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Gibberellin metabolism and regulation of α-amylase isoenzymes in higher plants

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Abstract

So far 52 gibberellins (GA) have been isolated from fungi and higher plants. Structural elucidation, biogenetic sequence of the intermediates and the nature of regulating enzymes involved in the biosynthesis of GA from their precursors, are well established. The pathways from mevalonic acid (MVA) to GA_{12}—aldehyde are common in fungi and higher plants and then differ, depending upon the order of hydroxylation. The structural requirement for specific biological function of GA is well established. Numerous conventional methods and newer approaches are used for tentative identification and quantitation of GA metabolites. 2β-hydroxylation and glycosylation of GAs are correlated with seed development, maturation and storage of GAs in inactive forms. The subsequent release of active GAs during germination follows the enzymatic hydrolysis of the endogenous ones. However, environmental stresses have deleterious effects on GA metabolism; in γ-irradiated seeds reduced GA formation during germination adversely affects GA-controlled metabolic processes such as seedling growth and development of alpha-amylase isoenzymes. Physiological aspects of the role of GA in breaking dormancy, germination and in protein, carbohydrate and lipid metabolism have been discussed. The hormonal role of GA in biogenesis of alpha-amylase and its isoenzymes have been outlined in detail. Recently, mediation of GAs in organelle biogenesis and in the formation of subcellular biomembranes, has been envisaged.

Key words: Gibberellins biogenesis, environmental stress, physiological role, alpha-amylase, isoenzymes.

1. Introduction

Gibberellins (GAs), comprising a large family of naturally occurring diterpenoid acids, have a hormonal function in higher plants and are essential for normal growth and development. They were originally isolated as secondary metabolites from *Gibberella fujikuroi*, the causative fungus of the 'Bakanne' disease of rice. Though intensive studies on various aspects of GA metabolism including their turnover, biosynthesis and regulation have been made in recent years, their mode of action at molecular level is not well understood.

GAs display a wide spectrum of structural array in higher plants and 52 derivatives, isolated to date, are denoted by the trivial names GA_1 to GA_{52}. Systematic nomenclature of naturally occurring GAs is based on their gibbane or gibberellane skeleton with steric configuration of cyclic diterpene (−) kaurene (Fig. 1) and on their biological

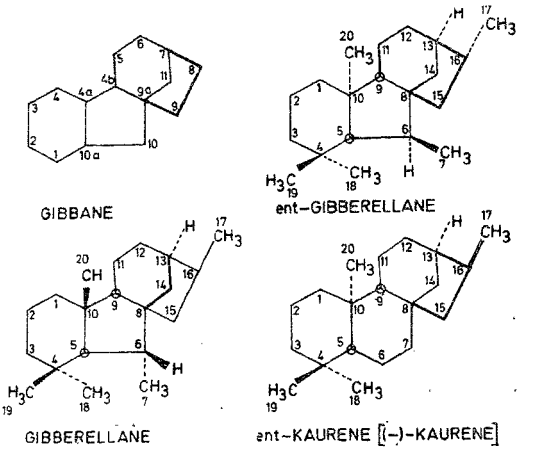


FIG. 1. The structures of gibbane, gibberellane, ent-gibberellane and ent-kaurene. Heavy lines or wedges indicate bonds lying above the plane of the ring system; broken lines indicate bonds lying below this plane[1].

properties. GAs are broadly classified into two distinct groups, depending upon the number and position of hydroxyl groups. The C_{19} GAs have one COOH group in position 7 (gibberellane numbering) and a lactone configuration in the A ring with one C atom less; whereas C_{20} GAs have full complement of diterpenoid C atoms. If GA has a single functional hydroxyl group, it is always in 3 and 13 positions in fungi and higher plants, respectively[1]. For physiological experiments with plants, ^{14}C-GAs with high specific activity are prepared by growing *Gibberella fujikuroi* in a medium containing ^{14}C-acetate[2]. Large amount of ^{3}H-GA_3 can be produced during catalytic reduction through the exchange of tritium of ^{3}H-GA_1[3]. Several chemical processes, involved in conversion of relatively inactive C_{20} GAs into biologically active C_{19} GAs, have been suggested using cell free fungal systems[4]. Recently, Bearder *et al*[5] have envisaged a biological sequential mechanism for GA synthesis, involving Baeyer-Villiger type oxidation, hydrolysis and lactonisation. However, the reaction, responsible for loss of the angular C in the conversion of C_{20} into C_{19} GA, remains unresolved. So far, the absolute stereo-

chemistry of only few chemically related GAs (like GA_3, GA_{12}, GA_{15}, etc.) has been established, rest are assigned by analogy.

