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COMMENTARY

REGULATION OF POLYAMINE BIOSYNTHESIS

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The polyamines, putrescine, spermidine and spermine, are simple aliphatic amines exhibiting a wide range of biological activity [1–4]. The problem to be faced in elucidating their biological role is which of the many actions they exert *in vitro* are manifest in the intact cell. For many groups of compounds, designated as regulators of cell metabolism, much of the biological specificity is contained within the chemical structure of the molecule. The specificity of cyclic nucleotides and steroid hormones for their target proteins, the distinction between α and β receptors for catecholamines, and the differences between E and F prostaglandins illustrate the stringent structural restraints which are often observed in the execution of a specific biological effect. The polyamines, however, are small highly charged molecules of exceedingly simple structure, capable of interacting with a wide range of different cell components, and there is little within the chemical composition of these molecules which is likely to instill any high degree of biological specificity. If the polyamines, therefore, do exert specific regulatory functions *in vivo*, they are determined presumably by factors other than their molecular configuration and ionic deposition. The situation for the polyamines is very similar to that which exists for the histones. For such functionally elusive molecules operating within the intact cell, biological activity will be particularly susceptible to temporal influences and spatial restraints imposed by the subcellular architecture. When and where the polyamines are produced and bound and the relationship of these parameters to other cellular events and constituents are likely to be predominant factors in producing biological effects. The biosynthesis of polyamines is known to be a finely modulated process, and changes in the rates of polyamine production are known to be restricted to relatively narrow "time windows". This in itself imposes limitations upon the range of potential biological actions that could be exerted by the polyamines, and a precise delineation of these time slots in intact cells would contribute immeasurably to determining the biological role of these compounds.

There are four enzymes involved in polyamine biosynthesis: two decarboxylases and two synthases (Fig. 1). Putrescine is formed following decarboxylation of ornithine by ornithine decarboxylase. In most eukaryotic cells this enzyme is present only in low concentrations and is rate limiting in the biosynthesis of spermidine. Ornithine decarboxylase has been purified from a number of different sources [5–9]; it is a typical cytosol decarboxylase with an absolute

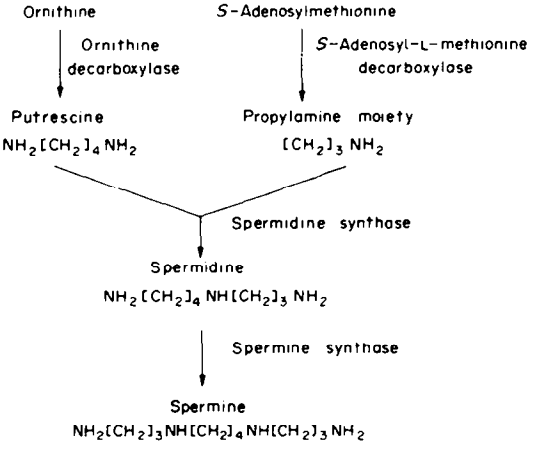

Fig. 1. Biosynthesis of polyamines.

requirement for pyridoxal phosphate. It is also stimulated by sulfhydryl compounds. In tissues and cultured cells, ornithine decarboxylase exhibits a short half-life, usually 10–20 min, and is rapidly inducible by a wide variety of different stimuli.

Putrescine is incorporated directly into spermidine and the propylamine moiety is derived from methionine. The sequence involves the synthesis of *S*-adenosylmethionine from methionine followed by decarboxylation of *S*-adenosylmethionine and transfer of the propylamine moiety to putrescine.

S-adenosyl-L-methionine decarboxylase from higher organisms is stimulated by putrescine and similar diamines [10]. This is in sharp contrast to the corresponding enzyme from bacterial sources, which shows a stringent requirement for magnesium ions, and the enzyme from lower eukaryotes such as protozoa which is not influenced by either diamines or magnesium [11, 12]. *S*-adenosyl-L-methionine decarboxylase has been purified and is the only mammalian decarboxylase requiring pyruvate as cofactor [13, 14]. It has a short half-life (35–60 min), and rapid inductibility has been observed in a number of different systems. However, inducibility for *S*-adenosyl-L-methionine decarboxylase is less pronounced and less prevalent in mammalian organisms than it is for ornithine decarboxylase.

Spermidine synthase transfers the propylamine moiety from decarboxylated *S*-adenosyl-L-methionine to putrescine. A similar enzyme, spermine

synthase, adds another propylamine moiety to spermidine to yield spermine. Spermidine synthase may form a functional complex with *S*-adenosyl-L- methionine decarboxylase since the two enzymes were initially found to be difficult to separate from each other during purification [15]. Both synthases are more stable than the decarboxylases although, recently, spermidine synthase in the mammary gland has been shown to be inducible by insulin [16].

Functionally, the polyamines may be regarded as a group of compounds acting as organic cations. They offer a selective advantage over inorganic cations in that intracellular synthesis is possible. In many systems their effects are not qualitatively different from that of the divalent cations, particularly Mg^{2+} and in a wide range of *in vitro* systems studied, the effectiveness of the polyamines follows a cationic progression, i.e. spermine, the strongest base, is the most effective, followed by spermidine and then by putrescine. Some studies, however, have indicated precise structural demands for a specific polyamine. For example, there is a stringent requirement for the 4-carbon chain of putrescine in the stabilization of DNA in λ phage heads. Amines with more than five or fewer than three $-CH_2$ groups were ineffective [17].

The relationships between polyamines and nucleic acids have attracted considerable attention and have been reviewed extensively [1–4]. Reactions in the sequence DNA → RNA → protein in which the polyamines have been implicated include, at the transcriptional level, strand selection and chain initiation, extension or termination, and at the translational level, preparation of tRNA, aminacylation, message binding to the 30S subunit, and ribosomal subunit assembly. As Cohen [1] has pointed out, the polyamines could play a role in almost every step from DNA to RNA and protein.

