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REVIEW ARTICLE

Recent advances in the biochemistry of polyamines in eukaryotes

Anthony E. PEGG

Department of Physiology and Cancer Research Center, The Pennsylvania State University College of Medicine, Hershey, PA 17033, U.S.A.

Introduction

The field of polyamine biochemistry has undergone an enormous expansion in the past 3 years. Much of this explosion of interest is due to the new research opportunities offered by the availability of potent and specific inhibitors of the key enzyme ornithine decarboxylase (ODC). These inhibitors, particularly 2-difluoromethylornithine (DFMO), have been used extensively not only to investigate the roles of polyamines in cell growth and differentiation but also in a variety of ways to advance understanding of the regulation of polyamine levels and the enzymes involved in their metabolism. In this brief Review I have attempted to summarize the major advances made in these areas. The emphasis is on work published in the past 3 years and the great majority of the references quoted come from this period. Earlier studies are fully covered in previous reviews (see Pegg & McCann, 1982; Pegg *et al.*, 1982*a*; Jänne *et al.*, 1983; Tabor & Tabor, 1984*a*, and references in these reviews) and full documentation of these investigations are given in these references. Only studies in eukaryotes are described since a comprehensive review of polyamines in micro-organisms has been provided by Tabor & Tabor (1985).

The mammalian polyamine biosynthesis pathway is shown in Fig. 1. Ornithine, which is formed by the action of arginase, is converted into putrescine by the action of ODC. Putrescine is converted into spermidine by the action of an aminopropyltransferase called spermidine synthase. A second aminopropyltransferase termed spermine synthase adds an additional propylamine moiety to spermidine, forming spermine. The source of these propylamine groups is decarboxylated *S*-adenosylmethionine (AdoMet) which is produced by the action of *S*-adenosylmethionine decarboxylase (AdoMetDC). The other product of the aminopropyltransferase reactions is 5′-methylthioadenosine (MTA).

MTA is broken down by the action of MTA phosphorylase (EC 2.4.2.28) and the resulting adenine is salvaged and returned to the purine nucleoside pool. The 5-methylthioribose 1-phosphate is converted back to methionine. This metabolism occurs via a series of reactions which are not fully characterized but involve

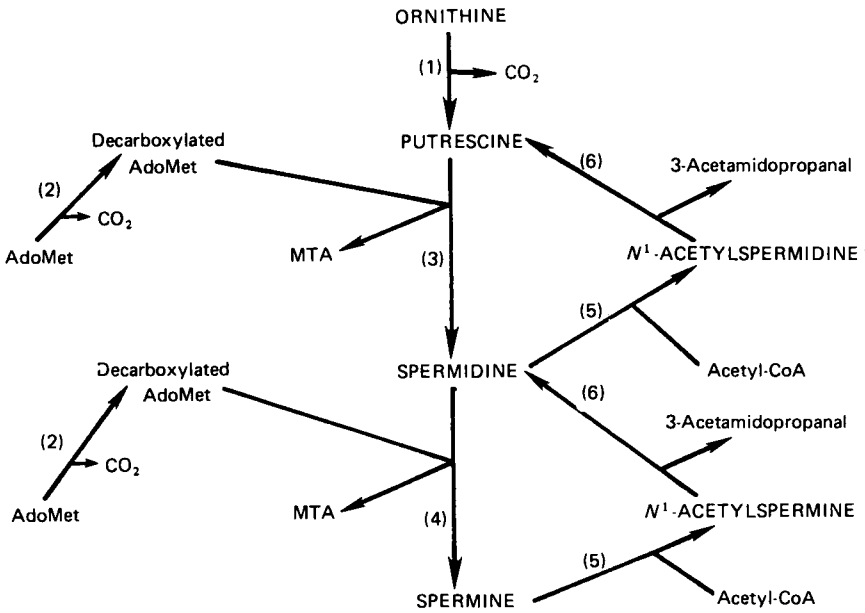

Fig. 1. Pathway for biosynthesis and interconversion of polyamines

The enzymes involved are: 1, ODC (EC 4.1.1.17); 2, AdoMetDC (EC 4.1.1.50); 3, spermidine synthase (EC 2.5.1.16); 4, spermine synthase (EC 2.5.1.22); 5, SAT; 6, polyamine oxidase (FAD-dependent). Enzymes 5 and 6 do not have EC numbers assigned to them.

Abbreviations used: ODC, L-ornithine decarboxylase; DFMO, DL-2-difluoromethylornithine; AdoMet, *S*-adenosylmethionine; AdoMetDC, *S*-adenosylmethionine decarboxylase; MTA, 5′-methylthioadenosine; SAT, spermidine/spermine-N^1-acetyltransferase; RIA, radioimmunoassay; MGBG, methylglyoxal bis(guanylhydrazone); AdoDATO, *S*-adenosyl-1,8-diamino-3-thio-octane; $AdoS^+(CH_3)_2$, *S*-methyl-5′-methylthioadenosine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; *m*-AMSA, 4′-(9-acridinylamino)methanesulphon-*m*-anisidide.

Table 1. Inhibitors of enzymes involved in biosynthesis and metabolism of polyamines

References to publications concerning these inhibitors are given in the text. It should be noted that a considerable number of other inhibitors of ODC have been reported (Danzin *et al.*, 1983*a*; Bey *et al.*, 1983; Sjoerdsma & Schechter, 1984).

Enzyme	Inhibitor
L-Ornithine decarboxylase (ODC)	DL-2-Difluoromethylornithine (DFMO)
	2-Fluoromethyl-(*E*)-dehydro-ornithine
	(2*R*,5*R*)-6-Heptyne-2,5-diamine, also described as (2*R*,5*R*)-*δ*-methylacetylenicputrescine (MAP)
S-Adenosylmethionine decarboxylase (AdoMetDC)	Methylglyoxal bis(guanylhydrazone) (MGBG)
	Ethylglyoxal bis(guanylhydrazone)
Spermidine synthase	*S*-Adenosyl-1,8-diamino-3-thio-octane (AdoDATO)
	Cyclohexylamine
Spermine synthase	*S*-Methyl-5′-methylthioadenosine [AdoS$^+$(CH$_3$)$_2$]
Spermidine/spermine-N^1-acetyltransferase (SAT)	*N*-[2-(*S*-Coenzyme A)acetyl]*sym*-norspermidine amide
Polyamine oxidase (FAD-dependent)	N^1-Methyl-N^2-(2,3-butadienyl)-1,4-butanediamine
	N^1,N^2-Bis-(2,3-butadienyl)-1,4-butanediamine
N^8-Acetylspermidine deacetylase	7-Amino-2-heptanone
5′-Methylthioadenosine phosphorylase	5′-Deoxy-5′-chloroformycin

the sequential production of 1-phospho-5-*S*-methylthioribofuranoside, 1-phospho-5-*S*-methylthioribulose and 2-oxo-4-*S*-methylthiobutyrate as intermediates (Schlenk, 1983; Trackman & Abeles, 1983; Ghoda *et al.*, 1984). Virtually all mammalian cells tested contain all of the enzymes of the polyamine biosynthesis pathway, but a fraction of the tumour lines lack MTA phosphorylase and simply excrete the MTA produced in polyamine biosynthesis (Kamatani *et al.*, 1981; Iizasa & Carson, 1985). This presumably leads to an increased need for methionine and purine nucleosides, since the normally efficient salvage of the MTA molecule does not occur in these cells. The possibility of exploiting this phenomenon for chemotherapy has been suggested (Kamatani *et al.*, 1981; Savarese *et al.*, 1983). The biochemistry of MTA has been reviewed by Williams-Ashman *et al.* (1982) and by Schlenk (1983).

As can be seen from Fig. 1, AdoMet serves as the precursor of polyamines in addition to its role as a substrate for many transmethylation reactions. AdoMetDC serves as a critical branch point between these pathways. Once AdoMet has been decarboxylated it is committed to polyamine synthesis because the decarboxylated form is virtually inactive as a methyltransferase substrate (Pegg, 1984*a*; Pegg *et al.*, 1985*a*). In whole animals the substantial use of AdoMet by the liver for transmethylations results in most of the total disposition of AdoMet occurring via methyl-transfer reactions (Eloranta & Kajander, 1984; Finkelstein & Martin, 1984; Guilidori *et al.*, 1984). However, in isolated cell culture the situation is different. The relative consumption of AdoMet via polyamine synthesis and transmethylation was found to vary according to the growth cycle in lymphoblasts (Iizasa & Carson, 1985). The rate of MTA synthesis was 1.4 times greater than that of transmethylation during the period after stimulation by serum prior to the onset of exponential growth. Over the period corresponding to one generation the total transmethylation was about 2.6 times the total amount of MTA production.

