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Functions of Cytokinins [and Discussion]

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Functions of cytokinins

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Mechanisms of action of cytokinins at the cellular and molecular levels are still unknown. Biological functions of cytokinins are presented through specific bioassay systems which are regarded as standard (delay of senescence of leaf tissue and stimulation of cell division) and which have been or may be biochemically investigated. These 'biochemical functions' of cytokinins are reviewed.

The biochemical significance of the specific occurrence of cytokinins in transfer RNA molecules is discussed with respect to the question of the incorporation of labelled cytokinins into RNA molecules. Also, the significance of the cytokinin binding protein recently isolated from higher plant ribosomes is discussed.

1. Introduction

Cytokinin is a 'generic name for substances which promote cell division' of plant cells (Skoog *et al.* 1965) and cytokinin activity generally refers to this particular biological activity measured under certain conditions of bioassay. This definition is restrictive in the sense that cytokinins affect many aspects of plant growth and development like flowering, germination, branching, fruiting and senescence (see the reviews by Skoog & Schmitz (1972) and Hall (1973)). On the basis that these biological effects on whole plants can be manipulated experimentally in the laboratory with exogenously added cytokinins and on the increasing evidence of their natural occurrence in plants – occurrence which can be correlated with their biological activities – cytokinins are considered as a class of plant hormones just like auxins and gibberellins (see Thimann (1972) for a critical assessment of the concept of plant hormones).

However, if biological responses of a great variety of plant tissues and plant cells to applied cytokinins are well defined (e.g. senescence, cell replication and differentiation) 22 years after the isolation of kinetin by Skoog, Miller and coworkers (Miller *et al.* 1956), their modes of action at the cellular and molecular levels are almost unknown. Under these circumstances, if it can be postulated that cytokinins may regulate DNA replication, transcription, post-transcriptional events, translation or post-translational processes, two questions need to be answered before one can propose a testable hypothesis of cytokinin action:

(1) Which biochemical processes or cellular events, among the biochemical events that occur in the course of the expression of the cytokinin biological activity, are regulated by cytokinins?

(2) How do the cytokinins, as a signal, modify or initiate these biochemical events?

In this short review, a number of biochemical investigations of well defined biological responses to cytokinins will be presented and discussed with regard to the answers they can provide to the first question essentially, as the question 'how' cannot be answered until we know 'what' cytokinins do.

2. Biological and biochemical functions of cytokinins

(a) *Cytokinin-delayed senescence of leaf tissues*

Detached leaves undergo rapid ageing manifested by an obvious yellowing due to chlorophyll loss. Chibnall (1939) observed that protein loss also occurred during this process and that senescence was prevented if adventitious roots developed on the petiole. This observation led Chibnall to propose that a hormone originating in the roots was responsible for maintenance of balanced protein metabolism in the leaves.

Richmond & Lang (1957) were able to delay senescence of *Xanthium* leaves with an application of kinetin. This observation was subsequently confirmed by several investigators (for review, see Kende 1971) and provided the basis of a rapid cytokinin bioassay in which cytokinin activity, as measured by the chlorophyll retention induced in the cytokinin treated leaf disks, was linearly related to the logarithm of the cytokinin concentration (Osborne & McCalla 1961). This simple and rapid biological response to cytokinin was also extensively studied at the biochemical level.

Richmond & Lang (1957), Wollgiehn (1961) and Osborne (1962) showed respectively that the protein level and the RNA levels declined more slowly in kinetin-treated leaves than in untreated control leaves. It was then hypothesized that cytokinin delayed senescence by stimulating protein and RNA synthesis. Indeed, enhanced incorporation of labelled amino acid into proteins and of labelled phosphate into RNAs was observed in the kinetin treated leaves, as compared with the untreated leaves (see Kende (1971) for a detailed review). Senescence of leaves, as a physiological process, is also characterized by increased levels of free amino acids and by an increase of hydrolytic activities like proteases (Shibaoka & Thimann 1970) and RNases (Srivastava 1968; Udwardy *et al.* 1969). Therefore, from the enhanced incorporations of precursors into proteins and RNAs in cytokinin-treated leaves, it cannot be simply concluded that cytokinin stimulated RNA or protein synthesis, as in the senescing untreated leaves 'proteolysis and RNA breakdown reduce the rate of precursor incorporation because (1) label already incorporated is lost again at a faster rate and (2) expanded precursor pools contribute to increased isotopic dilution of added radioactive precursors' (Kende 1971).