Regulation of ornithine decarboxylase activity

The rate-limiting step in the synthesis of polyamines in most instances is the activity of ornithine decarboxylase. When cells are stimulated to proliferate, there is a large increase in ornithine decarboxylase activity. The same basic pattern of response is observed in many different cell types. There is a slight delay or a lag period of about 1 hr, then a sharp increase in ornithine decaboxylase activity with a peak occurring around 4 hr after which the activity declines sharply. During this period when enzyme activity is declining, it is refractory to further stimulation. Although the refractory period has not been studied in any detail, it is, nevertheless, a vital component of the response since it ensures that the increased production of polyamines is restricted to a narrow time period. Eight to ten hr after stimulation, activity returns close to the initial level. This pattern of response is remarkably consistent, given the wide diversity of organisms, agents and procedures which produce it. It is observed, observed, for example, in regenerating rat liver after partial hepatectomy [18], during development of the chick embryo [19, 20], in the epidermis after application of tumor promoting agents [21], during the cell cycle [22, 23], and after virus infection of cells [24]. Most hormones increase ornithine decarboxylase in their target tissues, and it is particularly intriguing that the pattern of enzyme response is almost identical for both steroid and polypeptide hormones. The luteinizing hormone stimulation of ornithine decarboxylase in the ovary is identical to that of estradiol stimulation of the same enzyme in the uterus [25, 26]. The implication is that, although these two types of hormones initially interact with their respective target tissues in fundamentally different ways, there are components of the overall response which are common to both. Stimulation of ornithine decarboxylase, therefore, is acquiring the status of a biological universal in the anabolic response of a cell.

Although the overall pattern of response of ornithine decarboxylase is well established, there are numerous variations in how this is accomplished. Increases in activity are brought about primarily by alterations in the rate of protein synthesis, although there is increasing evidence that changes in the rate of degradation also occur, at least in some cell cultures. In many instances synthesis of new mRNA is required briefly. When inhibitors of RNA synthesis, such as actinomycin, D, are added shortly before or at the same time as the stimulus, induction of the enzyme is prevented, whereas if it is added 2 hr later it has little or no effect [27, 28]. Based on calculations obtained with α-amanitin, the half-life for mRNA of ornithine decarboxylase is about 7 hr in rat liver [29]. The lag period of about 1 hr, which is often observed in the response of ornithine decarboxylase, is presumably occupied with the synthesis of new RNA.

A number of cell lines in tissue culture responding to serum, growth factors, amino acids or changes in osmolarity do so with an alteration in the half-life of ornithine decarboxylase from 10 min up to as much as 120 min within the first 4-hr period of stimulation [30–33]. Alterations in the rate of degradation are not sufficient to account for the increase in ornithine decarboxylase. Alterations in the rate of synthesis appear to be the predominant factor. Nonetheless, changes in synthesis and degradation occur almost simultaneously, suggesting that both processes may be coordinated. These half-lives have been obtained by assaying enzyme activity after inhibition of protein synthesis using cycloheximide and similar compounds. Since these inhibitors can also inhibit protein degradation as well as mRNA turnover [34–36], enzyme half-lives are discussed with appropriate reservations.

In cell cultures the half-life ($T_{1/2}$) of ornithine decarboxylase is a variable function, whereas in physiological systems the $T_{1/2}$ for the enzyme is relatively constant. Moreover, in the few instances where it has been studied, such as the rat liver, the $T_{1/2}$ remains constant at around 10 min during a period of induction [27]. This is sometimes interpreted as a major distinction between cultured cells and whole animal systems, but the enzyme in the liver can be stabilized by drugs such as α-hydrazino-ornithine and α-methylornithine [37, 38]. The machinery for altering the degradative rate of ornithine decarboxylase is clearly present in physiological systems even though it may not be extensively utilized under normal conditions.

Inhibition of ornithine decarboxylase by putrescine, spermidine and spermine and other related amines has been observed in a number of physiological systems, as well as in a variety of cell cultures [31, 39–41]. The

mechanisms involved in the action of these compounds are not clear. In rat liver, ornithine decarboxylase activity declined with a half-life comparable to that seen after general inhibition of protein synthesis [41]. Such results have been widely interpreted as suggestive of end product repression, i.e. that ornithine decarboxylase levels are regulated normally by endogenous polyamines [4]. A conservative appraisal of the available evidence, however, yields several points for concern.

First, there is the question of identity. Apart from one study [31], few attempts have been made to determine the nature of the effector molecule in repressing ornithine decarboxylase activity. In whole animal experiments, injected polyamines are broken down rapidly, particularly by oxidative deamination, and some of the possible intermediate metabolites are biologically very reactive with nucleic acids [42, 43]. Some sera used in cell culture experiments contain considerable amine oxidase activity; others do not. Without some indication of these factors the possibility that metabolites are involved in reducing ornithine decarboxylase activity cannot be excluded.

The second concern is with the assumption, implicit in the end product repression hypotheses, that exogenously administered polyamine is taken up and mixes uniformly (and exclusively) with the intracellular pools. This is debatable since polyamine on traversing the cell membrane is likely to bind to the first suitably charged molecule it encounters. The locations of such interactions are not likely to coincide with those of the endogenously produced amine. This is particularly relevant for spermidine which may be synthesized in the cell nucleus at sites far removed from ports of entry of exogenously applied amine. There is little doubt that some of the administered amine enters the endogenous pool since labeled putrescine, for example, can be converted into spermidine, but quantitative studies with adequate bookkeeping are scarce.