The aminopropyltransferase reactions which form spermidine and spermine are effectively irreversible but these polyamines can be converted back into putrescine by the combined actions of two enzymes, spermidine/spermine-N^1-acetyltransferase (SAT) and polyamine oxidase (FAD-dependent) (Fig. 1). SAT catalyses the conversion of spermine into N^1-acetylspermine which is then degraded by polyamine oxidase to form spermidine and 3-acetamidopropanal. Similarly, spermidine is a substrate for SAT which forms N^1-acetylspermidine and this is split by polyamine oxidase to form putrescine and 3-acetamidopropanal. The acetylase/oxidase system and either of the aminopropyltransferases can therefore be regarded as forming a cycle which leads to the production of MTA from decarboxylated AdoMet. The importance of these cycles and the extent to which they occur in unstimulated cells are unknown at present but it should be noted that their existence renders the use of labelling in the putrescine moiety unsatisfactory for studies of the half-lives of the polyamines.

The regulation of the polyamine biosynthesis and interconversion pathway shown in Fig. 1 is accomplished by changes in the activity of three of these enzymes: ODC, AdoMetDC and SAT. The other enzymes (spermidine synthase, spermine synthase and polyamine oxidase) appear to be regulated via the availability of their limiting substrates which are decarboxylated AdoMet and the acetylated polyamine derivatives. ODC, AdoMetDC and SAT can undergo large changes in their activity which appear to be brought about by alterations in the amount of enzyme protein present. All three of these enzymes have extremely short half-lives, which enables a new level of enzyme protein to be reached very rapidly after the application of an appropriate stimulus (Pegg *et al.*, 1982*a*). In addition AdoMetDC is activated by putrescine (Pegg, 1984*a*; Tabor & Tabor, 1984*b*). This activation ensures that the supply of decarboxylated AdoMet is increased when putrescine production by ODC is increased and that the putrescine is converted into spermidine. ODC is well known to be induced by a vast array of stimuli which lead to cell growth. Its induction appears to be a universal accompaniment of the stimulation of growth by hormones, drugs, regenerative stimuli and tumour promoters.

Much of the recent work in mammalian polyamine biochemistry has made use of inhibitors of the enzymes

which are responsible for polyamine synthesis and degradation and are described above. A summary of these inhibitors, their common abbreviations and the enzymic step at which they act is given in Table 1.

Inhibitors of ornithine decarboxylase

The availability of specific and potent inhibitors of ODC has provided an enormous stimulus to the field of mammalian polyamine biology and it is probable that some of these inhibitors will have major impact on pharmacology. More detailed references to the extensive studies which have been carried out with these compounds can be found in the earlier reviews by Heby (1981), Pegg & McCann (1982), Jänne *et al.* (1983), Sjoerdsma & Schechter (1984), Sjoerdsma *et al.* (1984), Tabor & Tabor (1984*a*) and by Williamson & Tyms (1984). These references describe in detail the effects of these inhibitors on cell growth and differentiation and their potential value as anti-tumour, anti-viral and anti-protozoal agents. Some of the recent work in which the inhibitors have been used to study ODC and on the development of more potent inhibitors is outlined here.

The first of these inhibitors, and still by far the most studied, is DFMO. DFMO, which was synthesized by Metcalf *et al.* (1978), is an enzyme-activated irreversible inhibitor of ODC. They reasoned that it should be recognized by ODC as a substrate and that its decarboxylation should lead to the generation of a highly reactive electrophilic intermediate which would react with a nucleophilic centre at the active site of the enzyme to form a covalent bond and irreversibly inactivate it. ODC is indeed inactivated by DFMO with kinetics which are consistent with this mechanism and the inactivation is accompanied by the release of CO_2 from the carboxyl group of DFMO (K. McGovern & A. E. Pegg, unpublished work) and the binding of the remainder of the DFMO molecule to the enzyme (Seely *et al.*, 1982*a*, *b*). The highly specific nature of the binding of DFMO to ODC has provided an important tool for further studies of the biochemistry of this enzyme. The reaction of ODC with [^{3}H]DFMO provides a valuable method to investigate the purity of ODC preparations (Seely *et al.*, 1982*b*), to titrate the number of active ODC molecules in tissue extracts (Seely *et al.*, 1982*a*, *c*; Erwin *et al.*, 1983), to identify the ODC protein spot on two-dimensional gels (Persson *et al.*, 1984; Seely *et al.*, 1985) and to provide a labelled ligand for RIA (Seely & Pegg, 1983*a*, *b*) and the screening of monoclonal antibodies (Pegg *et al.*, 1984).

The specific covalent binding of labelled DFMO to ODC also allows the autoradiographic localization of the enzyme. The majority of the ODC in the male mouse kidney was found to be present in the cells of the proximal tubules by this technique (Zagon *et al.*, 1983), which agrees with the result found by immunocytochemistry (Persson *et al.*, 1983). A fraction of the cellular ODC was found to be present in the nucleus (particularly in the nucleolar region) with the majority present in the cytosol (Zagon *et al.*, 1983; Persson *et al.*, 1983). The nuclear location of some of the ODC is consistent with the suggestion of Russell (1983) that ODC protein may serve as an activator of RNA polymerase 1. However, more detailed studies do not support this hypothesis. The ability of crude ODC preparations to stimulate RNA polymerase activity *in vitro* was lost on further purification and there were no antigenic similarities between ODC and the subunits of RNA polymerase 1 (Seely *et al.*, 1984).

The turnover of ODC protein could be investigated directly by the administration of labelled DFMO and measurement of the loss of the labelled ODC peak (Seely *et al.*, 1982*a*). No sign of any partial degradation products was found in these experiments.

A large number of other enzyme-activated irreversible inhibitors of ODC have now been synthesized (Bey *et al.*, 1983; Sjoerdsma & Schechter, 1984; Danzin *et al.*, 1983*a*). Some of these compounds may well prove to be superior to DFMO for certain purposes. DFMO is an extremely specific and quite potent inhibitor but it has certain disadvantages. Its K_i of 39 μM is quite high and the half-life of ODC at saturating concentrations of DFMO is 3.1 min (Metcalf *et al.*, 1978). Since ODC turns over very rapidly a high level of DFMO must be maintained in order to keep the enzyme activity low. A second problem is that DFMO is quite rapidly cleared from the body by excretion (Grove *et al.*, 1981) and its uptake into cells, which probably occurs by a diffusion mechanism rather than via an amino acid carrier (Erwin & Pegg, 1982; Bitonti *et al.*, 1985*a*), is quite slow. Some of the newer ODC inhibitors are improvements in these respects. 2-Fluoromethyl-(*E*)-dehydro-ornithine has a K_i of only 2.7 μM (Bey *et al.*, 1983) and the uptake problem can be overcome by the use of the methyl ester which is rapidly cleaved to form the amino acid within the cell (Sjoerdsma & Schechter, 1984; Bitonti *et al.*, 1985*a*). Another promising compound is (2*R*,5*R*)-6-heptyne-2,5-diamine [also described as (2*R*,5*R*)-δ-methylacetylenic-putrescine or MAP] which enters mammalian cells relatively well and has a K_i of 3 μM and a rate constant for inactivation equivalent to a half life of 1.7 min at saturating concentrations (Danzin *et al.*, 1983*a*; Mamont *et al.*, 1984*a*). Both the methylester of 2-fluoromethyl-dehydro-ornithine and (2*R*,5*R*)-MAP are more active antitumour agents than DFMO in animal models (Sjoerdsma & Schechter, 1984; Bartholeyns *et al.*, 1984; Mamont *et al.*, 1984*a*).

Enzymology/molecular biology of ornithine decarboxylase

Major advances in the study of ODC have been made possible by the production of monospecific, high affinity antibodies to the enzyme and by the use of DFMO as a tool to label the enzyme specifically and to promote amplification of its gene. These approaches have led to the successful cloning of the ODC gene and the derivation of its complete amino acid sequence.

