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Short Communication

Apparent Changes in Rate of Kaurene Biosynthesis During the Development of Pea Seeds[1]

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The seeds of several species have been shown to accumulate, during certain stages of development, much greater quantities of gibberellin (GA) than are found in the vegetative parts of older plants (2, 4, 12, 14, 15, 17, 19). Since the first definitive discovery that immature seeds are abundant sources of GA (15), there has been much active interest in the relationship between GA content and growth of seeds and fruits (2, 4, 12, 19), the possible role of GA's stored in seeds in the subsequent growth of seedlings developing from them (3, 11), and the utility of cell-free enzyme extracts from immature seeds in investigations of GA biosynthesis (1, 6, 7, 8, 9, 16, 18).

Substantial progress toward the development of cell-free systems from immature seeds capable of GA biosynthesis from mevalonic acid (MVA) first was made when Graebe *et al.* (9) reported on the biosynthesis of (−)-kaurene, a known intermediate in GA biosynthesis in *Fusarium moniliforme* (5,9) and presumably also in higher plants, from MVA in cell-free extracts of *Echinocystis macrocarpa* Greene endosperm-nucellus. Subsequently, Robinson and West (18) showed that MVA also was incorporated into (−)-kaurene and other diterpenes in germinating seeds of *Ricinus communis* L. Dennis and West (7) developed a cell-free system from immature *Echinocystis* seeds in which (−)-kaurene, (−)-kaurenol, (−)-kaurenal and (−)-kaurenoic acid were synthesized irreversibly and in sequence from MVA. Anderson and Moore (1) reported on (−)-kaurene production from MVA in cell-free extracts from immature pea (*Pisum sativum* L.) seeds, and Graebe (8) has demonstrated more recently the synthesis of (−)-kaurene, in addition to squalene, phytoene and some other isoprenoid compounds, in cell-free preparations from immature pea seeds and pods. The purpose of this investigation was to compare the apparent capacities for (−)-kaurene biosynthesis from MVA in immature pea seeds at various stages of development, and to describe some optimum conditions for the enzymic assay.

The methods and materials used in these experiments were similar to those of Anderson and Moore (1). Alaska peas (*Pisum sativum* L.; W. Atlee Burpee Company, Riverside, California) were grown in a greenhouse where the light and temperature regime consisted of a 16 hr photoperiod, at approximately 20 to 27° and a light intensity of 800 to 1000 ft-c, and an 8-hr nyctoperiod at approximately 16 to 18°. The natural light intensity was supplemented with and the photoperiod extended by Gro-Lux fluorescent lamps. Samples of 75 or more immature seeds were harvested at intervals from 10 to 16 days after anthesis and were homogenized immediately in cold 0.1 M potassium phosphate buffer (1 g fresh wt/ml buffer), pH 7.1, containing 50 μg/ml each of penicillin G and streptomycin sulfate using a cold mortar and pestle. The homogenates were centrifuged at 40 000*g* for 15 min and the supernatant was used as the enzyme extract. Fresh enzyme extracts were prepared for each experiment with the exception of the experiments represented in Fig. 5 and 6, in which the enzyme extracts were frozen in liquid nitrogen and stored at −70° for 2 months prior to use. A complete reaction mixture routinely contained 0.05 μmole of MVA-2-^{14}C (specific radioactivity 5.02 mc/mmole; CalBiochem; converted from MVA-2-^{14}C lactone by treating overnight with 100% excess NaOH), 0.75 ml enzyme extract [representing the enzyme extracted from about 3.5 seeds, or approximately 2.75 mg of micro-Kjeldahl determined nitrogen (1)], 0.5 μmole

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of ATP, 3 μmoles $MnCl_2$, and 0.6 ml of 0.1 M potassium phosphate buffer at pH 7.1 in a total volume of 1.6 ml. Reaction mixtures were incubated for 75 min at 30°. The reactions were stopped by adding 3 ml of acetone, and the reaction mixtures were extracted twice with 1 ml portions of benzene. The radioactive (−)-kaurene-^{14}C (hereafter referred to simply as kaurene) was located by scanning thin-layer chromatograms, developed in hexane (1), on a Packard Radiochromatogram Scanner, Model 7201. The appropriate bands of silica gel were removed from chromatograms and the radioactivity measured on a Packard Liquid Scintillation Spectrometer, Model 3375, with an average counting efficiency of 85 %. Each point on the graphs represents the mean dpm obtained from at least duplicate reaction mixtures. The results of each experiment were fully confirmed in at least 1 duplicate experiment conducted on a different occasion.

The rate of incorporation of MVA into kaurene was directly proportional to enzyme concentration through approximately 0.75 ml of enzyme extract (Fig. 1), which was the amount used in all subsequent experiments, and the reaction rate was constant through 90 min at 30° (Fig. 2). Based on the data shown in Fig. 3, a nearly saturating concentration of 0.03 mM MVA was selected for use in all other experiments. Incorporation of MVA into kaurene is dependent upon ATP, and the concentration of ATP very critically affects the rate of incorporation (Fig. 4). The results with ATP agree generally with those of Graebe (8) for a similar system.

