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Studies on Cytokinins in Young *Lupinus* Seeds with Paper Chromatography

By

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It was shown that young seeds of *Lupinus luteus* contained some unknown cytokinins besides dihydrozeatin, which strongly stimulated the growth of cultured tobacco tissue (*Nicotiana tabacum* cv. Wisconsin No. 38). These unknown cytokinins were purified partially and studied with paper chromatography and bioassay. One of them is water-soluble and hydrolysis of it yields more active water-soluble cytokinin, but neither dihydrozeatin nor its ribonucleoside. This unknown cytokinin is not dihydrozeatin ribonucleotide, and may be a novel cytokinin. Another unknown cytokinin is not water-soluble, but extractable with *n*-butanol. On paper chromatograms this cytokinin locates closely to dihydrozeatin ribonucleoside, and gives a positive color reaction specific to pentose. Hydrolysis of it yields an active component indistinguishable from dihydrozeatin. It seems likely that this cytokinin is dihydrozeatin ribonucleoside.

Introduction

Since the first isolation of native cytokinin, zeatin, from immature corn kernels by Letham (1963)[8], it has been demonstrated that such native cytokinins are of wide-spread occurrence in various plants, and reports indicating the important roles of these cytokinins in plant growth have been accumulated (see Skoog & Armstrong, 1970[11]). One of such natural cytokinins is dihydrozeatin which was isolated from young *Lupinus* seeds (Matsubara & Koshimizu, 1966[5]) and identified as (-)-N^6-(4-hydroxy-3-methylbutylamino)-purine[2]. Noticeable effects of this cytokinin on plant growth were also reported (Matsu bara et al., 1968[7]).

During the course of the isolation of dihydrozeatin from young *Lupinus* seeds, more than two fractions with strong cytokinin activity were obtained in charcoal treatment followed by elution. From a fraction eluted with a mixture of pyridine, ethanol and NH_4OH, dihydrozeatin was isolated[2], while another fraction eluted with 70 % acetone has remained to be examined. Very strong cytokinin activity of this fraction aroused us to carry out additional study.

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Materials and Methods

Young seeds (6.5 kg) of *Lupinus luteus* L. harvested on 30 days after flowering were extracted three times with 6.5 liters of 80 % ethanol, and filtered through double layers of filter paper. The ethanol solution was evaporated under 50°C *in vacuo* until all of the ethanol had evaporated. This crude extract was used for bioassay and further purification as shown in Figures 2 and 4, which were based on the methods reported previously by Matsubara & Koshimizu (1966)[5], Koshimizu et al. (1967)[2] and Matsubara et al. (1970)[8].

In paper chromatography, the crude extract and other preparations were streaked on sheets of Toyo No. 51 filter paper, and developed ascendingly 25 cm with following solvent systems : (A) 0.02 M boric acid (pH 8.4), (B) *n*-butanol : formic acid : water (80 : 15 : 15), (C) water, and (D) water-saturated *n*-butanol. For bioassay a chromatogram was cut into 10 (or 11 in some experiments shown in Figure 7) transverse sections which were eluted with water. Eluates were added to the culture medium for tobacco callus bioassay.

The fresh weight increase of tobacco callus (*Nicotiana tabacum* cv. Wisconsin No. 38) which was isolated originally from stem pith and maintained as stock cultures was used for all cytokinin assays following the methods of Linsmaier & Skoog (1965)[4] modified by Matsubara et al. (1970)[8]. A preparation to be tested was added to Linsmaier & Skoog's basal medium containing 2 mg/l IAA[4]. The medium was adjusted to pH 5.6, divided by 12 ml into 10 test tubes (18×180 mm) and autoclaved at 1.0 kg/cm² for 15 minutes, unless otherwise stated. An explant of tobacco callus (ca. 5 mg in fresh weight) which was cut out of stock culture was planted on the agar surface in each test tube. Cytokinin activity was determined by measuring average fresh weight of calluses after 30 day culture in the dark at 27°C. All the experiments were repeated at least three times.

Results

Preparation of the active fractions……The crude extract of young *Lupinus* seeds stimulated the growth of tobacco callus at concentrations equivalent to 2–50 g of fresh seeds per liter of medium (Table 1). The maximum growth stimulation was observed at 20 g equivalents per liter.

When the crude extract corresponding to 20 g fresh seeds was paper-chromatographed with 0.02 M boric acid (pH 8.4) (solvent A) and the location of cytokinin activity was investigated, two main peaks of the activity were observed (Figure 1). The fast-moving cytokinin was at Rf 0.7–1.0, and the slower-moving cytokinin at Rf 0.4–0.6 where dihydrozeatin co-chromatographed migrated. From this result it is clear that the crude extract contains unknown cyto-

Table 1. Effect of the crude ethanol extract of young *Lupinus* seeds on the growth of tobacco callus. The extract was tested at concentrations equivalent to 2–50 g fresh seeds per liter of medium.