Monospecific antibodies to ODC were obtained by Kameji *et al.* (1982) for the homogeneous rat liver protein and by Persson (1982), Isomaa *et al.* (1983) and Seely & Pegg (1983*a*) for mouse kidney ODC. These antisera cross-react with ODC from all mammalian species tested so far. They can be used to precipitate all of the ODC activity present in cell extracts, indicating that there are no antigenically distinct forms of the enzyme, and they can be used for the identification of ODC in autoradiographs or Western blots after separation by polyacrylamide-gel electrophoresis. Such investigations indicated that there are two forms of ODC in mouse kidney but only one form was found in rat liver and hepatoma extracts (Persson *et al.*, 1984; Seely *et al.*, 1985). The antisera have been used for RIA of ODC protein. A particularly useful method for such RIA is to use the ODC protein labelled by reaction with [^{3}H]DFMO as the

labelled ligand, since the specificity of this interaction makes it unnecessary to use completely homogenous protein for the labelling (Seely & Pegg 1983*a*, *b*). Monoclonal antibodies to ODC have also been produced by Matsufuji *et al.* (1984) and by Pegg *et al.* (1984).

The development of RIA techniques for the assay of ODC protein has permitted more incisive investigations of the mechanism by which the large and rapid fluctuations in ODC activity occur. No evidence whatsoever that ODC is regulated by post-translational modifications of the enzyme protein or by changes in the content of activating or inhibitory factors was obtained. In contrast, there was an excellent correlation between the amount of ODC protein and the enzymic activity, indicating the regulation occurs by changes in the rate of synthesis or degradation of this protein (Seely & Pegg, 1983*a*, *b*; Isomaa *et al.*, 1983; Erwin *et al.*, 1983). Under conditions where ODC activity is greatly enhanced, such as in the mouse kidney after androgen administration, the elevated amounts of ODC protein arise from both an increase in the rate of synthesis (Persson *et al.*, 1984) and from a decrease in the rate of degradation (Seely *et al.*, 1982*a*; Isomaa *et al.*, 1983). A major part, but possibly not all, of the increased rate of synthesis occurs by virtue of a large rise in the amount of ODC mRNA (Berger *et al.*, 1984; Kontula *et al.*, 1984). Striking increases in the level of ODC mRNA have also been observed in other situations where ODC activity and protein are induced (Kameji *et al.*, 1984; Kahana & Nathans, 1984; McCologue *et al.*, 1984; Alhonen-Hongisto *et al.*, 1985; Persson *et al.*,1985; Pohjanpelto *et al.*, 1985*a*).

ODC is a very minor component of the total soluble protein in most cells (Seely *et al.*, 1982*b*, *c*; Pegg *et al.*, 1982*a*; Kameji *et al.*, 1982). For example, ODC represents about 1 part in 60000000 of the soluble protein in uninduced rat liver (Pegg *et al.*, 1982*a*) and even after maximal induction by a combination of inducers ODC protein is only 1 part in 350000 (Kameji *et al.*, 1982). It was therefore necessary to obtain enriched sources for the isolation of mRNA and cloning of cDNA. This was accomplished in two ways. The mouse kidney after maximal stimulation with androgens contains much higher amounts of ODC protein than do other mammalian tissues and ODC can amount to up to 1 part in 8000 (Persson, 1982; Seely *et al.*, 1982*a*, *b*, *c*; Isomaa *et al.*, 1983). The synthesis rate of ODC in such mouse kidneys reaches values as high as 1% of the total soluble protein synthesis because of the rapid turnover of ODC protein (Persson *et al.*, 1984). It was therefore feasible to obtain ODC cDNA clones using mRNA from mouse kidney (Kontula *et al.*, 1984; Berger *et al.*, 1984). The reason for the very large amounts of ODC in male mouse kidney is completely unknown, although the use of the putrescine produced as a component of a pheromone or territory marking substance is one possibility. Profound behavioural effects such as the burying of dead conspecifics by rats are known to be instigated by the presence of putrescine or cadaverine (Pinel *et al.*, 1981).

The second approach used was to amplify the ODC gene by exposure of cells to DFMO. Overproduction of ODC protein as a result of such gene amplification occurs rather readily in cells treated with DFMO (McConlogue *et al.*, 1984; Kahana & Nathans, 1984; Alhonen-Hongisto *et al.*, 1985). Perhaps the most striking example is the mouse S49 lymphoma cell line variant D4.1 obtained in this way by McConlogue & Coffino (1983). In D4.1 cells there is a 300-fold increase in ODC production and synthesis of ODC protein amounts to 15% of the total protein made. The use of such cell lines for isolation of mRNA renders the task of cDNA production relatively simple and full-length cDNA copies have been obtained from them (Kahana & Nathans, 1984; McConlogue *et al.*, 1984).

These cDNA probes have been used to demonstrate that there is a family of ODC genes as detected on Southern blots and that one of these is greatly amplified in the overproducing cell lines produced by exposure to DFMO (Berger *et al.*, 1984; McConlogue *et al.*, 1984; Kahana & Nathans, 1984; Alhonen-Hongisto *et al.*, 1985; Pohjanpelto *et al.*, 1985*a*). Amplification of the ODC gene can also be achieved by starving cells which lack arginase for ornithine (Pohjanpelto *et al.*, 1985*a*). There may be multiple active ODC genes (Alhonen-Hongisto *et al.*, 1985) but some of the other members of the gene family indicated by Southern blot analysis may well represent pseudogenes. A full analysis of these fragments which requires sequencing the genomic clones has not yet been carried out.

With one exception, all of the cDNA probes for ODC obtained in different laboratories cross-hybridize and have similar restriction maps, indicating that they correspond to the same or very closely related mRNAs. The exception is the plasmid 1440 which was claimed by Berger *et al.* (1984) to correspond to ODC. This identification was made solely on the basis of translation of mRNA isolated by hybridization with this plasmid. The translation products reacted with antibodies to ODC but no competition experiments with authentic ODC protein were carried out. This question will only be resolved by more detailed studies, including sequencing, but the multiple forms of mouse kidney ODC differ only very slightly in charge and seem more likely to result from phosphorylation or minor differences between closely related genes than from such strikingly different gene products.

Two forms of ODC mRNA with sizes of 2.2 kb and 2.7 kb are observed on Northern blots (Berger *et al.*, 1984; Kontula *et al.*, 1984; McConlogue *et al.*, 1984). These two forms probably reflect the presence of two possible polyadenylation sites in the 3′ non-coding segment.

Although translation *in vitro* of ODC mRNA has been achieved (Berger *et al.*, 1984; Kontula *et al.*, 1984; McConlogue *et al.* 1984; Kameji *et al.*, 1984; Kahana & Nathans, 1984) there is general agreement that ODC mRNA is translated very poorly *in vitro* in the reticulocyte lysate system. The reason for this is unclear but may relate to the very long 5′ leader sequence which is present in the mRNA. Kahana & Nathans (1985) point out that in their cDNA clones this sequence is 737 nucleotides long and contains 4 ATG triplets each of which is followed by an in-phase termination codon. This could have regulatory significance, but puzzlingly the 5′ leader sequence obtained by Gupta & Coffino (1985) is substantially different from that found by Kahana & Nathans (1985) even though the coding sequence is identical. It is possible therefore that part of the 5′ sequence represents a cloning artifact.

ODC activity declines very quickly in cells in response to exogenous putrescine or polyamines (Canellakis *et al.*, 1979; Pegg & McCann, 1982) This decline appears to be brought about by both an increased rate of degradation

of the protein (see below) and a decreased rate of synthesis. However, there is no change in the content of ODC mRNA (G. Sertich & A. E. Pegg, unpublished work). This suggests that the translation of ODC mRNA may be affected by the polyamine content. Since the standard reticulocyte lysate system contains polyamines at concentrations which are optimized for other mRNAs it would appear to be worthwhile to study the possibility that the translation of ODC mRNA *in vitro* might be greatly affected by varying the polyamine content of the system.

The increased synthesis of ODC protein in response to short-term exposure to ODC inhibitors is also not accompanied by any change in the mRNA content (Persson *et al.*, 1985). There are a number of reports in which it was found that the increase in the level of ODC mRNA was not as large as the increased rate of synthesis of the protein after stimulation by various agents (Berger *et al.*, 1984; Kontula *et al.*, 1984; Pohjanpelto *et al.*, 1985*a*). Overall it is clear that the synthesis of ODC protein can be regulated at the level of transcription of the ODC gene and at the level of translation of the ODC mRNA.