2. Occurrence, transport and storage of GA

2.1. *Occurrence*

In higher plants, GAs are synthesized at diverse sites including endosperm and cotyledons of immature seeds, scutellum, embryonic axis, shoot apex, root tip, etc.[1] The complete GA-biosynthetic pathway may be operating in plastids, since GA-like substances can be extracted from this organelle. Since chloroplast membrane is impermeable to MVA[6], this precursor may be synthesized in chloroplasts. This statement is supported by demonstrating the presence of MVA-activating enzyme and biosynthesis of ent-kaurene in chloroplasts, isolated from non-aqueous media[7]. It is suggested that the etioplasts have access to cytoplasmic MVA, but lose this property when they fix CO_2[8]. Phytochromes present in the membrane of barley etioplasts are shown to regulate GA levels. This is attributed to red-light stimulated increase in the membrane permeability[9] or to the release of 'bound' GAs from the membrane[10].

2.2. *Transport*

GAs are transported from roots to longer distances both *via* xylem and phloem, specifically by sieve tubes. They are transported passively with the flow of water and are assimilated. However, the short distance transport of GA from the sites of synthesis to the site of action is shown to be non-polar and slow by donor-receptor technique[1]. Very little is known about the occurrence and nature of GA-receptors. Musgrave *et al*[11] have shown by determining the distribution pattern of applied ^3H-GA_1 in peas that growing portion of the axis is a GA-target tissue. A positive correlation between GA uptake in barley aleurone layers and its physiological potency are reported, though binding sites in cellular compartments are not localized[12]. A specific GA receptor and two non-covalent GA protein complexes have been isolated from pea seedlings treated with ^3H-GA_1, though their physiological significance is not clear[13].

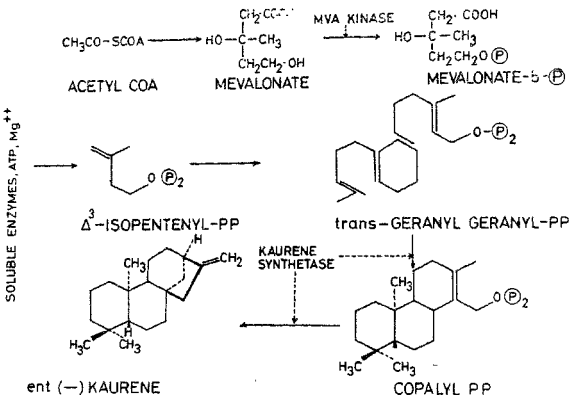
2.3. *Storage*

Active terminal GAs like GA_1, GA_3 and GA_5, present during maturation, are conjugated mainly with sugar through a hydroxyl group and are stored as stable but biologically inactive complexes in dormant seeds. 2β-hydroxylation of GAs, *e.g.*, $GA_1 \rightarrow GA_8$ in *Phaseolus vulgaris* and $GA_{20} \rightarrow GA_{29}$ in *Pisum sativum*, results in marked reduction in their biological activity[14] and thus plays an important role in metabolic control and seed development. An amphoteric conjugate of ^3H-GA_1, metabolically bound to a peptide chain is isolated from barley aleurone layers[15]. Several such inert acetyl, glucosyl as well as glucosidic conjugates, in which sugar is linked to the 7-carboxyl group of GA, with different relative biological activities are isolated or synthesized chemically and characterised[16]. These are cleaved by endogenous β-glucosidases and released as free functionally active GAs during early seedling growth[17]. Thus, glucosyl ester formation

and hydrolysis of GAs regulate their levels during seed maturation and early germination. Recently, a sulphur containing deactivation product of GA_3, gibberethione, is isolated from immature seeds of *Pharbitis nil*[19].

3. Biogenesis of GA

GAs are occurring in diverse forms in nature and no single biosynthetic pathway exists in fungi and higher plants. The steps up to the synthesis of GA_{12}-aldehyde, the first intermediate with entgibberelline skeleton, are common in both fungi and higher plants, as established by *Fusarium moniliforme* and by cell free systems from immature seeds of *Cucurbitaceae*, *Ricinus communis* and *Pisum sativum*[10, 20, 21]; thereafter the pathways diverge. The current understanding of intermediates and interconversions involved in GA biosynthesis has been extensively reviewed by Hedden *et al*[22]. Initially (Fig. 2)



ıG. 2. Outline of the gibberellin-biogenetic pathway from acetyl-CoA and mevalonate to (−) kaurene ıe conversions are mediated by soluble enzymes having a requirement for Mg^{++} and ATP[1].