As an alternative hypothesis, the importance of proteolysis in the process of senescence was investigated in two ways. Kuraishi (1968) showed that in [^{14}C]leucine prelabelled disks of *Brassica* leaves, transferred to solutions with or without a cytokinin, the radioactivity of the labelled proteins was lost more slowly in the presence of cytokinin than in its absence. Tavares & Kende (1970) studied the effect of benzyladenine on protein synthesis and breakdown by measuring the loss of radioactivity, the loss of total protein and the evolution of the specific activity of proteins of corn leaf disks prelabelled with [^{14}C]leucine. After transferring the disks to media containing a high concentration of [^{12}C]leucine, with or without benzyladenine, Tavares & Kende found that losses of radioactivity and protein were less in the presence of cytokinin than in its absence, but the specific activity of the protein remained constant in the cytokinin-treated and untreated disks. They concluded that protein synthesis did not occur to a great extent and that it was definitely not stimulated by benzyladenine.

However, some protein synthesis seems necessary to the normal course of senescence. Martin & Thimann (1972) showed that cycloheximide prevented senescence of oat leaves in the same way as kinetin did, and under these conditions the amounts of two proteases which normally appeared in the untreated leaves were greatly decreased. They concluded that synthesis of

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these proteases preceded senescence and that kinetin might delay senescence by 'its ability to suppress protease formation'. This last conclusion was mainly based on the fact that kinetin was shown to counteract the effect of the amino acid serine which stimulated senescence and promoted the synthesis of the described proteases. Senescence may not occur exactly in the same way in all plant tissues, as Takegami (1975*a*) reported that cycloheximide and actinomycin-D accelerated the senescence of tobacco leaf disks and interfered with the anti-senescence effect of benzyladenine. The disagreement between these results and Martin & Thimann's findings may be due to the difference in the material used.

With regard to the relations between leaf senescence and RNA metabolism, I mentioned above that RNase activities were shown to increase in senescing detached leaves. Cytokinin treatment of excised leaves, while delaying senescence, was shown to prevent this increase in RNase activities (Dove 1972, quoted by Hall 1973) and to maintain the ribosome content of the tissue (Shaw & Manocha 1965; Berridge & Ralph 1969). Takegami (1975*b*) indicated that the loss of rRNA in senescing tobacco leaf disks was essentially due to the loss of chloroplast rRNA. Furthermore, cytokinin can prevent senescence of radish leaf disks even when RNA synthesis is effectively inhibited by 5-fluorouracil as shown by Paranjothy & Wareing (1971). Thus, it seems likely that RNA level is maintained in cytokinin-treated excised tissue more by preventing an increase of RNase activities than by stimulating RNA synthesis.

(*b*) *Cytokinin-promoted cell division*

Cytokinins, by definition, promote cell division in plant tissue cultures and their specificity with regard to cell division is no longer questioned as it is well established that they act as a specific trigger of mitosis (Das *et al.* 1956; Jouanneau & Tandeau de Marsac 1973; Fosket & Short 1973). It is likely that they fulfil the same function in plants, on the basis of a correlation between rate of tissue growth and level of measurable cytokinin activity (Hall 1973). Cytokinins also promote cell differentiation and organogenesis in plant tissue cultures. This property causes cytokinins to be widely used in the developing field of plant protoplasts, cells and tissue cultures (Reinert & Bajaj 1977). Knowledge of the mechanisms by which cytokinins control cell division would certainly facilitate their utilization.

Surprisingly, few biological systems have been investigated at the biochemical level with regard to their cytokinin-stimulated growth: only tobacco pith tissue and derived suspension cultures (Das *et al.* 1956; Jouanneau 1966), cotyledon-derived soybean tissue and suspension cultures (Fosket & Torrey 1969), and pea root cortical explants (Phillips & Torrey 1973).