In agreement with the results of Anderson and Moore (1), Mn^{2+} stimulated the incorporation of MVA into kaurene much more than Mg^{2+} (Fig. 5). This finding is consistent also with the report by Loomis and Battaile (10) that Mn^{2+} is a better activator than Mg^{2+} of mevalonic kinase from pumpkin seedlings, and the finding by Nandi and Porter (13) that Mn^{2+} is a better activator than Mg^{2+} for the carrot root enzyme which catalyzes the synthesis of geranylgeranyl pyrophosphate from isopentenyl and farnesyl pyrophosphates. These systems all are

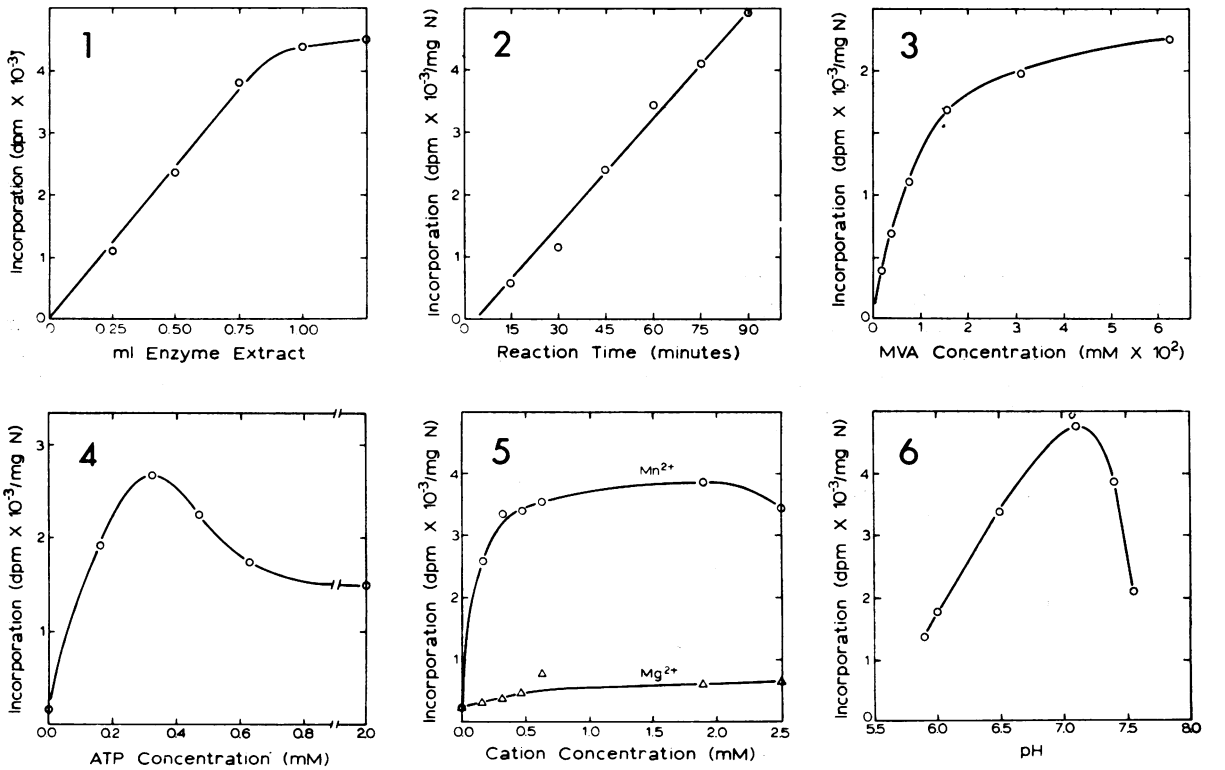

FIG. 1. Incorporation of mevalonate-^{14}C into (-)-kaurene-^{14}C at various enzyme concentrations. One ml of extract contained 3.28 mg nitrogen determined by a micro-Kjeldahl method. The experiments represented in Fig. 1 to 6 were done with seeds which were harvested 12 to 14 days after anthesis.

FIG. 2. Time-course of incorporation of mevalonate-^{14}C into (-)-kaurene-^{14}C.

FIG. 3. Effect of substrate concentration on the conversion of mevalonate-^{14}C to (-)-kaurene-^{14}C.

FIG. 4. Effect of ATP concentration on the incorporation of mevalonate-^{14}C into (-)-kaurene-^{14}C.

FIG. 5. Effects of Mn^{2+} and Mg^{2+} on the conversion of mevalonate-^{14}C to (-)-kaurene-^{14}C. Each cation was added as the chloride salt.

FIG. 6. Effect of hydrogen-ion concentration on the incorporation of mevalonate-^{14}C into (-)-kaurene-^{14}C.

inhibited by high concentrations of Mn^{2+}. Graebe (8) reported that Mg^{2+} stimulated kaurene production, whereas Mn^{2+} in the presence of Mg^{2+} did not stimulate. We have found by using successively higher concentrations of each cation that the Mg^{2+} and Mn^{2+} curves converge at a concentration of about 4 mM because of the decreasing effectiveness of Mn^{2+} above about 2 mM.