ıosphorylation of MVA to MVA-5-phosphate (MVAP) is catalysed by cytoplasmic VA-kinase, a rate-limiting enzyme for the formation of various polyprenoid comunds in plants and microorganisms. The subsequent phosphorylation of MVAP d the sequential formation of pyrophosphate (PP) intermediates, are catalysed by a ies of kinases, including phosphomevalonate kinase. Further, prenyl transferases a group catalyse the sequential condensations of isopentenyl-PP (IPP) and longer enyl-PP to produce pools of farnesyl-PP (FPP, C_{15}) and geranyl geranyl-PP (GGPP,

C_{20}) as the major products of chain elongation. Two forms of geranyl transferase (I and II), which catalyse the specific formation of FPP and GGPP from IPP, have been isolated together from castor bean seedlings[23]. They undergo protein-protein interaction, which modulates their catalytic properties. However, Shinika *et al*[24] have purified GGPP synthetase from pumpkin fruit, which is free of FPP synthetase and catalyses the condensation of IPP with either FPP or GPP to give GGPP as the final product. All these soluble enzymes have been isolated, purified and characterised. They have pH optimum between 7·8 and 8·0, utilise only phosphate or pyrophosphate as donors and require Mg^{++} for activation[25]. The final cyclization of GGPP to tetracyclic diterpene ent (−) kaurene proceeds *via* a two stage mechanism. The reaction is catalysed by a high molecular weight enzyme complex, kaurene synthetase (KS)[26]. The enzyme can be resolved into two catalytic activities (A and B) with substrate specificity and activated by divalent metal ions[27]. Activity A catalyses the conversion of GGPP to copalyl PP (CPP), a bicyclic intermediate, probably through a proton initiated cyclization[26]. Activity B mediates further cyclization, where pyrophosphate is lost from CPP, followed by rearrangement of the resulting carbonium ion to produce ent (−) kaurene. This reaction is a potential site of regulation of GA biosynthetic pathway in higher plants, since GGPP is a branch point metabolite in the production of diterpenoids and carotenoids[20].

In a second part, ent (−) kaurene is metabolized *via* a series of oxidative steps: the biosynthetic sequence, ent kaurene → kaurenol → kaurenal → kaurenoic acid, has been established by refeeding the intermediates and isolation of the end products. Kaurenoic acid on further hydroxylation in 7β position forms the last product in the series, namely, 7-α (OH) kaurenoic acid[21]. All these steps are catalysed by the microsomal preparation in *E. macrocarpa* seeds and require oxygen, ATP and NADPH as cofactors, suggesting that they are mixed function oxidases[19], having electron transport system similar to that found in liver. The presence of cytochrome P_{450} and a high molecular weight ent-kaurene carrier protein in high speed supernatant from the cotyledons of *P. sativum*[28], is analogous to the sterol carrier protein found in rat liver homogenates[29]. These compounds exhibit GA activities and stimulate α-amylase formation in embryoless barley seeds[1]. Further, by contraction of B ring from a 6 to 5 carbon ring with C_7 being extruded, 7-α (OH) kaurenoic acid is converted to a relatively inactive GA configuration with aldehyde group in C^7 position[21]. The rate limiting step appears to be the removal of a hydrogen from the ent 6-position. The resulting high energy intermediate, *e.g.*, carbonium ion could undergo rearrangement to give GA_{12}-aldehyde[22].

Lastly (Fig. 3), GA_{12}-aldehyde is sequentially oxidized *in vitro* in higher plants at C_7 to form GA_{24}, followed by 3β hydroxylation to give GA_{36}. Further oxidation at C_{20} to the carboxylic acid yields GA_{13}, which is then hydroxylated at the 2β position to the final product GA_{43}[30]. The conversion is catalysed by the soluble enzymes which are distinct from the membrane-bound microsomal oxidase responsible for GA_{12}-aldehyde formation from ent-kaurene[31]. Conversion of GA_{12}-aldehyde through GA_{14} to GA_4 is the first demonstration of *in vitro* transformation of C_{20} to C_{19} GA by 3 β-hydroxyla-

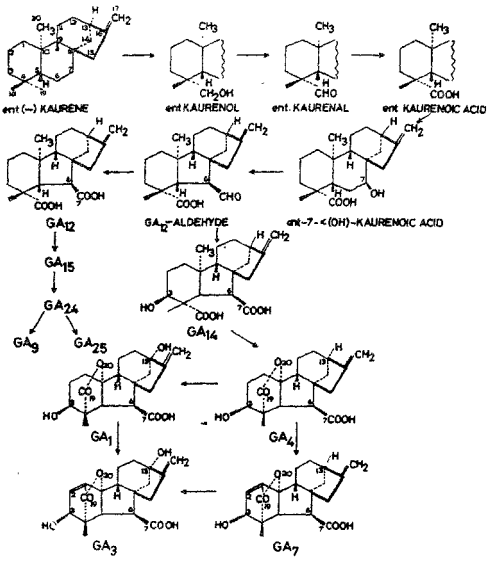


FIG. 3. Sequence of conversion pathway from ent(−) kaurene to gibberellins, in *F. moniliforme* and in higher plants.

