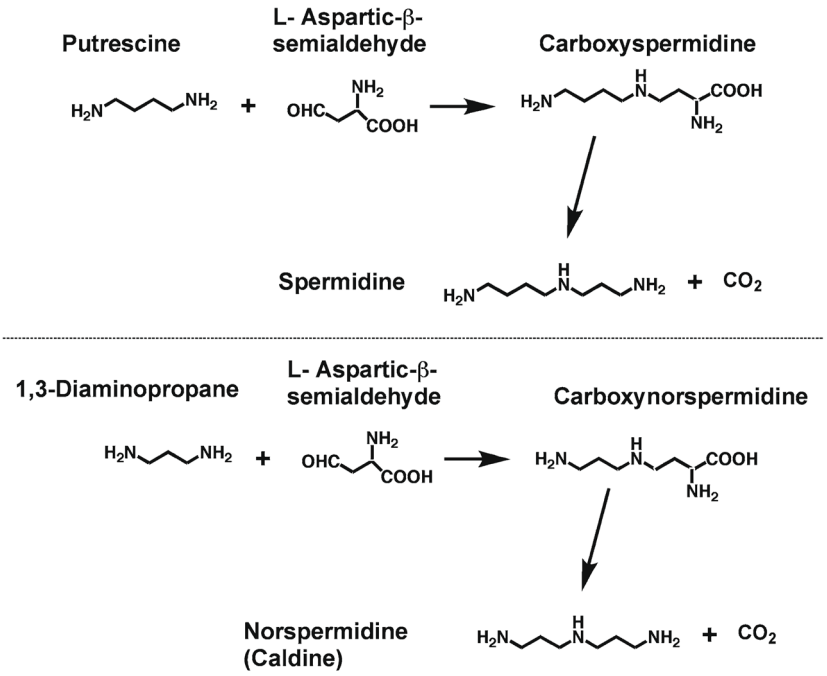

Fig 4.

Condensation reactions forming polyamines from L-aspartic-β-semialdehyde. Reaction of L-aspartic–β-semialdehyde with putrescine or 1,3-diaminopropane by carboxynorspermidine synthase forms carboxyspermidine or carboxynorspermidine, which are decarboxylated by carboxynorspermidine decarboxylase to form spermidine or *sym*-norspermidine, respectively.

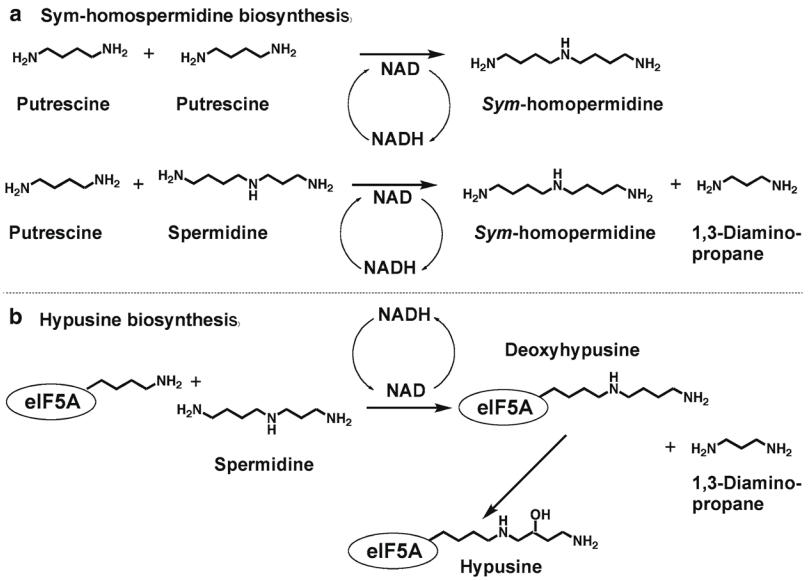

Fig 5.

Synthesis of *sym*-homospermine and of hypusine. *sym*-Homospermine can be formed from two molecules of putrescine or from putrescine and spermidine as shown in (**a**). In both cases, NAD is needed for hydrogen extraction, but is regenerated in the second half of the reaction. The reaction using spermidine and putrescine, which also generates 1,3-diaminopropane, is similar to that used for the hypusine modification of eIF5A shown in (**b**). In this case, a lysine residue in eIF5A is used instead of putrescine forming deoxyhypusine in eIF5A and free 1,3-diaminopropane. A second enzyme hydroxylates the deoxyhypusine to form the complete hypusine modification (see Chapters 12 and 13 for more details of the reactions forming hypusine).