Third, the addition of high concentrations of amines (10^{-3} to 10^{-5} M) increases the intracellular polyamine levels. However, inhibition of ornithine decarboxylase induction is also observed with much lower doses. Spermidine is particularly potent and can produce a 50 per cent inhibition at 10^{-7} M [9]. These low concentrations are unlikely to contribute significantly to the intracellular concentrations of spermidine, a fact which has prompted Canellakis *et al.* [9] to postulate that the polyamines are acting on membrane receptors. It is not clear whether this is compatible with the repression of enzyme induction by endogenously generated amines. It is also worth noting that, in those cells where changes in ornithine and *S*-adenosyl-L-methionine decarboxylases are synchronized, the rate of spermidine production 2–4 hr after cell stimulation is probably much higher than the amount of spermidine taken up into the cell. If end product repression were exerted by putrescine and spermidine in these systems, then ornithine decarboxylase would be expected to peak much earlier than 4 hr.

Fourth, specificity of the effect of exogenous amines in preventing the induction of ornithine decarboxylase has not been established unequivocally. As mentioned elsewhere in this report, spermidine and spermine are potent inhibitors of *S*-adenosyl-L-methionine decarboxylase as well as ornithine decarboxylase induction in HeLa cells. In another example, the regenerating rat liver, a single injection of diaminopropane inhibited the same enzyme by 50 per cent [44]. This was interpreted as a consequence of a much more effective inhibition of ornithine decarboxylase. On the other hand, the differential sensitivities of the two decarboxylases to diaminopropane may arise from the difference in half-lives of the enzymes relative to the clearance rate of the drug. Thus far the evidence does not eliminate the possibility that inhibition of ornithine decarboxylase by polyamines represents a selective inhibition of rapidly turning over proteins. Since such proteins constitute only a small fraction of the total cellular protein, the failure to observe an effect of polyamines on overall protein synthesis is not informative.

The fifth point of concern is that inhibition of ornithine decarboxylase by the polyamines is complex. At high doses (10^{-2} to 10^{-5} M), putrescine or spermidine induces the formation of an antizyme in some cell lines but not in others [45]. An antizyme has been defined as a protein whose synthesis is induced by the proximal or distal products of the enzyme it inhibits [46]. The antizyme for ornithine decarboxylase is a heat labile trypsin-sensitive protein with an apparent molecular weight of 26,500. The antizyme is specific for ornithine decarboxylase and induction of it is not blocked by actinomycin D. Antizymes to ornithine decarboxylase described thus far have short half-lines ranging from 18 min in rat liver to 60 min in neuroblastoma cells. These half-lives are strikingly similar to those of the decarboxylase derived form the same source. The antizyme derived from one source binds with ornithine decarboxylase from a different source. The enzyme-inhibitor complex is stable through Sephadex G-75 chromatography. After treatment with 10% ammonium sulphate, however, the enzyme and inhibitor can be dissociated and separated on Sephadex. The interaction between ornithine decarboxylase and antizyme is not stoichiometric, since in the presence of excess antizyme a greater than equivalent amount of enzyme is required for neutralization [9].

The physiological significance of antizymes remains to be established. At issue is the question of whether increased endogenous polyamine production during a period of elevated decarboxylase activity is sufficient to induce antizyme formation. It should be noted that antizyme production after amine addition follows the same time course as the induction of ornithine decarboxylase. However, the rate of endogenous amine production is not increased until 1–2 hr after cells stimulation, so the time courses for enzyme activity and antizyme formation under physiological conditions will be out of phase. The possibility arises that antizymes are involved in the shutdown of enzyme activity, and it should be recalled that little or nothing is known about the mechanisms leading into or out of the refractory period or ornithine decarboxylase.

With respect to the validity of the concept of end product repression of ornithine decarboxylase, the difficulties raised above are by no means insurmountable. The different amines may not all be acting in the same way and the mode of action of each amine

may differ according to the dose and conditions used.

Fluctuations in ornithine decarboxylase activity are generally assumed to reflect the relative rates of synthesis and degradation of the protein. Two kinds of studies, however, indicate that there are multiple forms of the enzyme. First, different forms of the enzyme have been observed during purification. In the slime mold, *Physarum polycephalum*, putrescine formation involves two physically and kinetically distinct forms of ornithine decarboxylase [47, 48]. These enzymes differ in their stability and in their affinity for pyridoxal phosphate. Multiple forms of ornithine decarboxylase have also been demonstrated in rat liver and 3T3 cells [49, 50]. Evidence for heterogeneity of ornithine decarboxylase has also been derived from studies with antibodies and antizymes. Even when added in excess they do not remove all the enzyme activity present in a non-purified cytosol cell fraction. Antibody, however, does inhibit the activity of more purified fractions. These studies also indicate that there is more than one form of ornithine decarboxylase, but it is uncertain whether these different forms are preparative artefacts, different gene products or inconvertable subunits.

A needed resource at this time is the availability of specific antibodies and antizymes for studying molecular mechanisms of ornithine decarboxylase synthesis in cell-free systems. Although enzyme antibodies have been obtained [8, 51, 52], their scarcity would suggest technical difficulties in their preparation. Difficulties also have been encountered in the purification of antizymes. For the production of antibodies two problems are readily apparent. Purification efforts have emphasized the isolation of a single protein rather than the retention of enzyme activity. In most cases recovery of enzyme activity represents less than 3 per cent of the initial value. Accordingly, the isolated protein may simply represent greater survival value during purification rather than physiological significance. A second problem, concerning which there is little or no information available, is the lability of purified ornithine decarboxylase when returned to a physiological environment for the purposes of generating antibodies. If the protein is unstable, the chances of generating antibodies may be reduced although it should be noted that antibodies to at least one other rapidly turning-over enzyme, tyrosine aminotransferase, are readily obtainable.