A continuing puzzle in the ODC field is the role of the inhibitory protein of M_r 22000 discovered by Canellakis and his colleagues, which they have named antizyme (Canellakis *et al.*, 1979). The content of antizyme increases very rapidly in cells exposed to exogenous polyamines and the protein binds quite tightly to ODC and inhibits its activity (Canellakis *et al.*, 1979). However, the obvious possibility that the accumulation of inactive ODC–antizyme complexes may be responsible for the rapid fall in ODC activity under such conditions does not appear to be correct. Very little build-up of the inactive complex was observed by Seely & Pegg (1983*b*) using a RIA technique which detects ODC in both the free and complexed form. Similarly, Fujita *et al.* (1982) found only a small accumulation of inactive ODC in rat liver after treatment with 1,3-diaminopropane.

The decline in ODC protein after exposure to exogenous di- or polyamines occurs more rapidly than the fall when protein synthesis is blocked by cycloheximide (Canellakis *et al.*, 1979; Seely & Pegg, 1983*b*; Persson *et al.*, 1984; Murakami *et al.*, 1985). This observation has led to the suggestion that the physiological role of antizyme may involve the degradation of the ODC protein. If the rate-limiting step in this degradation involves the formation of the ODC–antizyme complex the lack of accumulation of this complex can be explained (Seely & Pegg, 1983*b*). There appears to be a good correlation between the reciprocal of the half life of ODC activity in the presence of cycloheximide and the proportion of ODC present as a complex with antizyme (Murakami & Hayashi, 1985). Further evidence favouring this hypothesis was obtained using the HTC variant cell line, HMO_A (Murakami *et al.*, 1985). These cells, which were isolated by McCann, Mamont and colleagues because of their resistance to ornithine decarboxylase inhibitors, have an elevated level of ODC due to a substantial increase in its half-life (Pritchard *et al.*, 1982 Murakami *et al.*, 1985). A human cell line resistant to DFMO containing elevated levels of ODC with a greatly increased half-life has also been reported (Pösö *et al.*, 1984). Such cell variants provide a useful system in which the critical factors for the degradation of ODC may be studied.

The ultimate test of the hypothesis that the antizyme–ODC complex is on the pathway by which degradation occurs requires the development of a cell-free system in which this process takes place. This has not yet been achieved, but more recent procedures for the purification of antizyme by immunoprecipitation of the complex (Seely & Pegg, 1983*b*) or by conventional methods (Kitani & Fujisawa, 1984) should help to accomplish it. Rat liver also contains a macromolecular inhibitor of the antizyme which can be used to reverse the inhibition of ODC, but its physiological importance is totally unknown (Fujita *et al.*, 1982).

The amino acid sequence of mouse ODC has been deduced from the nucleotide sequence of cDNA (Gupta & Coffino, 1985; Kahana & Nathans, 1985). The enzyme consists of 461 amino acids and has a predicted isoelectric point of 5.1 and M_r of about 51000, which agrees well with values obtained for the purified mouse kidney protein (Seely *et al.*, 1982*b*). The sequence contains 12 cysteine residues which may account for the strict requirement for high concentrations of thiol reducing agents to maintain enzymic activity (Pegg & Williams-Ashman, 1981). The sequence offers no obvious clues as to the reasons for the rapid turnover of the ornithine decarboxylase protein. It contains 19 arginines [it should be noted that the statement in the text of Gupta & Coffino (1985) of 15 arginines is incorrect] and 29 lysines, but there are only three pairs of adjacent basic amino acids which may have particular importance in proteolytic cleavage.

During purification or storage of mouse kidney ODC there is a loss of a small fragment of the molecule (M_r about 2000) without affecting the enzymic activity (Persson *et al.*, 1984). This loss could involve the cleavage at either end but indicates that these regions are unlikely to contain critical portions of the active site. The location of the active site is not fully established, but studies in which the protein labelled by reaction with [^{3}H]DFMO has been digested with proteinases and reagents cleaving at specific amino acid residues suggest that the DFMO is bound to the lysine residue at position 298 (L. Persson, K. McGovern & A. E. Pegg, unpublished work). This lysine occurs in the sequence Val-Trp-Lys-Glu-Gln-Pro-Gly-Ser (residues 296–303) which has certain similarities to the sequence Val-His-Lys-Gln-Gln-Ala-Gly-Gln which is known to be the active site of the *E. coli* biodegradative ornithine decarboxylase (Morris & Boeker, 1983). Except for the first 40 residues, the *N*-terminal two-thirds of the mouse ODC contains predominantly hydrophobic clusters and the majority of the hydrophilic clusters are in the remaining one-third at the *C*-terminal end. The lysine at 298 is in a region just after this transition which corresponds to maximum hydrophilicity.

It is also close to the sequence starting at amino acid 302 of Gly-Ser-Asp-Asp-Glu-Asp-Glu-Ser-Asn-Glu which might be expected to be a substrate site for casein kinase 2. ODC does contain phosphate and rat heart ODC is apparently phosphorylated by casein kinase 2 at a site contained within a CNBr fragment which could correspond to this region (Meggio *et al.*, 1984). Although this modification may therefore occur close to the active site the phosphorylation did not affect the ODC activity at all and no evidence that ODC was regulated by phosphorylation by the nuclear kinase N2 was obtained (Seely *et al.*, 1984). The phosphorylation could account

for the heterogeneity of mouse kidney ODC on analysis by two-dimensional gel electrophoresis, when two forms differing very slightly in charge but not in M_r are seen (Isomaa *et al.*, 1983; Persson *et al.*, 1984; Seely *et al.*, 1985). Both forms are enzymically active and the physiological significance, if any, of this modification is unclear.

There have been various reports of multiple forms of ODC based on heterogeneity on chromatography on DEAE-cellulose (most recently by Flamigni *et al.*, 1984; Laitinen *et al.*, 1985 and Mitchell *et al.*, 1985). This analytical method does not rule out the possibility that the apparent multiple forms are due to artifactual interactions with the high level of pyridoxal phosphate used in ODC extraction buffers or to the complex of ODC with other proteins. No heterogeneity of ODC with respect to size, and only two (or possibly three) forms differing very slightly in charge, have been observed when analysed more rigorously by gel electrophoresis (Isomaa *et al.*, 1983; Persson *et al.*, 1984; Seely *et al.*, 1985; Laitinen *et al.*, 1985).

S-Adenosylmethionine decarboxylase (AdoMetDC)

AdoMetDC has been the subject of two quite recent reviews which deal in detail with the structure and regulation of the activity of this interesting enzyme which contains a covalently bound pyruvate prosthetic group (Pegg, 1984*a*; Tabor & Tabor, 1984*b*). The enzyme, which has a subunit M_r of 32000, has been purified from a variety of mammalian sources and high-affinity monospecific antibodies have been raised to enzyme from rat prostate (Shirahata *et al.*, 1985; Shirahata & Pegg, 1985) and bovine lymphocytes (Seyfried *et al.*, 1982). These antibodies have been used to show that both the rate of synthesis and the half-life of the protein are increased when bovine lymphocytes were stimulated with mitogens (Seyfried *et al.*, 1982).

There is no irreversible inhibitor comparable to DFMO which can be used to titrate and label AdoMetDC, but a suitable procedure has recently been developed. The Schiff base between labelled decarboxylated AdoMet and the pyruvate prosthetic group was reduced by sodium cyanoborohydride to give a stable covalent bond. This procedure labelled the 32000 M_r subunit stoichiometrically and provided a labelled ligand for RIA (Shirahata *et al.*, 1985).

Polyamine depletion brought about by DFMO and other inhibitors leads to a large increase in AdoMetDC activity; conversely, high levels of polyamines reduce AdoMetDC activity (Mamont *et al.*, 1982; Pegg, 1984*a*,*b*; Tabor & Tabor, 1984*b*). It was found using both RIA and the titration method described above that the increased activity of AdoMetDC in cells treated with DFMO was due to a parallel rise in the amount of enzyme protein. Similarly, the fall in AdoMetDC on application of exogenous polyamines was due to the loss of enzyme protein (Shirahata *et al.*, 1985). More detailed studies of the effects of DFMO on AdoMetDC in the rat prostate indicated that the increased activity is due both to a stabilization of the active protein, whose half life is increased by 2–3-fold (Shirahata & Pegg, 1985), and to an increased rate of synthesis which is brought about by a 6-fold increase in the amount of mRNA (A. Shirahata & A. E. Pegg, unpublished work). In contrast, the well-known increase in AdoMetDC produced by methylglyoxal bis(guanylhydrazone) (MGBG) was entirely due to stabilization of the protein as previously reported (Pegg *et al.*, 1982*a*, Pegg, 1984*b*).