tion pathway in higher plants. Interconversion of GA_4 to GA_1 and to GA_{34} are mediated by a single hydroxylation reaction[32]. A biogenetic sequence for GA_{14}, GA_4, GA_7, GA_1 and GA_3, in which GAs are both 3*β and* 13*β-hydroxylated*, is well established using cell free systems[33]. In dormant seeds GA_4 and GA_7, more non-polar and inactive GAs, are predominant. These are oxygenated to more polar and biologically active GA_1 and GA_3 during germination[34]. In the fungi, both 3*β*-hydroxylation and non-3-hydroxylation pathways for the synthesis of GAs from GA_{12}-aldehyde are established using a number of GA-producing strains of *G. fujikuroi* and their mutants. In mutant R-9, 13-hydroxylation reaction is blocked and as a result, GA_1 and GA_3 are not formed. Similarly, in mutant B1-41a, GA_{15} and GA_{24} do not act as precursors of GA_9 and since 3*β*-hydroxylation pathway does not operate, GA_{20} is not metabolised[35]. All these hydroxylation processes are mediated by hydroxylases, having specific require-

ment for reduced co-substrates and are inhibited by EDTA. Some of them are purified and characterised[36].

4. Structure–function relationships of GA

Two hypotheses have been postulated to explain the structure—function relationship of GA, which depends upon several factors like transport, biosynthetic pattern and catabolism[37]. The biological activity of GA directly depends upon the degree to which it fits to hypothetical receptor molecule or site in the cell. It is, mainly, associated with 3β-OH, 13-α-OH and γ-lactone structure; GAs, having a 19, 10 or 19, 20 lactonic bridge at the receptor site, usually show substantial biopotency[38]. The modification in the usual hydroxyl configuration by substitution with 20-carboxyl or methyl group completely destroys the activity, whereas molecular rearrangement in lactone ring partially reduces GA activity. Four major decomposition products of GA_3 (iso-GA_3, allo-GA_3, epialo-GA_3 and $\triangle 9$ (11)-dehydro GA_3), exhibit partial or total loss of activity[38]. This further ascertains the structural requirement for biological activity Secondly, efficiency of GA interconversion of inactive to active metabolites (*e.g.*, $GA_{13} \rightarrow GA_3$) also controls the biopotency of GA derivatives. However, the assessment of the relationship depends upon the bioassay methods employed. Dwarf rice bioassay method responds to all GAs except GA_{21}, GA_{25} and GA_{26} because of their rapid interconversion in rice seedlings, whereas barley aleurone responds to only limited number of GAs[13].

5. Identification and estimation of GAs

GAs are generally extracted from fungal or plant systems with appropriate organic solvents and buffers. They are further purified by partition column chromatography[39] and by agar diffusion technique[40]. Treatment with polyvinyl-pyrrolidine is highly effective in purification of GA from the extracts of vegetative tissues, presumably by selective removal of inhibitory impurities such as phenolic compounds, abscicic acid and glucosides of active GAs[41].

Separation, identification and characterisation of minute quantities of GA metabolites in plants have been achieved by employing sensitive techniques based on TLC, GLC, fluorimetry, paper chromatography, silica gel adsorption, etc[42]. Isotope dilution method is shown to correlate well with bioassay methods[43]. Deuterium-labeled substrates are used as internal standard for the determination of native GAs. In metabolic studies, these can be distinguished from the labeled metabolites by using doubly labeled substrates with tritium, and quantified by GLC and mass-spectroscopy[24]. More recently, using GLC with 3 liquid phases, all GAs and their glucosides have been separated and characterised[45]. Similarly, a conclusive identification and sequence of interconversions of GA derivatives present in sub-microgram quantities have been achieved by combined gas liquid radio chromatography–mass spectrometry[46] and radioimmunoassay technique[47]. X-ray analysis and NMR spectroscopy are useful in determining the stereochemistry of natural GAs[48].

A number of bioassay methods for quantitative analysis of GA activity are developed. The barley aleurone α-amylase bioassay, though laborious and time consuming, is one of the most sensitive one[49]. In addition to widely employed dwarf mutant tests, rapid bioassay methods using wheat, rice or barley endosperms, are routinely used; GA activity is measured in terms of alpha-amylase production and reducing sugars released in the medium. Similarly, total proteins released from barley aleurones are measured to determine GA-like activity over a wide range of concentrations[50]. Attempts have been made to use simple systems such as pale malt[51] and soluble starch[52] as substrates for rapid assay of GA activity. The methods based on stimulation of pea shoot growth, lithia or cucumber hypocotyl growth or retardation of senescence in leaf discs of *Taraxacum officinale* and *Rumex obtusifolius*, have been reported for detection of GAs[53].