Regulation of S-*adenosyl*-L-*methionine decarboxylase*

S-adenosyl-L-methionine decarboxylase has not received the same attention as ornithine decarboxylase although it is an equally fascinating enzyme. In some tissues such as the mammary gland, it may be rate-limiting in polyamine biosynthesis [53]. In many other situations, it becomes rate-limiting in the synthesis of spermidine after ornithine decarboxylase has been stimulated and putrescine levels are increased. *S*-adenosyl-L-methionine decarboxylase differs from ornithine decarboxylase in requiring pyruvate as cofactor and putrescine stimulates the enzyme partly by lowering the K_M for the substrate and partly by protecting it against inactivation [14, 54]. Spermine inhibits the enzyme at physiological concentrations by reducing the maximum velocity. *S*-adenosyl-L-methionine decarboxylase is similar to ornithine decarboxylase in that it has a short half-life and, at least in some cells, is rapidly inducible. Like ornithine decarboxylase, stabilization of the enzyme by increasing the half-life is rarely observed in physiological systems but can be produced by a substrate analogue inhibitor such as methylglyoxal bis(guanylhydrazone) (MGBG) [55]. The subcellular distribution of ornithine and *S*-adenosyl-L-methionine decarboxylase may be different. Ornithine decarboxylase is a cytosol enzyme, but experiments using cells enucleated with cytochalasin B indicate that nearly half of the *S*-adenosyl-L-methionine decarboxylase is located in the cell nucleus [56].

Between different systems there is wide variation in the mechanisms by which the overall pattern of ornithine decarboxylase response to stimulation is accomplished. Nevertheless, the end result, namely a transient increase in putrescine levels, is the same. It is subsequent to the production of putrescine that most variations in the eventual progression of polyamine biosynthesis are observed. One distinction can be drawn, for example, between cultured cells and whole animal systems. In cells in culture the alignment of the metabolic pathways is usually directed toward spermidine formation, that is, ornithine and *S*-adenosyl-L-methionine decarboxylases are increased in concert and the putrescine formed is converted rapidly into spermidine. However, a coordinated response of the two enzymes in physiological systems is a comparative rarity. Alterations in *S*-adenosyl-L-methionine decarboxylase, if they occur at all, may be quite out of phase with those of ornithine decarboxylase. The sequence has been delineated most clearly in regenerating rat liver [4]. After partial hepatectomy, ornithine decarboxylase and putrescine levels are elevated markedly within 4–6 hr. Increased putrescine activates *S*-adenosyl-L-methionine decarboxylase, increasing spermidine formation. Increases in spermidine lecels are evident 12–16 hr after partial hepatectomy, although *S*-adenosyl-L-methionine decarboxylase may not be increased until the following day. The situation in cell cultures is quite different. In HeLa cells striking similarities between ornithine and *S*-adenosyl-L-methionine decarboxylases are observed (D. V. Maudsley, unpublished observations). On cell stimulation the activity of both enzymes is increased in unison and peaks around the same time. Alterations in activity involve changes in the rates of both synthesis and degradation. Both enzymes are refractory and they have the same recovery times. They exhibit similar sensitivities to inhibitors of RNA and protein synthesis. Induction of both enzymes is inhibited potently by spermidine or spermine. In this system *S*-adenosyl-L-methionine decarboxylase does not appear to be critically dependent on putrescine since changes in enzyme level occur before any significant changes in putrescine concentration. These observations support the view that the genetic expression of those two enzymes and their intracellular regulation share many features in common [57]. It is possible that the expression of *S*-adenosyl-L-methionine decarboxylase inducibility in many physiological systems is specifically repressed and that in cells in tissue culture this regulatory

element is lost.

Putrescine can be cleared from the cell, bound, converted to spermidine, or otherwise metabolized. Conversion to spermidine is the option exercised by cells in culture. On the other hand in regenerating rat liver, it is likely that only, a small percentage of the putrescine generated is converted to spermidine, although accurate estimates are not available. Between these two extremes numerous variations in the fate of putrescine in physiological systems are known to exist. The question inevitably arises as to why there is so much diversity in polyamine biosynthesis.

Conceptually it is convenient to regard the sequence, putrescine → spermidine → spermine as a metabolic transition in which increasing molecular complexity is coupled with increasing cationic strength. As mentioned earlier, however, specificity of action within the intact cell is only partially determined by the molecular configuration and ionic deposition. A number of other factors are important in determining biological activity. In the case of the regenerating rat liver, putrescine levels are elevated by 4 hr, spermidine by 16 hr and spermine by 64 hr [4]. It follows that the cellular environment in which putrescine is generated is likely to be quite different from that in which spermine is produced. The essential point, however, is that the sum of the characteristics of the individual components, i.e. temporal, spatial, ionic, structural, etc., which collectively determine the biological activity of these amines within the cell is likely to be substantially different for each compound. Putrescine, spermidine and spermine, therefore, should, be regarded as representatives of a group of compounds each of which may, under certain circumstances, exert a specific function in the regulation of cellular activity. These actions could be quite different for the different amines. The metabolic conversion of putrescine to spermidine and spermine, therefore, is seen as an evolutionary event. The differential distribution of these amines, their associated enzymes, and variations in the regulatory influences affecting their metabolism presumably reflect different growth or metabolic patterns requiring different regulatory organic cations. For example, the transformation of spermidine to spermine is largely a characteristic of eukaryotic cells [1]. Similarly, it has been suggested that the putrescine activation of *S*-adenosyl-L-methionine decarboxylase in higher organisms is an evolutionary change associated with the appearance of a specific spermine synthase [12]. Delineating the evolutionary sequence in which the various regulatory influences have developed represents a major challenge to our understanding of the process.