Prostate mRNA, which was enriched for AdoMetDC by pretreatment of the rats with DFMO, and further purified by immunoprecipitation of polysomes, appeared to be at least 10% pure AdoMetDC mRNA when translated in the reticulocyte lysate system. This material is currently being used for cloning of the cDNA for AdoMetDC. Translations *in vitro* show that the protein is made as a precursor of M_r 37000 which is converted to the active enzyme subunit of 32000 (A. Shirahata & A. E. Pegg, unpublished work). This presumably generates the pyruvate at the *N*-terminus as described by Recsei & Snell (1984) for bacterial enzymes.

At present there are no good inhibitors of AdoMetDC which can be used to influence polyamine metabolism by blocking this enzyme specifically (Kolb *et al.*, 1982; Pegg & Jacobs, 1983). The only nucleoside which had appreciable inhibitory activity was *S*-methyl-5′-methylthioadenosine [$AdoS^+(CH_3)_2$] which had a K_i of 2 μM (Kolb *et al.*, 1982) or 6 μM (Pegg & Jacobs, 1983), but this nucleoside is a much more potent inhibitor of spermine synthase (see below). Although MGBG is well known as an inhibitor of AdoMetDC it has a wide variety of other effects on cells (Pegg & McCann, 1982). These include the ability to cause severe mitochondrial damage (Pleshkewych *et al.*, 1983; Nikula *et al.*, 1985), to inhibit diamine oxidase (Pegg & McCann, 1982; Jänne & Morris, 1984; Kallio *et al.*, 1984), to interfere with polyamine transport (Jänne *et al.*, 1983; Wiseman *et al.*, 1983; Kramer *et al.*, 1985) and to induce SAT (Persson & Pegg, 1984; Pegg *et al.*, 1985*b*; Karvonen & Pösö, 1984). Furthermore, the substantial increase in AdoMetDC brought about by MGBG because it stabilizes the enzyme (Pegg *et al.*, 1982*a*; Pegg, 1984*b*; Tabor & Tabor, 1984*b*) reduces its effectiveness as an inhibitor *in vivo*. Although it may be possible to prevent the mitochondrial damage by addition of carnitine, as reported by Nikula *et al.* (1985), the multitude of effects relating to polyamine metabolism makes it extremely difficult to interpret experiments with this drug. In particular, it should be noted that the decrease in spermidine and spermine and the increase in putrescine brought about by MGBG may well be mediated principally by the acetylase/oxidase pathway via induction of SAT (Pegg *et al.*, 1985*b*). Also, the inhibition of intestinal diamine oxidase may contribute to the rise in putrescine (Kallio *et al.*, 1984). In some respects ethylglyoxal bis(guanylhydrazone), which is a more potent inhibitor *in vitro* (Pegg & Jacobs, 1983; Jänne & Morris, 1984; Seppänen *et al.*, 1984), may be preferable but it is unlikely to be free from all of these side effects and in fact is known to inhibit diamine oxidase (Jänne & Morris, 1984) and to induce SAT (A. E. Pegg & B. G. Erwin, unpublished work).

Spermidine synthase and spermine synthase

The aminopropyltransferases have been fully characterized from bovine brain and from rat prostate and liver (Samejima & Yamanoha, 1982; Yamanoha *et al.*, 1984; Raina *et al.*, 1984). Spermidine synthase consists of two subunits of M_r about 36000 and spermine synthase has two subunits of M_r 44000. Detailed kinetic studies of both the spermine synthase and the spermidine synthase reactions have now been carried out using homogeneous preparations of these enzymes (Samejima & Yamanoha, 1982; Pajula, 1983; Raina *et al.*, 1984). These have

confirmed earlier reports that these enzymes have K_m values for decarboxylated AdoMet of 0.1–1.1 μM and that it is likely that they are regulated by the availability of this substrate and their competition for it. The profound inhibition of spermine synthase by its product MTA is now established (Pajula, 1983) and the rate of spermine synthesis is likely to be affected by the accumulation of MTA in those cells which lack MTA phosphorylase (Kamatani *et al.*, 1981; Iizasa & Carson, 1985).

The most potent and specific inhibitor of spermidine synthase is AdoDATO, a mechanism-based inhibitor, which has an I_{50} of less than 20 nM under assay conditions approximately comparable to those *in vivo* (Pegg *et al.*, 1982*b*, 1983). AdoDATO does not significantly inhibit spermine synthase. AdoDATO causes a depletion of spermidine but an increase in spermine in cells treated with it (Pegg *et al.*, 1982*b*). This result is in agreement with the concept that the two aminopropyltransferases compete for the available decarboxylated AdoMet. In the presence of AdoDATO the spermine synthase is favoured in this competition.

Another substance which has been used to inhibit spermidine synthase is cyclohexylamine (Hibasami *et al.*, 1980; Pegg *et al.*, 1983). [It should be noted that this compound as sold by the Sigma Chemical Co. is incorrectly identified by them and by workers publishing results with it as dicyclohexylamine (Batchelor *et al.*, 1986)]. The specificity of cyclohexylamine as an inhibitor of spermidine synthesis remains to be established but it is quite a potent inhibitor which is apparently well taken up by cells.

Spermine synthase is strongly inhibited by $AdoS^+(CH_3)_2$, which had an I_{50} of 8 μM in assays containing 5 μM-decarboxylated AdoMet, which is considerably above physiological substrate concentration (Pegg & Coward, 1985). This inhibition was quite specific for spermine synthase as spermidine synthase was inhibited by only 34% with 1 mM-$AdoS^+(CH_3)_2$. When SV-3T3 cells were treated with $AdoS^+(CH_3)_2$ at doses of 50–400 μM there was a complete inhibition of spermine synthesis and a corresponding increase in spermidine. At higher doses the concentration of both spermine and spermidine decreased, suggesting that the inhibition of AdoMetDC also occurred at these concentrations of $AdoS^+(CH_3)_2$ (Pegg & Coward, 1985).

Acetylation and interconversion of polyamines

Acetylation of polyamines can be catalysed by both the predominantly nuclear histone acetylase (Libby, 1983) and by SAT which is a cytosolic enzyme. These enzymes are completely different and have been distinguished by their substrate specificity (Della Ragione & Pegg, 1983, 1984), the use of specific antibodies (Persson & Pegg, 1984) and their differential response to inhibitors (Erwin *et al.*, 1984). The possibility that polyamines inhibit the acetylation of histones (or vice versa) *in vivo* because they are substrates for the same nuclear acetylase has been raised (Dod *et al.*, 1982; Libby, 1983) but little firm information is available. Virtually all of the product of the acetylation of spermidine by the nuclear enzyme is N^8-acetylspermidine (Seiler *et al.*, 1981; Erwin *et al.*, 1984). The only known fate of this compound is deacetylation (Blankenship & Marchant, 1984; Della Ragione & Pegg, 1984) which regenerates spermidine and the significance of this acetylation is unclear. Some information on this point may be obtained by the use of 7-amino-2-heptanone which has been reported to be a potent competitive inhibitor (K_i of 2.2 μM) of the deacetylase (Mamont *et al.*, 1984*b*).

Detailed studies of the specificity, kinetics and active site of SAT have been carried out using homogeneous preparations (Della Ragione & Pegg, 1983; Della Ragione *et al.*, 1983). SAT does not act at all on histones and only acetylates substrates which have the structure R-NH-$(CH_2)_3NH_2$. It therefore forms only N^1-acetylspermidine and no N^8-acetylspermidine when acting on spermidine. Similarly SAT does not acetylate *sym*-homospermidine at all but *sym*-norspermidine is an excellent substrate.