Investigators employing the tobacco pith system or derived cultures (Pätau *et al.* 1957; Simard 1971; Jouanneau & Tandeau de Marsac 1973) and the soybean tissue system (Fosket & Short 1973) concluded that although cytokinin is required for cell proliferation, it is not required for DNA synthesis. Phillips & Torrey (1973) found that kinetin was necessary for DNA synthesis in cells of cortical explants of pea root segments. This latter system seems also to respond differently from the tobacco pith system with regard to RNA synthesis. Recently, Shinninger & Polley (1977) reported cytokinin-enhanced rates of RNA synthesis preceding DNA synthesis in pea root segments, while Zwar (1973) and Klambt (1974) had concluded that cytokinin did not regulate RNA synthesis in tobacco pith tissue.

Protein synthesis has been particularly investigated in the soybean and tobacco pith systems; early results obtained in the senescence system (§ 2*a*) pointed to a possible control of protein synthesis by cytokinins and this hypothesis received further support from the discovery of the

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distribution of cytokinins in tRNA molecules and from the report of their specific binding to plant ribosomes (see this and the preceding section). Jouanneau (1970) did not observe any quantitative effect of cytokinins on protein synthesis in suspension cultures of tobacco in relation to cytokinin-induced cell division, although he reported discrete and qualitative changes in the distribution of soluble proteins in cytokinin-treated cells compared with untreated cells. However, cytokinin induction of mitotic activity in partly synchronized tobacco cell suspensions required that protein synthesis remained efficient (Jouanneau 1975). When protein synthesis was inhibited by the addition of 5-methyltryptophan, during the premitotic period no mitosis occurred and after the restoration of protein synthesis by tryptophan addition, cytokinin had to be added again in order to restore mitosis. Fosket and coworkers approached the question of the rate of protein synthesis in soybean cells, as a possible mechanism by which cytokinins could promote cell division, through the examination of their polyribosome content, assuming that polyribosome content is directly correlated with the rate of protein synthesis. At first, Short *et al.* (1974) observed that cytokinin treatment rapidly stimulated polyribosome formation in cytokinin-requiring soybean tissue and that mitotic activity could be correlated with a high polyribosome content. This finding was reassessed recently with soybean cells grown as suspension cultures (Fosket *et al.* 1977). Although it was confirmed that cytokinin treatment stimulated polyribosome formation, it appeared that it was not a specific effect, as transfer of cells from stationary phase suspension cultures to fresh medium lacking cytokinin also stimulated polyribosome formation which was then correlated with no mitotic activity. Thus, if cytokinin stimulates polyribosome formation and mitotic activity, polyribosome formation (as induced by transfer of the cells) is not a sufficient condition for the induction of cell division. Also, polyribosome formation as measured by the ratio polysomes : monosomes was not correlated with qualitative changes in the spectrum of proteins synthesized by soybean cells, but cytokinins brought about significant qualitative changes in the spectrum of proteins synthesized. These results are in close agreement with Jouanneau's results and it can be concluded that cytokinin treatment allows synthesis of specific proteins which permit the cells to divide.

3. Mode of action of cytokinins

(*a*) *The incorporation of cytokinins into RNA*

The study of the incorporation of cytokinins into RNA stems from the fact that adenine is a metabolite (among others) of benzyladenine in plant tissues. In the first study of the metabolism *in vivo* of [8-^{14}C]benzyladenine in *Xanthium* leaf disks, McCalla *et al.* (1962) were led to investigate the nature of labelled compounds derived from benzyladenine found in polynucleotides. These were adenylic and guanylic acids, but a very small amount of labelled compound appeared to be similar to benzyladenylic acid. On this evidence, it was considered that benzyladenine could be incorporated into nucleic acids.

In 1966, Fox, using [benzyl-^{14}C]benzyladenine, reported a preferential incorporation of benzyladenine into sRNA of tobacco and soybean tissues. This work was completed by a more detailed study on the distribution of radioactivity into different sRNA fractions (Fox & Chen 1967). At the same time, cytokinins were shown to be part of tRNA molecules (Hall *et al.* 1966) and located specifically at the position adjacent to the 3′ end of the anticodon (Biemann *et al.* 1966). Although the functions of such hypermodified nucleosides (Hall 1971) in tRNA were not known at that time, it was speculated on the basis of these features that cytokinins

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might control protein synthesis through the formation of a specific transfer RNA (Fox & Chen 1968).

Subsequently, different attempts to measure the incorporation of exogenous cytokinins into plant tissue tRNA led to conflicting results (see Kende (1971) for a detailed review) and a controversy developed as to whether the presence of cytokinins in tRNA was related to their mode of action in regulation of growth.