Pharmacological manipulations of polyamine biosynthesis

Drug inhibition of polyamine biosynthesis has been reviewed recently and thoroughly by Jänne *et al.* [4]. In this section the discussion will focus on some of the problems encountered and the difficulties inherent in deducing physiological functions from pharmacological manipulations.

In formulating an approach to the inhibition of polyamine biosynthesis two factors should be considered. First, much of the cellular polyamine is manufactured within a short period of time. In the case of putrescine, for example, maximum production rates are restricted to a 2 to 4-hr period approximately correlating with changes in ornithine decarboxylase. It is during these time periods that inhibitors need to be effective. Second, in a typical sequence, the rate-limiting step in polyamine synthesis in a stimulated cell changes from mRNA synthesis for ornithine decarboxylase to translation within 90 min. Enzyme activity and putrescine levels then determine the amount of spermidine formed. As putrescine levels continue to increase, they eventually covershoot the amount of propylamine moiety produced by *S*-adenosyl-L-methionine decarboxylase. The activity of this enzyme then becomes the limiting factor in the production of spermidine. Subsequent events are not clear since little is known about the factors regulating the conversion of spermidine to spermine. In the sequence outlined, the rate-limiting step in polyamine metabolism constitutes a moving target and should be considered in drug studies.

In the light of these considerations, the customary practice of flooding the tissue or cell with inhibitor for extended periods of time inevitably creates problems of specificity emanating from overexposure of the cell to the drug. Intuitively one suspects that some of these problems could be alleviated if application of the inhibitor were timed to coincide better with the vulnerability of the system.

The logical approach to inhibition of polyamine biosynthesis is obviously to inhibit ornithine decarboxylase of *S*-adenosyl-L-methionine decarboxylase but this has proven difficult, in part, because of the high rate of turnover of these proteins. Inhibition through interference with the cofactors, pyridoxal phosphate for ornithine decarboxylase or pyruvate for *S*-adenosyl-L-methionine decarboxylase, is not regarded as being a specific approach. However, most of the cellular putrescine or spermidine is produced during brief spurts in activity of the biosynthetic enzymes. During such times, ornithine decarboxylase synthesis is placing heavier demands upon both substrate and cofactor pools. It is possible, therefore, that the specificity of inhibitors which affect the cofactors, compete with substrate, or interact in a non-competitive manner could be increased if their application, as mentioned above, were timed to coincide better with the induction of the enzyme proteins.

The K_M of ornithine decarboxylase for ornithine is typically 0.1 mM, whereas ornithine levels are usually higher than 1 mM. In most cells, therefore, it is unlikely that ornithine availability is a limiting factor in polyamine formation. This situation may change when ornithine decarboxylase is elevated to very high levels. Under these circumstances substrate limitation may occur [57], suggesting that most of the cytosol ornithine is decarboxylated to putrescine and the supply of ornithine to this pool is now a limiting factor in putrescine formation. This limitation may not be of any consequence in the functioning of polyamines since by the time it occurs the rate-limiting step has probably shifted to the activity of *S*-adenosyl-L-methionine decarboxylase, i.e. putres-

cine is already present in excess. However, the results do imply that "spare" ornithine decarboxylase activity exists, and this concept has direct relevance to the pharmacological strategy adopted for manipulating polyamine metabolism. If only a fraction of the total ornithine decarboxylase is used in synthesizing putrescine, it will require an almost complete inhibition of the enzyme to significantly lower putrescine levels.

Inhibitors of ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase can be divided into compounds which act directly or indirectly on the enzymes. The major inhibitors acting directly on ornithine decarboxylase are substrate analogs such as α-methylornithine or α-hydrazino-ornithine. For *S*-adenosyl-L-methionine decarboxylase, the most effective compounds are MGBG and, more recently, MBAG [1, 1'-(methylethanediyidine dinitrilo)-bis-(3-aminoguanidine)] [58]. Some of these compounds require high doses and their specificity has not been rigorously established. Three of them, α-methylornithine, α-hydrazino-ornithine and MGBG, have a major disadvantage in that they stabilize the decarboxylases by prolonging their half-lives. The effect of stabilization of the protein on the catalytic activity is not known.

An alternative approach to the problem of inhibiting polyamine biosynthesis is through inhibition of enzyme synthesis. This could be accomplished either by inhibition of specific mRNA production or by inhibiting translation. In rat liver, the half-lives of the mRNAs for ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase appear to be longer by several hours than the half-lives of the enzyme proteins [29]. Furthermore, production of new message is not always obligatory in the induction of the enzymes. Inhibition of mRNA synthesis, therefore, will have a delayed effect upon enzyme production.

The most potent inhibitors of polyamine biosynthesis in intact cells are, in fact, those compounds, such as cycloheximide, which inhibit the translational phase of protein synthesis, although the toxicity renders them of little value except for short-term experiments. In view of the lack of information on the steps involved in the synthesis of these enzymes, the possibility of tailoring an agent to specifically inhibit enzyme synthesis is not great at this time.

Although a reduction in ornithine decarboxylase activity is observed with inhibitors of protein synthesis the critical question is whether putrescine levels are similarly reduced. When cycloheximide is added to HeLa cells at the time of stimulation, both the induction of ornithine decarboxylase and the increase in putrescine levels are prevented. If cycloheximide is added 2 hr after stimulation, rather than at the same time or slightly before, enzyme activity declines but putrescine levels continue to increase for a further 2–4 hr albeit at a slower rate than in the control cells. A similar picture is obtained when cycloheximide is given at 4 hr. When the inhibitor is added to the system at 6 hr, i.e. when ornithine decarboxylase activity is declining, it has little effect upon either enzyme activity or on putrescine levels (unpublished observations).