6. Inhibitors of GA biogenesis

A number of synthetic compounds including, 2-chloroethyl trimethyl ammonium chloride (CCC), 2'-isopropyl-4'-(trimethylammonium chloride)-5'-methyl phenyl piperidine-1-carboxylate (AMO-1618), tributyl-2, 4-dichlorobenzyl phosphonium chloride (Phosphon D), etc., are known growth retardants[1]. Some hydrazonium analogues of CCC also have the same potency for growth retardation in wheat[54]. CCC and phosphon D interact with GA_3 in a specific, non-competitive way for the sites of action[1]. The available evidence indicates that AMO-1618 inhibits *in vitro* incorporation of GGPP into (−) kaurene in castor beans[55]. Quarternary ammonium compounds derived from α-ionone and isophorones are more potent inhibitors of GA biosynthesis than AMO-1618, with a similar site of action[56]. CCC is shown to inhibit cyclization of GGPP to (−) kaurene, causing the accumulation of the latter in *F. moniliforme* culture[57]. However, Paleg *et al*[58] have indicated that CCC inhibits acetate to mevalonate conversion, thereby blocking the metabolism of barley embryos grown in soluble starch. Dennis *et al*[59] have suggested that the sites of action of these two potent growth retardants on GA_3 may be different and AMO-1618 may have a multiple action as shown schematically in Fig. 4. Further, these inhibitors uncouple oxidative phosphorylation in pea seedlings, thus reducing the availability of ATP for cell division and GA synthesis[60]. A bitter principle cucurbitacins

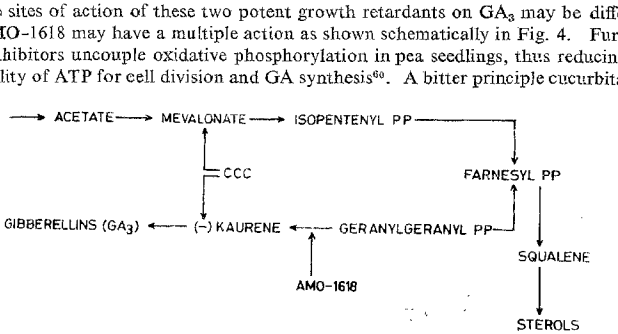


FIG. 4. Schematic representation of sites of action of growth retardants on GA biosynthetic pathway

isolated from cucurbitaceae has also a pronounced anti GA activity and reduces GA_3-induced plant growth[61]. Thus, these retardants, having high specificity of action limited to one single step, can serve as very useful tools in studies of GA physiology. Treatment with CCC results in increased cold-hardiness of winter wheat, cabbage, and peas[62]. This may be attributed to CCC-induced reduction in GA levels, which controls leaf size and stem growth, both of which are retarded in cold temperatures.

7. Effects of environmental stress on GA metabolism

Environmental factors such as light, photoperiod, vernalization, stratification and radiation bring about quantitative and qualitative changes in GA levels in higher plants. Involvement of endogenous GAs in photomorphogenic events have been proved convincingly. Inhibitory effects of red light on stem growth in peas is attributed to the depression of endogenous GA levels in tissues and such physiological dwarfism can be reversed by exogenous GA_3[63]. On the other hand, exposure to red light stimulates the synthesis of extractable but transient GA_3 in etiolated barley[64] and wheat[65] leaves. Elimination of this GA by incubation of leaf sections with AMO–1618 or CCC, suggests that new synthesis of hormone is occurring. The appearance of diffusible GA is increased in unilaterally illuminated or geotropically stimulated sunflower shoot tips, due to increased GA biosynthesis and transport[66]. GA-induced inhibition and promotion of sprouting in aerial tubers is directly correlated with photoperiodic treatment and tuber growth stage[67]. The phytochrome-mediated dormancy of celery seeds responds to GA_3, and treated seeds may germinate in dark at high temperatures[68]. GA synthesis is inhibited and biologically active acidic GAs are converted into relatively inactive polar GAs or GA-glycosides in cold-hardened wheat[69]. However, cold treatment triggers GA synthesis in tulip and plum seeds[70]. This suggests that possible interacting effect of vernalization on GA metabolism may be a complex phenomenon. It is shown that mechanical stress given by moderate shaking also retards seedling growth and GA production in *P. vulgaris*[71].

Ionizing radiations induce numerous histological and cytological changes in plants. Though DNA is shown as the primary site of damage[72], the lesion and/or depletion of the meristem polulation, the site of auxin and GA synthesis during germination of seeds, is strikingly radio-sensitive[73]. Stimulation of mitotic activity in suboptical region of meristem and spindle orientation, preceding the cell elongation, are the earliest effects of GA treatment[74]. Treatment with exogenous GA_3 reverses the inhibition of seedling growth in sub-lethally irradiated wheat[75] by stimulating the synthesis of mRNA[76] or of polydisperse RNA fraction and thereby triggering α-amylase synthesis[77]. High doses of gamma irradiation induces physiological dormancy. Loss of viability in irradiated seeds is attributed to the metabolic disturbances in the biosynthetic events leading to protein synthesis[78] which controls the intracellular GA concentration. GA is degraded by gamma-irradiation *in vivo* and *in vitro*[79]. Incorporation of radioactive precursors in GA_3, the most bioactive GA derivative in wheat, is reduced in gamma-irradiated wheat during germniation and the response is dose-dependent[80]. Initiations of RNA, DNA and protein syntheses are delayed during early stages of germination in irradiated wheat[81].