The presence of cytokinins in tRNA (see Hall (1971) for a detailed review of their occurrence) was clarified when Hall and coworkers demonstrated that N^6(Δ^2-isopentenyl)adenosine in tRNA was synthesized by attachment of the isopentenyl group from Δ^2-isopentenylpyrophosphate to the appropriate adenosine residue of preformed tRNA (Kline *et al.* 1969) but the question of cytokinin incorporation into RNA still remained open.

Recent work clearly established that benzyladenine was incorporated as such (without any transbenzylation reaction) (Walker *et al.* 1974). This was based on the ^3H : ^{14}C ratio of benzyladenosine recovered from labelled tobacco callus tRNA extracted from callus tissue grown in the presence of benzyladenine labelled in the phenyl ring with ^3H and at the 8-position of the purine ring with ^{14}C. Incorporation was further demonstrated by the facts that the nucleoside 2′ and 3′-monophosphates of benzyladenine and the nucleoside 3′-monophosphate of benzyladenine were characterized from alkaline hydrolysis products (Jouanneau *et al.* 1977) and from ribonuclease T_2 digests (Armstrong *et al.* 1976) of RNA. However, in these two studies, benzyladenine was shown to be incorporated also in other RNA molecules than tRNA molecules. The significance of cytokinin incorporation into RNA is not known, even if the incorporation of benzyladenine into tRNA is probably not linked to its growth promoting effect, on the evidence that cytokinin-requiring tobacco tissue grown on benzyladenine is perfectly capable of synthesizing the usual complement of cytokinins in its tRNA molecules (Burrows *et al.* 1971). Incorporation of benzyladenine into rRNA at a frequency of one molecule for 25000–100000 adenine residues may be the result of transcriptional errors, and the demonstration of the formation of benzyladenosine, 5′mono-, di- and triphosphates in tobacco cells (Laloue *et al.* 1974) provides biochemical support for such an incorporation.

(*b*) *Cytokinin receptor sites*

On the basis that cytokinin-delayed senescence of excised leaf tissue was associated with an apparent increase of protein synthesis *in vivo* (§ 2*a*) and that in an in-vitro system with chloroplasts, kinetin seemed to stimulate protein synthesis in less than 5 min (Davies & Cocking 1967), Berridge *et al.* (1970) investigated the interactions of cytokinins with ribosomes isolated from Chinese cabbage leaves: they found that benzyladenine and kinetin exhibited equilibrium binding to the ribosomes at cytokinin concentrations in good agreement with the concentrations at which they exhibit biological activity. However, they failed to demonstrate any effect of cytokinins in a protein synthesizing system *in vitro* developed from these Chinese cabbage leaves (Berridge *et al.* 1970, Berridge *et al.* 1972).

The fact that higher plant ribosomes had specific affinity sites which bind cytokinins at low concentration was confirmed by Fox & Erion (1975) who showed that this binding was saturated at one cytokinin molecule per ribosome and that the cytokinin binding site was associated with a ribosomal protein which could be removed from the ribosomes by 0.5 M KCl. This ribosomal cytokinin-binding protein was recently isolated and purified almost to homogeneity (Fox & Erion 1977). The binding appears to be specific for N^6-substituted purines

exhibiting cytokinin activity with, however, the exception of zeatin. Except for these correlations, there is no evidence so far that binding of cytokinins to ribosomes is related to their biological actions. Binding sites on ribosomes may be just 'normally responsive to the cytokinin moiety present in tRNA' (Berridge *et al.* 1970).

Within this line of investigation, Takegami & Yoshida (1975) isolated and purified a soluble cytokinin binding protein from tobacco leaves. This cytokinin binding protein seems to interact specifically with 40 *S* ribosomal subunits of ribosomes in the presence of cytokinin *in vitro* (Takegami & Yoshida 1977). On the basis of its molecular mass (4000–5000) this soluble cytokinin binding protein appears to be different from the ribosomal cytokinin binding protein isolated by Fox & Erion (1977).