These results illustrate some limitations that can be anticipated even if specific inhibitors of enzyme synthesis were available. First, the time at which the inhibitor is added to the system is important. If it is added a long time prior to a period of enzyme induction, presumably it could be metabolized or bound elsewhere, thereby reducing the effective concentration and lowering the overall specificity. If the inhibitor is administered when enzyme activity has already been induced, then it will be only partially effective in lowering putrescine levels or even completely ineffective. Maximum effectiveness and specificity, therefore, are critically dependent upon the time point application of the inhibitor to the system. The second point is that when the inhibitor is added at certain times, a divergence between ornithine decarboxylase activity and putrescine levels may arise. One contributing factor for this, at least in HeLa cells, is that the $T_{1/2}$ for ornithine decarboxylase has gone from 10–15 min to 60–75 min during the first 2-hr period of stimulation. When cycloheximide is added at 2 hr, there is still sufficient enzyme available over the next 2–3 hr to contribute significantly to the putrescine levels.

Consider an enzyme with a $T_{1/2}$ of 10 min. One hour after inhibition of protein synthesis 1.5 per cent of the initial activity remains. With a $T_{1/2}$ of 75 min 55 per cent of the activity is still present 1 hr after inhibition. This represents approximately a 35-fold difference in potential putrescine production between the two systems. Inhibitors of enzyme synthesis, therefore, will have a more immediate effect upon putrescine production when the enzyme half-life is short and is unchanged during a period of induction. When increases in enzyme activity involve alterations in the rate of both synthesis and degradation and the half-life is increased, then protein synthesis inhibitors will have a less immediate effect on putrescine levels.

Apart from compounds which are known inhibitors of protein synthesis, the most effective group of compounds for inhibiting the decarboxylases are the polyamines and other related compounds. Enzyme activity usually decays at a rate similar to that seen after inhibition of protein synthesis. This may be a direct effect on translation or due to the production of an antizyme. As discussed earlier, the exact mechanism by which this effect is brought about is not known. The disadvantage with these amine repressors, such as diaminopropane, is that they may mimic the actions of the naturally occurring polyamines.

Pharmacological inhibition and biological function

In relating pharmacological manipulations of polyamine metabolism to biological function, certain criteria need to be satisfied. The drugs must be shown to be specific, a rigorous requirement which is rarely met. Polyamine metabolism in the intact cell must be shown to be altered and compatible with the mode of action of the drug. The biological disturbance produced by the drug should be shown to be sensitive to polyamines. An additional criterion, that of reversal of the effect of the drug by addition of exogenous amine, is of limited value unless it has also been shown that amine metabolites are not involved in reversing the effect and that amine taken up by the cell mixes uniformly with the endogenous pool.

Most attempts to define the biological role of the

polyamines have relied heavily on correlations between some index of polyamine metabolism and various parameters of DNA, RNA and protein metabolism. The potential of this approach is limited, in part, because the validity of the parameters used to make the correlations has not been established. DNA, RNA and protein synthesis are conglomerate indices that are imprecisely defined when compared with a specific molecular entity such as spermidine or ornithine decarboxylase. Protein synthesis, for example, normally measured using labeled precursors, conceals a wide heterogeneity of turnover rates. Accordingly, we have the paradoxical situation that compounds such as diaminopane do not affect protein synthesis [44] despite the fact that they may well be acting as repressors of ornithine decarboxylase synthesis. The question that needs to be answered is what is the effect of these compounds, not on overall protein synthesis, but on other rapidly turning over proteins with mechanisms of inductions similar to ornithine decarboxylase. In some cell systems, *S*-adenosyl-L-methionine decarboxylase is a possible control. Tyrosine aminotransferase, which has many similarities to ornithine decarboxylase, would appear to be another obvious enzyme to use to establish the specificity of these agents in inhibiting ornithine decarboxylase.

The uncertainty of which parameters to use for correlative purposes is also noticeable in studies on the relationship between polyamines and DNA or RNA metabolism. In bovine lymphocytes, for example, no correlation between the kinetics of RNA and polyamine accumulation following lectin activation was observed [59]. Other workers, however, using, rRNA as a basis for correlation suggested that a relationship to polyamines did exist [60]. Similarly, α-methylornithine reduced polyamine levels in L1210 cells but did not affect DNA synthesis when total DNA levels were measured [61], whereas in rat hepatoma cells DNA synthesis was profoundly reduced by this compound when synthesis was monitored using [^{3}H]-thymidine incorporation [62].

The confusion over which parameters of macromolecular metabolism should be used is understandable given the complexities involved, but there is also no unanimity on which indices of polyamine metabolism are the most meaningful for correlative purposes. Emphasis has been placed on the activities of ornithine and *S*-adenosyl-L-methionine decarboxylases and on the measurement of total amine levels. None of these parameters, however, are entirely satisfactory. Enzyme activities are always determined *in vitro* and amine levels do not necessarily reflect production rates.

Although changes in ornithine decarboxylase broadly correlate with changes in putrescine levels, there are a number of reported instances where there is a lack of correlation between the two. In rats, α-hydrazino-ornithine reduced putrescine levels but increased enzyme activity [37]. In HTC cells, a substantial increase in ornithine decarboxylase was observed without any concomitant increase in putrescine levels [63]. The extent to which estimations of ornithine decarboxylase determined *in vitro* are an accurate reflection of putrescine production in the intact cell has not been rigorously established. Moreover, enzyme activity is usually measured after extraction and incubation of the cytosol fraction under conditions of constant temperature, pH and substrate and cofactor concentrations. These conditions may not always pertain to the whole cell, and it is conceivable that some of these factors may become limiting especially during a period of rapidly increasing enzyme activity. A major unknown at this point, for example, is the kinetics of ornithine availability to the substrate pool under conditions of high ornithine decarboxylase activity.