Dunham and Cherry[82] have shown that gamma-irradiation inhibits *de novo* synthesis of RNA polymerase, subsequently leading to reduced RNA formation in sugar beet tissues. Gamma irradiation also affects the transcription and translation processes leading to altered protein conformation[83]. Recently, it has been reported[84] that gamma-irradiation stimulates repair mechanism in barley aleurones and this is accelerated by GA treatment. The underlying mechanism which adversely affects the structure and function of GAs, is elucidated in irradiated wheat during germination. The activities of MVA kinase, ATPase and kaurene synthetase, involved in various intermediary steps leading to GA synthesis from precursor, are impaired due to radiation treatment of wheat (Fig. 5)[78]. Further, efficiency of interconversion of a less active GA to a highly active one is adversely affected. This results in accumulation of GA_4 and GA_7, which are biologically less active intermediate precursor products on GA biosynthetic pathway leading to GA_3 formation[78]. Differential requirements for GA_3 to stimulate the synthesis of total and of three major α-amylase isoenzymes (α1, α2, α3) are noted in germinating control and irradiated seeds. α_1-isoenzyme is the most radiolabile and does not appear in germinating wheat, irradiated at 200 krad. However, pre-treatment of seeds with GA triggers its formation (Fig. 6). This suggests that two aggregate systems

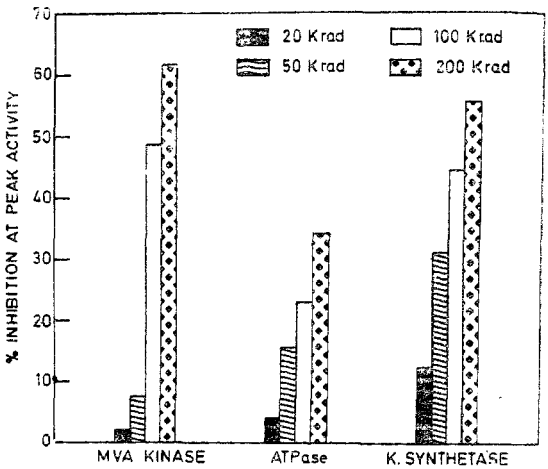


FIG. 5. Individual peak activities of the enzymes, reaching maximum during germination were measured for MVA kinase (at 4 hr), ATPase (at 7 hr) and kaurene synthetase (at 56 hr). The results are expressed as % of the activity in irradiated seeds taking control (in unirradiated seeds) as 100% activity.

differing in their radiosensitivity and response to GA_3 application, are operating for the synthesis of functional alpha-amylase molecule in germinating wheat[81].

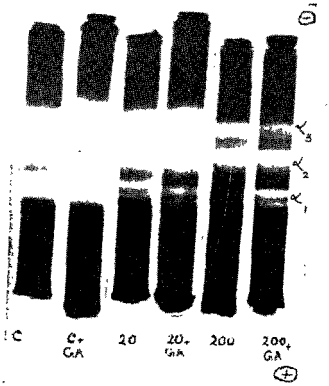


FIG. 6. Effect of GA treatment on the formation of alpha-amylase isoenzymes in irradiated wheat. Control and irradiated (20 or 200 krad) seeds were imbibed for 16 hr either with water or GA solution and germinated for 4 days. Zymograms depict the polyacrylamide gel electrophoretic pattern of the isoenzymes stained with starch + I_2 solution.

8. Physiological role of GA

In recent years, GAs have been implicated in several biochemical reactions and shown to play an important role in physiological processes of diverse nature. These include : breaking of dormancy, regulation of germination processes, control of synthesis and release of various hydrolysing enzymes, etc. The processes controlled and stimulated by GA in germinating grain, are summarised in Fig 7.

8.1. *Dormancy and germination*

Dormant seed embryos can be activated by the removal of seed coat, a physical barrier for cell expansion, by increasing O_2 supply and leaching out the endogenous inhibitors. Various environmental conditions like light, photoperiod, vernalization, etc., bring about changes in endogenous metabolism[85]. GAs are implicated in reversal of thermo- and photo-dormancy as well as osmotic dormancy imposed by mannitol[86]. Jarvis *et al*[87] have suggested that GA_3 breaks seed dormancy by derepression of dormant, repressed gene in embryonic cells. Increase in mitotic activity, DNA template availability for transcription and RNA polymerase activity in embryonic axis, are the earliest visible

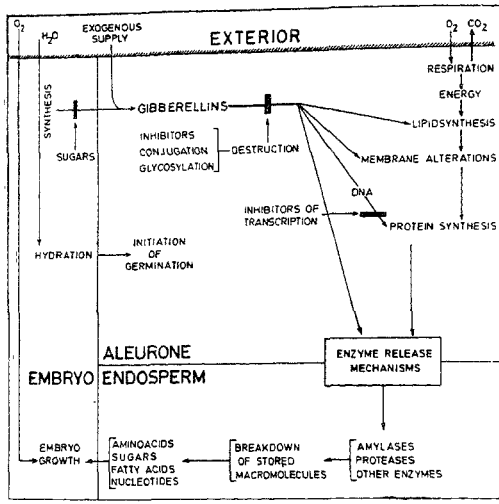


FIG. 7. Schematic representation of biochemical reactions responding to GA action in germinating grains.