The optimum pH for kaurene formation in the crude cell-free extracts is about 7.1, and the activity declines sharply above pH 7.3 (Fig. 6). The pH curve was obtained using 6 separate enzyme extracts all of which were prepared simultaneously from 1 randomized sample of seeds. Six phosphate buffers were prepared by mixing appropriate volumes of 0.1 M K_2HPO_4 and 0.1 M KH_2PO_4 until the desired H-ion concentrations were obtained. The pH of each reaction mixture was measured both before and after incubation and did not vary more than one-tenth of a pH unit during this time. Thus the graph represents activity obtained at the measured H-ion concentrations. It was interesting to note in other experiments that the saturating concentrations of both Mn^{2+} and ATP changed from about 0.4 mM to about 0.3 mM when the pH of the reaction mixtures was changed from 6.5 to 7.1.

The capacity to convert MVA to kaurene, as measured in cell-free extracts, varied markedly with stage of seed development (Fig. 7). Using enzyme extracts obtained from first-formed fruits at different stages of seed development, it was seen that the activity increased to a maximum at about 13 days after anthesis, or when the seeds had attained about half-maximal fresh weight, and then declined as the seeds approached maximum diameter and fresh weight. In other experiments some variation (about 1 day) was observed in the time sequence of the development of the seed as well as in the day upon which maximum incorporation of MVA into kaurene occurred. This variation was probably caused by fluctuations in the greenhouse temperature during the summer months. In these experiments peas were planted at 1- or 2-day intervals, and all the groups of seeds were harvested and assayed simultaneously. Only fruits which had developed from first-formed flowers were harvested. It was noted that the pods achieved their final length during the first 10 days after anthesis. On about the tenth day, the fruits were flat and the developing ovules were extremely small (< 50 mg/ovule). The maximum incorporation of MVA into kaurene was obtained, as already noted, on approximately the thirteenth day after anthesis. The seeds obtained maximum fresh weight on about the sixteenth day after anthesis. Hence the apparent maximum capacity for kaurene biosynthesis in immature seeds was attained after the pods had reached final length and before the developing ovules reached maximum fresh weight.

The conversion of MVA to kaurene is expressed in Fig. 7 as dpm incorporated per mg N; however,

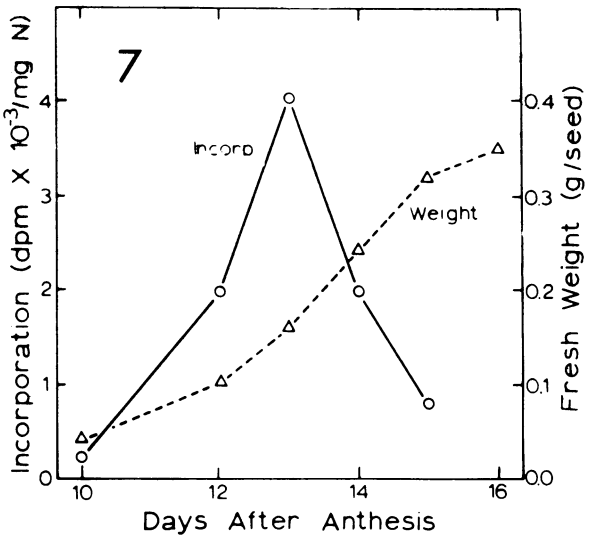

FIG. 7. Apparent changes in the rate of (-)-kaurene biosynthesis during the development of pea seeds.

the same relationship is also seen when the data are calculated as dpm incorporated per seed. Thus, the mean dpm incorporated per seed were 223, 725, 2400, 1717, and 1020 at 10, 12, 13, 14, and 15 days after anthesis, respectively.

It is interesting to compare these results on kaurene formation with the available data on the extractable GA content of seeds at various stages of development. In immature seeds of *Echinocystis macrocarpa* Greene, *Lupinus succulentus* Dougl. (4), *Phaseolus vulgaris* L. (4, 19), *Lupinus luteus* L. (14), and *Pharbitis nil* Chois. (12), the maximum amounts of extractable GA's are found when the seeds have attained about half their maximum fresh weights. Preliminary extraction data from our laboratory indicate the same result for pea seeds. Thus the quantity of extractable GA and the apparent rate of kaurene synthesis from MVA both appear to increase sharply during development of leguminous seeds and to reach maximum values when the seeds have attained about half-maximal fresh weight.

Graebe (8) obtained comparatively low incorporation of MVA into kaurene using cell-free enzyme extracts prepared from very young pea fruits and even lower incorporation with immature seeds from almost fully grown pods. A direct comparison of our results with Graebe's is difficult; however, our results, showing relatively low activity in very immature seeds, are consistent with Graebe's suggestion that the kaurene-synthesizing activity which he observed with very young fruits may reflect an active synthesis in the pod. Just as the growth curves for the legume pod and seed do not coincide in time (4), maturation of the seed lagging behind that of the pod, it seems likely that the time-courses of kaurene

and GA synthesis and GA accumulation in the pod and seed are similarly related.

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