Amine levels are also a limited index for correlative studies. Cell concentrations of spermidine, for example, are often 10–20 times higher than those of putrescine. Even when ornithine decarboxylase is markedly stimulated and the putrescine formed is converted to spermidine, it may not always be manifest by any detectable alteration in total spermidine concentration. This can create problems in interpretation. For example, diaminopropane lowered putrescine levels in synchronized Chinese hamster ovary cells with little change in spermidine or spermine [64]. Inhibition of putrescine synthesis was associated in the decreased DNA synthesis and it was suggested that putrescine was associated with DNA chain elongation. However, in CHO cells, changes in ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase during the cell cycle occur in concert [22], and it is probable that much of the putrescine formed is converted normally to spermidine. Inhibition of putrescine production by diaminopropane would be expected to reduce spermidine formation, even though it may not be detectable by measuring spermidine levels. Needed in these and similar studies are the effects of the inhibitors on the conversion rates of one amine to another and not just information on total cellular content.

Conclusions

An increase in ornithine decarboxylase activity is an invariant component of the integrated response of a cell stimulated to proliferate. This enzyme represents a suitable model system for studying the control of enzyme synthesis and degradation and provides a useful alternative to tyrosine aminotransferase. Future emphasis is likely to lie with the development of resources and techniques for studying the molecular mechanisms involved in the turnover of this enzyme in cell-free systems. This may eventually yield a specific means of manipulating ornithine decarboxylase activity in the whole cell. Such a means would constitute a useful adjunt for studying the polyamines since in most physiological systems ornithine decarboxylase is the rate-limiting step in polyamine metabolism.

At the present time much emphasis is being placed on a pharmacological approach to elucidating the biological function of the polyamines. Using present techniques, however, the outcome is likely to be disappointing. The characteristics of the key regulatory elements in polyamine metabolism, that is, rapidly inducible enzymes with short half-lives and brief periods of high activity, constitute formidable obstacles to achieving specificity of action with a drug. Using conventional modes of administration, overexposure of the cell to drug treatment is inevitable.

Perhaps the approach should be changed so that the system is exposed to short pulses of the drug timed to coincide with the brief spurts in polyamine biosynthesis.

An additional problem already encountered in pharmacological studies is the uncertainty of the validity of the correlative functions being used to try and established biological functions. These need refining or even redefining. The pivotal role of putrescine in polyamine metabolism underlines the importance of having methods and procedures which permit an accurate estimate of the formation of putrescine in the intact cell as well as its clearance rate and the rate of its conversion to spermidine and spermine. Labeling techniques, similar to those used extensively in studies on steroid dynamics, provide a possible means of refining the polyamine indices used for correlative purposes.

Beyond the formation of putrescine there is wide variation between different organisms in the subsequent direction of polyamine synthesis. This diversity may simply represent evolutionary selectivity for specific organic cations. If this is correct, then delineating the evolutionary sequence in which the various regulatory influences have developed constitutes a major challenge to our understanding of the process.