Conclusion

Most of the investigations at the biochemical level of the biological effects of cytokinins on senescing leaves and on cell division have focused on the hypothesis that cytokinins may regulate growth (i.e. cell division) or developmental processes (i.e. senescence) by controlling rates of protein synthesis. Such a hypothesis, as this review has attempted to illustrate, had emerged essentially from the following observations:

(i) cytokinins apparently stimulated bulk protein synthesis in cytokinin-treated senescing leaves;

(ii) cytokinins appeared to be a specific constituent of certain tRNA molecular species;

(iii) cytokinins were shown to be incorporated (specifically?) into RNA;

(iv) cytokinins were shown to bind to plant ribosomes.

So far the data available do not support the hypothesis that delaying of senescence in detached leaves or promotion of cell division by a cytokinin are just the result of an increased rate of protein synthesis.

However, in these biological systems which respond to a cytokinin, even if bulk protein synthesis is not modified it seems that qualitative changes in the spectrum of proteins synthesized are brought about by the cytokinin treatment (Jouanneau 1970; Fosket *et al.* 1977). The physiological significance and the specificity of these proteins have to be investigated in relation to the expressed biological activity which is induced by the cytokinin. This is a difficult task, but it is very likely that identification of specific or primary biochemical events controlled by cytokinin will depend upon the progress in the knowledge of the physiological responses themselves (e.g. cell division and plant tissue senescence).

In regard to this, cytokinins may be considered as 'probes' (Hall 1973) to study those physiological responses which are controlled by cytokinins. Specific inhibitors of cytokinin action should be useful in this respect. They should provide a tool to investigate the rôle of endogenous cytokinins in biological systems which do not normally require an exogenous supply of cytokinins. In addition, they are required to test the specificity of the interactions of cytokinins with any site supposedly involved in the expression of their activity.

Concerning this question of the mode of action of cytokinins, their incorporation in RNA involves the formation of the nucleoside 5′-triphosphate while binding to ribosomes seems to be a specific property of 'cytokinin bases'. These two hypothetical sites of action, even if incorporation into RNA is probably of no physiological significance, are quite contradictory in terms of 'biological activation' of cytokinins. Indeed, many workers have looked for cellular receptors to 'cytokinin bases', overlooking the possibility that other cytokinin metabolites may

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be physiologically significant. Recently, considerable attention has been given to cytokinin metabolism and new metabolites retaining the basic cytokinin structure (purine moiety and N^6-side chain) have been described. One cannot exclude the possibility that all the different and various biological properties of cytokinins are not related to such various cytokinin metabolites. One has just to think of all the biochemical processes controlled by adenine and its related metabolites. Attention should be given also to the possibility that a unique cytokinin metabolite may act upon different targets in plant cells like the nuclear cycle, membrane functions (cytokinins have been shown to influence plant cell permeability to different ions) (Marré *et al.* 1974), as well as chloroplast differentiation (cytokinins have been shown to control chloroplast differentiation independently of their action on cell division) (Seyer *et al.* 1975).

Therefore, in order to progress in the understanding of the mode of action of cytokinins at the molecular level:

(i) an answer should be given to the question of which form(s) of cytokinins (base, riboside or nucleotides) is or are involved in the expression of their activity;

(ii) research on cytokinin receptors should take into account the existence of these various metabolic forms of cytokinins;

(iii) attempts should be made to identify biological responses to cytokinins less integrated than the cell cycle response for example and showing a shorter lag period.

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Discussion

M. K. C. Menon (*Department of Botany and Microbiology, University College London, Gower Street, London WC1E 6BT, U.K.*). Since there are specific cell proteins synthesized at the receptor sites of competent moss protonemal cells (Erichsen *et al.* 1977; Sussman & Kende 1977) I wondered whether any evidence exists as to multiple protein synthesis due to cytokinin action in responding and non-responding cells.

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M. Laloue. I do not understand the question very well in the sense that in the moss system Dr Menon refers to, the specific cell proteins called caulonema-specific protein by Erichsen *et al.*, are not synthesized as a result of cytokinin action. These specific proteins seem to be part of the receptor sites of the competent protonemal cells.

Concerning the possibility of multiple protein synthesis due to cytokinin action in responding cells, Fosket *et al.* (1977) observed, after SDS electrophoretic separation, several qualitative differences in the proteins synthesized in cytokinin responding soybean cells.

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D. Klämbt (*University of Bonn, Germany*). There are no reasons for describing the well defined incorporation of cytokinins into tRNA and rRNA molecules as transcriptional errors. Errors in the process of nucleotidyl transfer are much more probable.

M. Laloue. This is indeed possible.

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