The acetylation of polyamines via SAT appears to be the limiting factor in the degradation and interconversion of the polyamines. N^1-Acetylspermidine and N^1-acetylspermine are rapidly degraded by polyamine oxidase (FAD-dependent). Although this oxidase will act on the non-acetylated polyamines under certain artificial conditions *in vitro*, it is clear that the acetylated derivatives are the true physiological substrates (Bolkenius & Seiler, 1981; Bolkenius *et al.*, 1985; Morgan, 1985). Since polyamine oxidase activity is usually vastly in excess of that of the SAT the cellular content of the acetylated polyamines is normally very low and frequently below the limit of detection (Seiler *et al.*, 1981; Pegg *et al.*, 1982*a*, Pegg & McCann, 1982). It has recently been proven conclusively that this oxidation of the N^1-acetylated derivatives occurs *in vivo* by administration of N^1-methyl-N^2-(2,3-butadienyl)-1,4-butanediamine or N^1,N^2-bis-(2,3-butadienyl)-1,4-butanediamine which are potent, enzyme-activated, irreversible inhibitors of polyamine oxidase (Bey *et al.*, 1985). Administration of these compounds to rodents or to HTC cells in culture leads to an accumulation of N^1-acetylspermidine and N^1-acetylspermine (Mamont *et al.*, 1984*b*; Bolkenius *et al.*, 1985; Seiler *et al.*, 1985; Seiler & Bolkenius, 1985). No significant toxic effects or effects on cell growth were observed, indicating that the degradation of the acetylated derivatives was not an essential reaction for normal cellular physiology under these conditions. The physiological function of polyamine oxidase therefore remains obscure but it may provide a salvage pathway by which the putrescine portion of the polyamine molecule can be retained under conditions where it is necessary to reduce the cellular content of the higher polyamines.

SAT is very highly inducible in response to a wide variety of toxic stimuli (Della Ragione & Pegg, 1984; Persson & Pegg, 1984). [For example, a 250-fold increase in the amount of SAT occurs in the liver of rats within 6 h of treatment with CCl_4.] These inductions are brought about by an increase in the amount of enzyme protein which occurs because of both a large increase in the rate of synthesis and in some cases by a decline in the rate of degradation (Persson & Pegg, 1984; Pegg *et al.*, 1985*b*; Karvonen & Pösö, 1984). In these respects SAT [which also has a very short half-life (Pegg *et al.*, 1982*a*; Persson & Pegg, 1984)] is similar to ODC. The suggestion that SAT is regulated by a phosphorylation/dephosphorylation mechanism was made by Matsui *et al.* (1982) based on studies in which the enzyme was apparently inactivated by alkaline phosphatase. However, this inhibition appears to be due to an effect of residual alkaline phosphatase on the substrate since the 3′ phosphate group on acetyl-CoA is essential for substrate

activity (Della Ragione *et al.*, 1983). Furthermore, such post-translational regulation is not supported by studies with antibodies in which there was a close correlation between activity and immunoreactive protein (Persson & Pegg, 1984).

SAT is also induced by administration of polyamines and their synthetic analogues (Persson & Pegg, 1984; Pegg *et al.*, 1985*b*; Pegg & Erwin, 1985). This induction can lead to a substantial breakdown of intracellular polyamines to putrescine. Administration of polyamine analogues which are potent inducers of SAT may provide a means to deplete the normal cellular polyamine content (Pegg & Erwin, 1985). One substance that has this effect is MGBG, which is a very potent inducer of SAT (Persson & Pegg, 1984; Karvonen & Pösö, 1984; Pegg *et al.*, 1985*b*). It is possible that much of the depletion of polyamines and increase in putrescine brought about by MGBG is due to its induction of the SAT/oxidase pathway rather than its inhibition of AdoMetDC (Pegg *et al.*, 1985*b*).

The mechanism underlying the induction of SAT by hepatotoxins and by exogenous polyamines is not known nor is the physiological importance of this induction. One possible unifying hypothesis would be that SAT is induced whenever the concentration of free polyamines in the cell exceeds a certain critical level and that its function is to reduce this level by acetylation of the excess. The damage produced by hepatotoxins such as CCl_4 may lead to the release of polyamines from bound intracellular sites and hence trigger the induction of SAT. Erwin *et al.* (1984) have reported that *N*-[2-(*S*-coenzyme A)acetyl]*sym*-norspermidine amide is a potent inhibitor of SAT. It is possible that this inhibitor can be used to demonstrate whether acetylation of polyamines is critical for the cell under conditions where SAT is induced. The additional use of the inhibitors of polyamine oxidase described above would be valuable in determining whether degradation of the acetylated polyamine is essential or merely a salvage mechanism.

It should also be pointed out that large inductions of SAT have beeen reported in cells in response to a variety of hormones and other stimuli, including liver regeneration or growth hormone (Della Ragione & Pegg, 1984), secretin or 3-isobutylxanthine (Danzin *et al.*, 1983*b*), phytohaemagglutinin (Matsui *et al.*, 1983), phorbol esters (Matsui-Yuasa *et al.*, 1984), 1,25-dihydroxyvitamin D_3 (Shinki *et al.*, 1985) and serum growth factors (Wallace *et al.*, 1985). In many cases these inductions may actually be understated in the articles because only a small percentage of the basal acetyltransferase activity found in cytosolic extracts of uninduced cells is actually immunoprecipitable SAT, whereas all of the induced activity reacts with the antibodies to SAT (Persson & Pegg, 1984; Pegg & Erwin, 1985; Pegg *et al.*, 1985*b*).

Catbolism of polyamines

The biochemistry underlying the final disposition of mammalian polyamines which involves both metabolic degradation and excretion is still relatively poorly understood. However, quite detailed studies have now been carried out on the identification and quantitation of polyamine catabolites (Seiler *et al.*, 1981, 1982, 1985; Seiler & Knodgen, 1983; Van Den Berg *et al.*, 1984; Bandle *et al.*, 1984). Urine contains a variety of oxidation products including N^8-(2-carboxyethyl)spermidine, spermic acid, putreanine, isoputreanine lactam [also referred to as *N*-(3-aminopropyl)pyrrolidin-2-one], γ-aminobutyric acid, pyrrolidin-2-one, β-alanine, the polyamines themselves and acetylated derivatives of the polyamines and their oxidation products.

Although a variety of mammalian enzymes are known which oxidize polyamines and putrescine (Seiler *et al.*, 1983, 1985; Morgan, 1985) some of these are extracellularly located and the extent to which they contribute to the breakdown of intracellular polyamines is unclear. It is a major source of confusion in the literature that a variety of enzymes oxidizing polyamines which are clearly different both in their substrate specificities and in their sites of action are frequently referred to by the same name of 'polyamine oxidase' or 'diamine oxidase'. The use of the terminology recently introduced by Seiler *et al.* (1983, 1985) of copper-containing amine oxidases which catalyse the oxidative deamination of the terminal amino groups into products which cannot be reconverted into polyamines ('terminal catabolism') and of polyamine oxidase (FAD-dependent) for the polyamine oxidase involved in interconversion is a step in the right direction. However, the class of enzymes catalysing terminal catabolism probably contains several members. It is not clear which of these is the major contributor to removal of polyamines or what is the preferred substrate. It is possible that putrescine may be the major substrate for such terminal oxidation under some conditions. The acetylase/oxidase pathway described above may facilitate polyamine catabolism by converting spermidine and spermine into putrescine. It is also possible that the acetylation of polyamines is used as a means to facilitate their excretion from the cell (Wallace *et al.*, 1985) and that the oxidase/acetylase pathway provides a way to mobilize spermine which may be predominantly bound in some compartmentalized form and is excreted in only very small amounts in urine.

Seiler *et al.* (1985) used aminoguanidine as an inhibitor of the copper-containing amine oxidases responsible for terminal catabolism of polyamines and N^1-methyl-N^2-(2,3-butadienyl)-1,4-butanediamine as an inhibitor of polyamine oxidase (FAD-dependent) to investigate the catabolism of polyamines in the rat. They found that about 40% of the polyamines which were eliminated were excreted in the urine as polyamines or acetylated derivatives and about 60% was catabolized by the copper-containing oxidases.

Mutants lacking polyamine biosynthetic enzymes

A strain of CHO cells which required either ornithine or polyamines for growth in cell-free media was found to lack arginase (Hölttä & Pohjanpelto, 1982). Most serum contains sufficient arginase activity to provide enough ornithine from arginine in the culture medium, but in serum-free media cellular arginase is essential for polyamine production unless ornithine is provided. Mutants truly deficient in ornithine decarboxylase have now been isolated in CHO cells using a suicide selection procedure with [^{3}H]ornithine (Steglich & Scheffler, 1982; Pohjanpelto *et al.*, 1985*b*). The P22 cells obtained in this way have no detectable ODC activity but contain normal amounts of ODC mRNA and immunoreactive ODC-protein with the same M_r (51000) as the wild type (Pohjanpelto *et al.*, 1985*b*). These cells have an absolute requirement for polyamines for growth.