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REFERENCES

1. S. S. Cohen, *Introduction to the Polyamines*, pp. 179. Prentice-Hall, Englewood Clifffs, New Jersey (1971).
2. U. Bachrach, *Function of Naturally Occurring Polyamines*, pp. 211. Academic Press, New York (1972).
3. C. W. Tabor and H. Tabor, *A, Rev. Biochem.* **45**, 285 (1976).
4. J. Jänne, H. Pösö and A. Raina, *Biochim. biophys. Acta* **473**, 241 (1978).
5. A. Raina and J. Jänne, *Acta chem. scand.* **22**, 2375 (1968).
6. S. J. Friedman, K. V. Halpern and E. S. Canellakis, *Biochim. biphys. Acta* **261**, 181 (1972).
7. M. Ono, H. Inoue, F. Suzuki and Y. Takeda, *Biochim. biophys. Acta* **284**, 285 (1972).
8. M. F. Obenrader and W. F. Prouty, *J. Biol. Chem.* **252**, 2866 (1977).
9. E. S. Canellakis, J. S. Heller, D. Kyriakidis, and K. Y. Chen, in *Advances in Polyamine Research*, Vol. 1, p. 17. Raven Press, New York (1978).
10. A. E. Pegg and H. G. Williams-Ashman, *Biochem. biophys. Res. Commun.* **30**, 76 (1968).
11. R. B. Wichner, C. W. Tabor and H. Tabor, *J. biol. Chem.* **245**, 2132 (1970).
12. H. Poso, P. Hannonen, J. J. Himberg and J. Jänne, *Biochem. biophys. Res. Commun.* **68**, 227 (1976).
13. A. E. Pegg, *Biochem. J.* **166**, 81 (1977).
14. A. E. Pegg, *Fedn Eur. Biochem. Soc. Lett.* **84**, 33 (1977).
15. P. Hannonen, J. Jänne and A. Raina, *Biochem. biophys. Res. Commun.* **46**, 341 (1972).
16. T. Oka, J. W. Perry and K. Kano, *Biochem. biophys. Res. Commun.* **79**, 979 (1977).
17. D. P. Harrison and V. C. Bode, *J. molec. Biol.* **96**, 461 (1975).
18. J. Jänne, *Acta physiol. scand.* **71**, suppl. 300 (1967).
19. A. Raina, *Acta physiol. scand.* **60**, suppl. 218 (1963).
20. C. M. Caldarera, B. Barbiroli and G. Moruzzi, *Biochem. J.* **97**, 84 (1965).
21. T. G. O'Brien, R. C. Simsiman and R. K. Boutwell, *Cancer Res.* **35**, 2426 (1975).
22. D. J. M. Fuller, E. W. Gerner and D. H. Russell, *J. cell. Physiol.* **93**, 81 (1977).
23. O. Heby, J. W. Gray, P. A. Lindl, L. J. Marton and C. B. Wilson, *Biochem. biophys. Res. Commun.* **71**, 99 (1976).
24. S. Don and U. Buchrach, *Cancer Res.* **35**, 3618 (1975).
25. S. Cohen, B. W. O'Malley and M. Stastny, *Science, N.Y.* **170**, 336 (1970).
26. Y. Kobayashi, J. Kupelian and D. V. Maudsley, *Science, N.Y.* **172**, 379 (1971).
27. D. H. Russell and S. H. Snyder, *Molec. Pharmac.* **5**, 253 (1969).
28. N. Fausto, *Biochim. biophys. Acta* **238**, 116 (1971).
29. A. Kallio, M. Lofman, H. Pösö and J. Jänne, *Fedn Eur. Biochem. Soc. Lett.* **73**, 229 (1977).
30. B. L. M. Hogan and S. Murden, *J. cell. Physiol.* **83**, 345 (1974).
31. J. L. Clark, *Biochemistry* **13**, 4668 (1974).
32. G. F. Munro, R. A. Miller, C. A. Bell and E. L. Verderber, *Biochim. biophys. Acta* **411**, 263 (1975).
33. W. F. Prouty, *J. cell. Physiol.* **89**, 65 (1976).
34. F. T. Kenny, *Science, N.Y.* **156**, 225 (1967).
35. K. L. Barker, K. L. Lee and F. T. Kenny, *Biochem. biophys. Res. Commun.* **43**, 1132 (1971).
36. C. D. Stiles, K. L. Lee and F. T. Kenny, *Proc. natn. Acad. Sci. U.S.A.* **73**, 2634 (1976).
37. S. I. Harik, M. D. Hollenberg and S. H. Snyder, *Molec. Pharmac.* **10**, 41 (1974).
38. P. P. McCann. C. Tardif, M-C. Duchesne and P. S. Mamont, *Biochem. biophys. Res. Res. Commun.* **76**, 893 (1977).
39. J. E. Kay and V. J. Lindsay, *Biochem. J.* **132**, 791 (1973).
40. T. R. Schrock, N. J. Oakman and N. L. R. Bucher, *Biochim. biophys. Acta* **204**, 564 (1970).
41. J. Jänne and E. Höltä, *Biochem. biophys. Res. Commun.* **61**, 449 (1974).
42. U. Bachrach, *Ann. N.Y. Acad. Sci.* **171**, 939 (1970).
43. B. W. Kimes and D. R. Morris, *Biochim. biophys. Acta* **228**, 223 (1971).
44. H. Pösö, A. Kallio, G. Scalabrino and J. Jänne, *Biochim. biophys. Acta* **496**, 288 (1977).
45. J. L. Clark and J. L. Fuller, *Biochem. biophys. Res. Commun.* **73**, 785 (1976).
46. J. S. Heller, W. F. Fong and E. S. Canellakis, *Proc. natn. Acad. Sci. U.S.A.* **73**, 1858 (1976).
47. J. L. A. Mitchell, H. A. Campbell and D. D. Carter, *Fedn Eur. Biochem. Soc. Lett.* **62**, 33 (1976).
48. J. L. A. Mitchell, S. N. Anderson, D. D. Carter, M. Sedory, J. F. Scott and D. A. Varland, in *Advances in Polyamine Research*, Vol. 1, p. 39. Raven Press, New York (1978).
49. M. F. Obenrader and W. F. Prouty, *J. biol. Chem.* **252**, 2860 (1977).
50. J. L. Fuller, S. Greenspan and J. L. Clark, in *Advances in Polyamine Research*, Vol. 1, p. 31. Raven Press, New York (1978).
51. E. Hölttä, *Biochim. biophys. Acta* **399**, 420 (1975).
52. T. C. Theoharides and Z. N. Canellakis, *J. biol Chem.* **251**, 1781 (1976).
53. T. Oka and J. W. Perry, *J. biol Chem.* **249**, 7647 (1974).
54. T. Oka, K. Kano and J. W. Perry, in *Advances in Polyamine Research*, Vol. 1, p. 59. Raven Press, New York (1978).
55. A. E. Pegg, A. Corti and H. G. Williams-Ashman, *Biochem. biophys. Res. Commun.* **52**, 696 (1973).
56. F. McCormick, *J. cell. Physiol.* **93**, 285 (1978).
57. D. V. Maudsley, J. Leif and J. King, in *Advances in Polyamine Research*, Vol. 1, p. 93. Raven Press, New York (1978).
58. A. E. Pegg and C. Conover, *Biochem. biophys. Res. Commun.* **69**, 766 (1976).
59. R. H. Fillingame and D. R. Morris, *Biochemistry* **12**, 4479 (1973).
60. J. E. Kay and V. J. Lindsay, *Expl Cell Res.* **77**, 428 (1973).

61. N. E. Newton and M. M. Abdel-Monem, *J. med. Chem.* **20**, 249 (1977).
62. P. S. Mamont, P. Böhlen, P. P. McCann, P. Bey, F. Schuber and C. Tardif, *Proc. natn. Acad. Sci. U.S.A.* **73**, 1626 (1976).
63. P. P. McCann, C. Tardif, P. S. Mamont and F. Schuber, *Biochem. biophys. Res. Commun.* **64**, 336 (1975).
64. P. S. Sunkara, N. R. Potu and K. Nishioka, *Biochem. biophys. Res. Commun.* **74**, 1125 (1977).