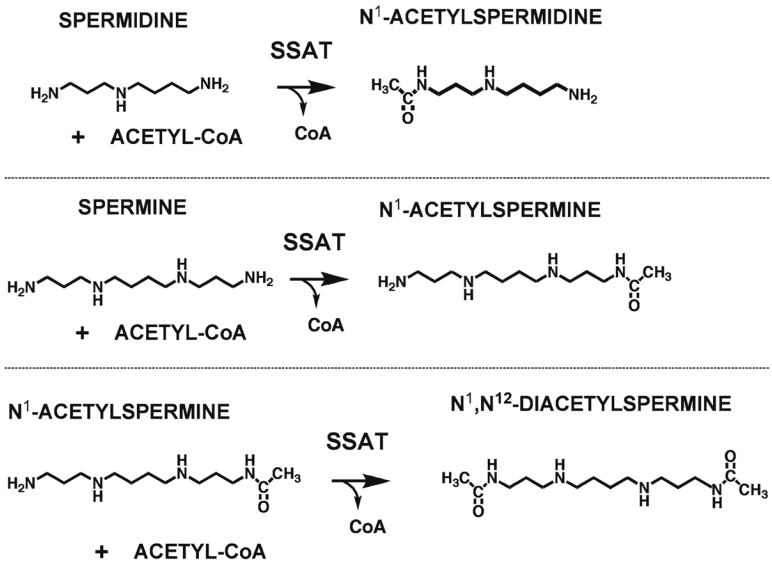

Fig 6.
Reactions catalyzed by SSAT.

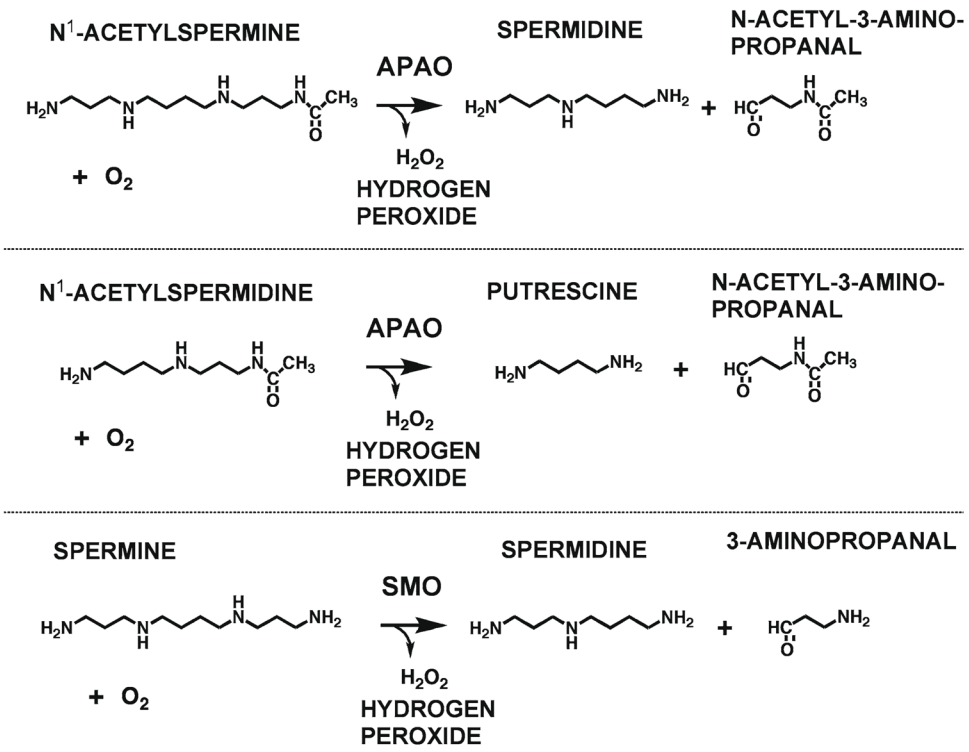

Fig 7.

Reactions catalyzed by APAO and SMO. APAO has a strong preference for N^1-acetylated polyamines formed by SSAT releasing *N*-acetyl-3-aminopropanal and SMO is specific for spermine releasing 3-aminopropanal. Both reactions also generate hydrogen peroxide.