Function of polyamines

The function of the polyamines in mammalian cells remains obscure although there is general agreement from studies with inhibitors and mutants that polyamines are essential for cell growth. The predominant effects of exposure of cultured cells to ODC inhibitors such as DFMO are a decline of putrescine and spermidine to almost undetectable levels and a large increase in the content of decarboxylated AdoMet. Spermine levels (on a per cell basis) are depleted only slightly. The lack of depletion of spermine is due to: (*a*) the continued synthesis of a small amount of putrescine which is completely converted to spermine because of the excess decarboxylated AdoMet; (*b*) the virtual cessation of cell growth and the lack of substantial degradation of spermine (Pegg & McCann, 1982)

Recently, it has been shown that a greater extent of spermine depletion can be achieved in a number of ways. Mamont *et al.* (1984*a*) report that (2*R*, 5*R*)-MAP, which is a more potent inhibitor of ODC than DFMO (Danzin *et al.*, 1983*a*), depletes all polyamines in HTC cells including spermine. Spermine was reduced to 40–50% of the normal value and there was a progressive loss of cell viability under these conditions. The combination of DFMO with a spermidine synthase inhibitor, AdoDATO, led to a 70–80% reduction in spermine in SV-3T3 cells and this decline was also accompanied by a progressive loss of viability (Pegg *et al.*, 1982*b*). Casero *et al.* (1984) and McGovern *et al.* (1986) have shown that various spermidine analogues produce only a transient increase in growth when applied to cells in which normal polyamine synthesis is blocked by DFMO. This transient burst of growth leads to a reduction in cellular spermine by increasing the cell number under conditions in which spermine synthesis cannot keep up. When spermine levels become reduced by more than 60–70%, growth ceases and a loss of cell viability ensues. Exposure to $N^{1,8}$-spermidine derivatives appears to reduce cellular polyamine levels by repression of both ODC and AdoMetDC activity and leads to more than 50% reduction of spermine and inhibition of cell growth (Porter *et al.*, 1985).

Although there are certain exceptions [such as the human tumour cells studied by Luk *et al.* (1982*a*, *b*), Sunkara *et al.* (1983) and Sano *et al.* (1984*a*)] it has been found in most cases that DFMO exerts cytostatic rather than cytotoxic effects towards mammalian cells (see references in Pegg & McCann, 1982; Mamont *et al.*, 1980, 1982, 1984*a*; Porter & Bergeron, 1983; Pegg *et al.*, 1982*b*; Pegg, 1984*b*). The reduction in cell growth rate can be completely overcome by addition of putrescine, spermidine or spermine.

There are several clear examples of the fact that cells treated with DFMO will grow at a normal rate if spermine is added (Mamont *et al.*, 1980; Porter & Bergeron, 1983; Rudkin *et al.*, 1984; Pegg, 1984*b*). Under these conditions spermine is virtually the only cellular polyamine although there is a small amount (up to 10% of normal) of spermidine present which is presumably produced by the acetylase/oxidase interconversion system. This implies that all of the cellular functions of polyamines can be fulfilled by spermine. However, as discussed above, spermine is not depleted in cells in which growth is arrested as a consequence of treatment with DFMO and the addition of exogenous spermidine will also support growth in these cells. Furthermore, recent studies in which the synthesis of spermine in SV-3T3 cells was selectively inhibited by the application of $AdoS^+(CH_3)_2$ revealed that these cells grew at a normal rate (Pegg & Coward, 1985). Such cells had elevated levels of spermidine but contained less than 20% of the normal spermine content.

These results indicate that either spermidine or spermine can support growth in mammalian cells. It is an interesting question as to why they contain a specific enzyme for the synthesis of spermine when it is not needed for growth. Two possible explanations are: (*a*) that spermine may have other functions in the cell not related to growth; and (*b*) spermine may act as storage form of the polyamines which is normally present in some bound or compartmentalized form. More detailed studies of the effects of the spermine synthase inhibitors on cellular physiology including differentiation would be of considerable interest.

The very large increase in the content of decarboxylated AdoMet which occurs in cells treated with DFMO is probably not directly responsible for the inhibition of cell growth. Analogues of spermidine which produce a reversal (albeit transient) of this effect did so at a time before there was a reduction of the decarboxylated AdoMet content to control values (Mamont *et al.*, 1982; Pegg, 1984*b*; McGovern *et al.*, 1986). Furthermore, the accumulation of decarboxylated AdoMet does not act as a trap for adenine. In fact there is an increase in the content of adenine nucleotides and of the other nucleoside di- and tri-phosphates (Heby *et al.*, 1984). This may reflect the slowing down of macromolecular synthesis. However, these studies of the effects of the accumulation of decarboxylated AdoMet in DFMO-treated cells have only been made in a few cell types and only growth rates were considered. The possibility that the large increases in the content of decarboxylated AdoMet might affect other cells and other cellular processes including differentiation (Heby, 1981; Pegg & McCann, 1982), perhaps by influencing methylation reactions, has not been ruled out.

The rise in decarboxylated AdoMet content in cells treated with DFMO is accompanied by an increase in another nucleoside which was first noticed but not identified by Wagner *et al.* (1982). We have recently identified this material as acetylated decarboxylated AdoMet and shown that it is synthesized by the same enzyme which acetylates histones (Pegg *et al.*, 1985*a*). Decarboxylated AdoMet concentrations similar to those which accumulate in DFMO-treated cells lead to substantial inhibition of histone acetylation *in vitro*. This provides another possible area in which the nucleoside could affect cellular physiology (Pegg *et al.*, 1985*a*). The formation of the acetylated derivative of decarboxylated AdoMet indicates that it can be metabolized in reactions other than those catalysed by the aminopropyltransferases.

The exact cause of the reduction in cell growth rate in cells in which spermidine is depleted by DFMO is still not fully understood. Although DNA synthesis in such cells is clearly inhibited, detailed studies by Rudkin *et al.* (1984) have shown that the earliest effect is seen on protein synthesis and that a normal polysomal profile requires the addition of spermidine or spermine. It is possible therefore that the changes in DNA synthesis are secondary to a reduction in the rate of protein synthesis.

There is a large body of literature indicating that polyamines can influence protein synthesis in a variety of ways affecting both the rate and the fidelity of translation (Pegg & McCann, 1982; Takemoto *et al.*, 1983; Rudkin *et al.*, 1984; Tabor & Tabor, 1984*a*). The cytostatic effect of polyamine biosynthesis inhibition could therefore be due to the reduced rate of synthesis of certain key proteins. The loss of cellular viability which accompanies the more complete depletion of spermine may be due to the requirement for polyamines to maintain normal chromatin structure, since polyamine depletion by starvation of mutants lacking ODC or by extensive treatment with DFMO caused major chromosome aberrations as well as other alterations in nuclear morphology and loss of actin filaments and microtubules (Pohjanpelto & Knuutila, 1982, 1984; Knuutila & Pohjanpelto, 1983; Pohjanpelto *et al.*, 1985*a*).

Post-translational modifications of proteins involving polyamines

The translation initiation factor eIF-4D contains the amino acid, hypusine, which is formed from lysine by an addition which originates from spermidine (Cooper *et al.*, 1983; Park *et al.*, 1984*a*). Hypusine is N^{ϵ}-(4-amino-2-hydroxybutyl)lysine and it is formed by the addition to lysine of the butylamine moiety from spermidine followed by hydroxylation (Park *et al.*, 1984*b*; Paz *et al.*, 1984). There is one molecule of hypusine per molecule of eIF-4D which has an M_r of 18000 and the tryptic peptides containing hypusine derived from eIF-4D from a variety of sources appear to be identical (Park *et al.*, 1984*a*). This conservation suggests an important function for this modification, but the role of this factor in protein synthesis and the function of the hypusine is not known. There seems to be a correlation between the rate of formation of hypusine and the rate of cellular growth (Torrelio *et al.*, 1984). At present, the 18000-M_r protein which has been identified as eIF-4D is the only protein known to contain hypusine (Cooper *et al.*, 1983; Chen, 1983). Hypusine has been found in a wide variety of tissues (Sano *et al.*, 1984*b*) but this is to be expected since eIF-4D is ubiquitous.

There has been much interest in the possibility that polyamines may become bound to proteins by transglutaminases. There is no doubt whatsoever that this reaction can take place *in vitro* and that certain extracellular proteins may contain polyamines which are covalently attached via the action of transglutaminases (Williams-Ashman *et al.*, 1980). Such reactions may have physiological significance under certain conditions. For example, it has been suggested that polyamines may regulate the formation of clots in seminal fluid by acting as competitive substrates for the transglutaminases secreted by the coagulating gland (Williams-Ashman, 1984).