and measurable effects of GA treatment[88]; although the exact inter-relationship, is still obscure. Although GA_3 has no effect on endomitotic DNA synthesis, it increases epicotyl DNA content and polyploidy of a cell[89], which is an important factor in the control of cell length[90]. GA increases total RNA content and enhances DNA-dependent nuclear RNA synthesis in isolated pea and sugar beet nuclei[91]. Application of GA stimulates protein synthesis in shoot and root meristems and in the developing vascular tissues of charlock seeds[92]. Increase in the synthesis of cell wall, with concurrent elongation of internodes in *Avena* stem, is proportional to GA_3 concentration[93]. This is attributed to stimulation of cell wall polysaccharide synthetase activity by GA_3, which in turn causes increased cell wall deposition[94]. GA_3 stimulates *de novo* synthesis and release of hydrolyzing enzymes including α-amylase, ribonuclease and protease in aleurone layers[95] and other enzymes involved in intermediary metabolism in cotyledons (Fig. 7). Importance of GA_3 has been recognised in regulating the mobilisation and absorption of the solutes produced by the action of these enzymes for growth and development of embryos. However, GA_3-induced inhibition in germination of various species has also been reported. Existence of two counteractive reactions (inhibition and

promotion of sprouting) activated by GA_3, are shown in the genus *Dioscorea*[96]. These reactions are related to light quality. In germinating *D. tokoro*, application of exogenous GA_3 inhibits the seedling growth in dark and red but promotes in blue and far red light[97].

8.2. *Balance of hormones for germination*

Though GAs are effective endogenous regulators, it is now fairly well established that appropriate balance between GA, growth promoters (cytokinins and indolacetic acid) and inhibitors (*e.g.*, abscisic acid (ABA)) is necessary for the regulation of growth and development of plant in normal and stress conditions[98]. Both ABA and GA are biosynthesised from MVA by common isoprenoid pathway[99] and during the later stages of growth, ABA accumulates in the seed[100]. All GA_3-stimulated germinative and enzymatic processes are strongly inhibited by ABA. ABA also blocks DNA and RNA syntheses and delays the onset of germination. Similarly, alpha-amylase and protease production in barley grains are inhibited by ABA and reversed by cytokinins but not by GA[101]. These observations on competitive interactions between cytokinins and GA and non-competitive interactions of ABA and GA[102], support the prevalent inhibitor promoter hypothesis[103]. Recently, Khan[98] has suggested several possible hormonal situations, which can control dormancy and germination, depending upon physiologically effective or non-effective levels of GA, cytokinins and inhibitors. Thus, GA plays a primary role in germination, whereas inhibitors and cytokinins are secondary and essentially preventive and permissive, respectively.

8.3. *Carbohydrate metabolism*

Besides the well defined primary action of GA in alpha-amylase formation for initiation of germination, the detailed characterisation of other carbohydrases is not achieved. However, GA is shown to increase *de novo* synthesis and release of laminarinase (β-(1 → 3) glucanase), alpha-glucosidase and diastase activities in germinating barley[104].

8.4. *Lipid metabolism*

Role of GA in lipid metabolism is elucidated by events occurring during lag phase of 2 to 8 hr preceding nearly germination, and therefore, cannot be considered as its primary action. GA_3 enhances the rate of ^{14}C-choline[105] and ^{32}P[106] incorporation into phospholipid moieties of semi-purified endoplasmic reticulum of aleurone layers of germinating barley[107]. This is attributed to the activation of two key transferases, namely, phosphoryl choline-cytidyl and phosphoryl choline glyceride, involved in lecithin biosynthesis[108]. Similarly, GA stimulates the activities of the enzymes of gluconeogenic pathway in castor bean seedlings[109].

8.5. *GA-induced changes in membrane properties*

GA is implicated in the biogenesis of endoplasmic reticulum[110], nuclear membrane[95], mitochondrial cristae[111], and binding of polysomes to the endoplasmic membranes[112]. GA_3 also stimulates extensive proliferation of cellular membranes during early germi

nation[113]. GA_3 is shown to enhance the synthesis of marker enzymes of glyoxysomal and mitochondrial (inner and outer) membranes in germinating almond seeds[114]. However, GA_3 is shown to inhibit cell-wall pentosan synthesis and membrane-bound arabinosyl transferase activity, present in barley aleurone layers[115]. A possible interaction between GA and phospholipids is shown to govern the permeability and transmembrane potential of liposomal membranes[116]. GA_3 also affects the phase transition temperature of liposomes as a function of concentration[117]. Recently, studies with dwarf maize mutant by electron microprobe analysis, have indicated that GA_3 selectively changes the permeability of aleurone plasmalemma and thereby regulates the influx of monovalent (Na^+, K^+, Cl^-) and divalent (Mg^{++}) ions[118]. Thus GA_3 alters the microenvironment in different cell compartments. The synergistic effects between GA and low pH compounds (*e.g.*, EDTA), may be *via* removal of inhibitors and/or alteration of membrane permeability through the chelating activity of EDTA[119]. However, acid growth hypothesis, which explains auxin-induced elongation growth by cell-wall acidification[120] is not applicable to GA_3-induced elongation in lettuce hypocotyl sections[121].