Incorporation of polyamines into intracellular proteins by transglutaminases may also occur. However, the metabolic incorporation of radioactivity from polyamines into some uncharacterized covalent attachment to protein has often been attributed to a transglutaminase-mediated event. This is not sufficient evidence without supporting evidence in which the actual structure of the attached polyamine has been analysed and shown to reside in a γ-glutamyl linkage. Hypusine production appears to account for the majority of the radioactive labelling of cellular proteins when cells are cultured in the presence of radioactive polyamines (Chen, 1983; Cooper *et al.*, 1983). Apart from the possibility of hypusine formation, a variety of artifactual incorporations can occur particularly if amine oxidases are present. These possibilities and the criteria which should be invoked to confirm rigorously the involvement of transglutaminases are discussed by Lorand & Conrad (1984). Few of the published reports describing polyamine incorporation by transglutaminases as a normal intracellular process provide this unequivocal evidence. A notable exception is the work of Beninati *et al.* (1985) which indicates that transglutaminase-mediated incorporation of polyamines into protein does occur in rat liver, kidney and testis.

Cancer chemotherapy

The possible use of DFMO and related ODC inhibitors as anti-cancer agents is reviewed by Sjoerdsma & Schechter (1984) and Jänne *et al.* (1983). The anti-proliferative action of DFMO suggests it may be effective in slowing tumour growth and it clearly does so in animal models. However, the general lack of cytotoxic effects also implies that it is unlikely to be effective clinically and this has been found to be the case. Unfortunately this applies even for those tumours such as lung small cell carcinoma in which DFMO was found to be cytotoxic (Luk *et al.*, 1982*a*). Although it is possible that the more potent ODC inhibitors such as (2*R*, 5*R*)-MAP, which are more effective as anti-tumour agents in animal models (Sjoerdsma & Schechter, 1984; Bartholeyns *et al.*, 1984), may also be more active in the treatment of human neoplasms it seems likely that the main role of the ODC inhibitors may be in combination chemotherapy. This is a complex topic which has been discussed by Marton *et al.* (1983) and by Jänne *et al.* (1983).

At the simplest level it is possible that the ODC inhibitors may be used to prevent or slow the rate of tumour regrowth between doses of therapy with radiation or other more toxic chemotherapeutic agents. It also appears that there may be additive or even synergistic actions between DFMO and other agents. The most promising such synergism seems to be between DFMO and certain interferons (Heston *et al.*, 1984; Rosenblum & Gutterman, 1984; Sunkara *et al.*, 1984), although the mechanism underlying this effect is entirely unknown. It was suggested that the combination of DFMO priming followed by administration of MGBG might enhance the anti-tumour activity of MGBG because of its increased cellular uptake (Jänne *et al.*, 1983). Although some exciting preliminary data was obtained, this approach has not proved successful in the clinical trials so far completed. This is probably because the major problem with MGBG as an anti-tumour agent is its toxicity and the uptake of the drug into tumour cells is not usually a limiting factor. It is not clear that the priming with DFMO selectively enhances the uptake into tumours and not into normal cells (Kramer *et al.*, 1985).

Finally, Marton and his colleagues have shown that polyamine depletion brought about by DFMO sensitizes cells to killing by certain anti-tumour agents such as the nitrosourea BCNU (Marton *et al.*, 1983; Sano *et al.*, 1984*a*). This synergism may result from the increased production of DNA cross-links by BCNU owing to the altered DNA structure in the absence of polyamines (Tofilon *et al.*, 1983). Similarly it has been found that the polyamine depletion brought about by DFMO increases the topoisomerase II-mediated DNA breakage which

occurs when the cells are treated with m-AMSA (Zwelling *et al.*, 1985). This could result from an increased intercalation of m-AMSA into the DNA or to an increase in topoisomerase binding affinity or binding sites. In either case the polyamine depletion appears to alter DNA or chromatin structure to increase the toxicity of the drug. These results suggest that there may be beneficial chemotherapeutic effects of combining polyamine biosynthesis inhibitors with antineoplastic agents which are known to bind to DNA. However, care must be used in selecting these agents since pretreatment with DFMO actually decreased sensitivity to certain other drugs, including *cis*-platinum (Marton *et al.*, 1983; Tofilon *et al.*, 1983). This decrease was correlated with a decline in the number of crosslinks brought about by *cis*-platinum (Tofilon *et al.*, 1983). These results emphasize that polyamine depletion may bring about significant changes in chromatin and DNA structure and that the effects of these changes may be complex.

Antiprotozoal effects of ODC inhibitors

DFMO inhibits the replication of a number of parasitic protozoa including various African trypanosomes, and *Eimeria tenella*, *Giardia lamblia*, *Plasmodium falciparum* and *Pneumocystis carinii* (McCann *et al.*, 1983; Sjoerdsma *et al.*, 1984). It is remarkably active against *Trypanosoma brucei brucei* infections in mice and acts against strains of *T. congolense* which are resistant to standard trypanocides (Bacchi *et al.*, 1983; McCann *et al.*, 1983; Schillinger & Gorton, 1984). DFMO has been found to be an effective agent in treating human sleeping sickness caused by African trypanosomes and is active against late stage trypanosomiasis even in the normally fatal melarsoprol-resistant cases (Sjoerdsma & Schechter, 1984; Sjoerdsma *et al.*, 1984).

The reason for the sensitivity of these organisms to DFMO is not entirely clear. The ODC from *T. brucei brucei* is no more sensitive to DFMO (K_i of 139 μM) than the ODC of mammalian cells (Bitonti *et al.*, 1985*b*). DFMO enters the parasites by passive diffusion so there is no selective uptake of the drug (Bitonti *et al.*, 1985*a*). The effects of DFMO on polyamine metabolism in the trypanosomes are similar to those found in other eukaryotes. Putrescine and spermidine are substantially depleted and there is a more than 1000-fold increase in decarboxylated AdoMet (Bacchi *et al.*, 1983). Spermine is not normally present in these parasites but it is found in small amounts after DFMO, presumably due to the increase in decarboxylated AdoMet driving any putrescine into spermine. There is a large decrease in the rate of nucleic acid synthesis in DFMO-treated *T. brucei brucei* and the depletion of polyamines induces morphological changes from the long slender parasitic forms to short stumpy forms with multiple nuclei and kinetoplasts.

These changes are consistent with the idea that the trypanosomes require polyamines for normal cell replication. It appears that the immune system can deal with the parasites when their replication is slowed by polyamine depletion. An intact immune response (but not T-cells, since nude mice can be cured) is required for the cures of trypanosomiasis with DFMO (de Gee *et al.*, 1983; Bitonti *et al.*, 1985*c*).

Another possible site of action is suggested by the recent findings that trypanosomes contain a glutathione reductase which requires a novel cofactor containing spermidine for activity. This cofactor, termed trypanothione, has been purified from the insect trypanosome, *Crithidia fasciculata*, and has been identified as N^1,N^8-bis-(L-γ-glutamyl-L-hemicystinyl-glycyl)spermidine (Fairlamb & Cerami, 1985; Fairlamb *et al.*, 1985. The synthesis of this cofactor may be blocked in cells in which spermidine is depleted. However, this cannot be the sole reason for sensitivity, since trypanothione is not present in *Eimeria tenella* (Fairlamb & Cerami, 1985), the growth of which is strongly inhibited by DFMO (McCann *et al.*, 1983).

The ODC activity and schizogony of *Plasmodium falciparum* which causes human malaria was inhibited by DFMO (McCann *et al.*, 1983; Whaun & Brown, 1985). The methyl ester of 2-fluoromethyldehydro-ornithine is even more effective than DFMO against the proliferation of the rodent malarial parasite *Plasmodium bergei* (Hollingdale *et al.*, 1985). This ODC inhibitor is also more potent than DFMO against trypanosomes, but (2*R*, 5*R*)-MAP is much less active (Sjoerdsma & Schechter, 1984; Bitonti *et al.*, 1985*b*). DFMO is an effective drug against pneumonia infections caused by *Pneumocystis carinii*, which is thought to be a sporozoon (Sjoerdsma *et al.*, 1984; Golden *et al.*, 1984).

Overall it is clear that DFMO, and probably other ODC inhibitors, have considerable promise for use against these diseases caused by protozoal parasites which represent major health problems in man. A more detailed understanding of the biochemistry and function of polyamines in these parasites and their mammalian hosts should enable them to be used with the maximal effect.

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