8.6. *Role of GA in alpha-amylase synthesis*

GA-induced alpha-amylase synthesis is one of the best understood hormone-controlled mechanisms in plants. In germinating barley and wheat, the enzyme is synthesised by the microsomal fraction[122] of the aleurone layers in response to GA[123]. GA-stimulated formation of polyribosomes is shown as a pre-requisite for the enzyme synthesis in aleurone layers. It has been observed that GA stimulates the synthesis of specific mRNA for α-amylase induction and accumulation of heterodisperse minor RNA fractions[124]. GA also activates some preformed RNA and inhibits the breakdown of mRNA. Carlson[125] has postulated that GA regulates at the post-transcriptional level. However, Chandra[126] has suggested that GA can bring about specific structural modifications of the transcribed RNAs, and consequently, a translational modulation of protein(s), with molecular heterogeneity follows[127]. Thus the effects of GA on synthesis, activation and stability of mRNA are not clearly distinguished.

Besides GA, many naturally occurring compounds including abscisic acid (ABA), helminthosporal, (−) kaurene, cyclic 3′–5′ AMP, and ATP as well as osmotic stress and concentration of hydrolytic products, are shown to regulate alpha-amylase synthesis[75]. ABA-inhibited alpha-amylase synthesis is counteracted by GA in barley[128] and mustard[129] seedlings. Photoinactivation of the enzyme in barley on exposure to light, is correlated with the chlorophyll content and a change in the endogenous GA-inhibitor balance[130].

The subcellular pathway of the release of alpha-amylase is not yet well defined. Evidence for enzyme packaging in lysosome-like bodies in GA-treated wheat aleurones is reported[131] but not confirmed in barley[132]. The hypothesis, that the enzyme is transported from the cytoplasm both inside and outside the cell by distinct secretory vesicles[133],

is not supported by cytological evidences. It is suggested[134] that alpha-amylase being a soluble enzyme, is directly released from the cytoplasm across the aleurone cell plasmalemma, without the involvement of discrete secretory organelles.

8.7. *Hormonal regulation of alpha-amylase isoenzymes*

Highly purified alpha-amylase is selectively obtained by complexing with glycogen, though two apparently independent binding sites on the enzyme are demonstrated for the formation of multimolecular complex[135]. Heterogeneity of α-amylases have been reported and 5 isoenzymes are separated from barley[136]. Isoenzymes are separated by starch or polyacrylamide gel electrophoresis, isoelectric focussing and by immunodiffusion methods[127]. Quantitative separation can be obtained by ion-exchange column chromatography using a linear salt gradient.

It has been shown that in barley aleurones, GA-induced alpha-amylase isoenzymes, synthesised *in vitro* and *in vivo*, are not similar[137]. Two sets of isoenzymes having different electrophoretic mobilities have been isolated from germinating barley[138]; GA-treatment accelerates the formation of all the isoenzymes[139]. Faster moving set of isoenzymes is comparable to green alpha-amylase of immature wheat kernels and their quantity varies at different stages of seed development. It is shown to be responsible for lowered amylograph viscosity and thus governs the ultimate quality of wheat[140].

Studies on physico-chemical properties of four alpha-amylase isoenzymes of malted wheat revealed that they have similar Km values, pH optima, activation energy and molecular weights, but different electrophoretic and immunological properties[141]. In barley, seven alpha-amylase isoenzymes are present differing only in their net electrical charges[142]. High acidic amino acid content of the enzyme explains their stability in acidic pH. However, their individual contribution in governing the rheological and bread making properties of wheat dough are not known. The failures to interconvert alpha-amylase isoenzymes or to implicate them as preparation artifacts, suggests that they have different genetic origin. The isoenzymes from barley, wheat and maize reflect some differences in their molecular characteristics, which result from base pair alterations in their structural genes[143]. Multiple *loci* controlling alpha- and beta-amylase isoenzymes have been reported in wheat[144]. Furuta *et al*[145] have concluded that no appreciable changes in the nuclear DNA content of A and B genomes of common wheat have occurred during the entire course of evolution. The genetic characteristics of alpha-amylase isoenzymes from genuine and reconstituted strains of tetra and hexaploid wheat are compared and used to analyse the genetic regulatory mechanism in plants[146]. The indirect fluorescent antibody technique is used to analyse the cellular and intracellular localization of the enzyme more precisely and this helps to screen mutation in plants[147].

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