Additives	Conc.	Fresh weight of tissue (mg)
	(g eq/l)	
Crude extract	0	16.9
	2	253.7
	5	401.3
	10	560.7
	20	701.9
	50	84.4
	(mg/l)	
Kinetin	0.03	401.1

kinins besides dihydrozeatin which was previously proved to be present in young *Lupinus* seeds[2].

In a separate experiment the eluates from the chromatogram were sterilized by Seitz-filter and added aseptically to autoclaved basal media shortly before solidification, because it was necessary to inspect whether or not the fast-moving unknown cytokinins shown above are artifact produced during autoclaving. Bioassay revealed qualitatively same distribution of cytokinin activity on the chromatogram as Figure 1. Hence, the fast-moving cytokinins are not formed by autoclaving.

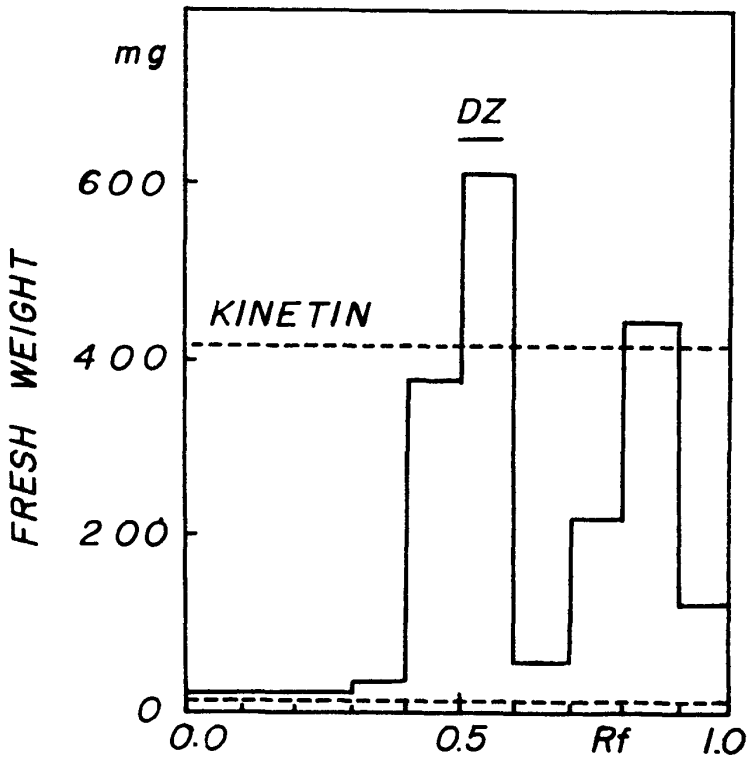

Figure 1. Location of cytokinin activity on paper chromatogram of the crude extact developed with 0.02 M boric acid (pH 8.4). Line at the top of the histogram indicates the locus of dihydrozeatin (DZ) co-chromatographed. The broken lines represent the fresh weight of tissue cultured on the basal medium with or without kinetin (0.03 mg/l).

In order to purify unknown cytokinins, the crude extract was fractionated further as shown in Figure 2 : by extraction with ethylacetate and *n*-butanol, and adsorption on charcoal and Dowex 50 W × 8 cation exchange column (H^+ and 50-100 mesh). Fractions 1-5 were prepared and tested for their cytokinin activity (Table 2).

The ethylacetate extract (Fraction 1), the filtrate from charcoal (Fraction 3) and the effluent from Dowex 50 column (Fraction 4) were inactive at 100 mg/l and inhibitory at higher concentrations. The aqueous fraction (Fraction 2) and the ammonia eluate from Dowex 50 column (Fraction 5) were stimulative, the former being considerably weaker than the latter. The cytokinin activity of the ammonia eluate was recognizable at a level as low as 1 mg/l and more remarkable with increasing concentration. Previous papers demonstrated that dihydrozeatin was easily extracted with *n*-butanol[5], and it was not eluted with 70 % acetone from charcoal, but preferentially eluted with a mixture of pyridine, ethanol and NH_4OH[2]. Therefore, in the present experiment dihydrozeatin should not be contained in 70 % acetone eluate from charcoal, but still adsorbed on charcoal (see Figure 2). Consequently it is thought that Fractions 2 and 5 do not contain dihydrozeatin but unknown cytokinins. The aqueous fraction (Fraction 2) and the ammonia eluate (Fraction 5) were purified further and studied with paper chromatography.

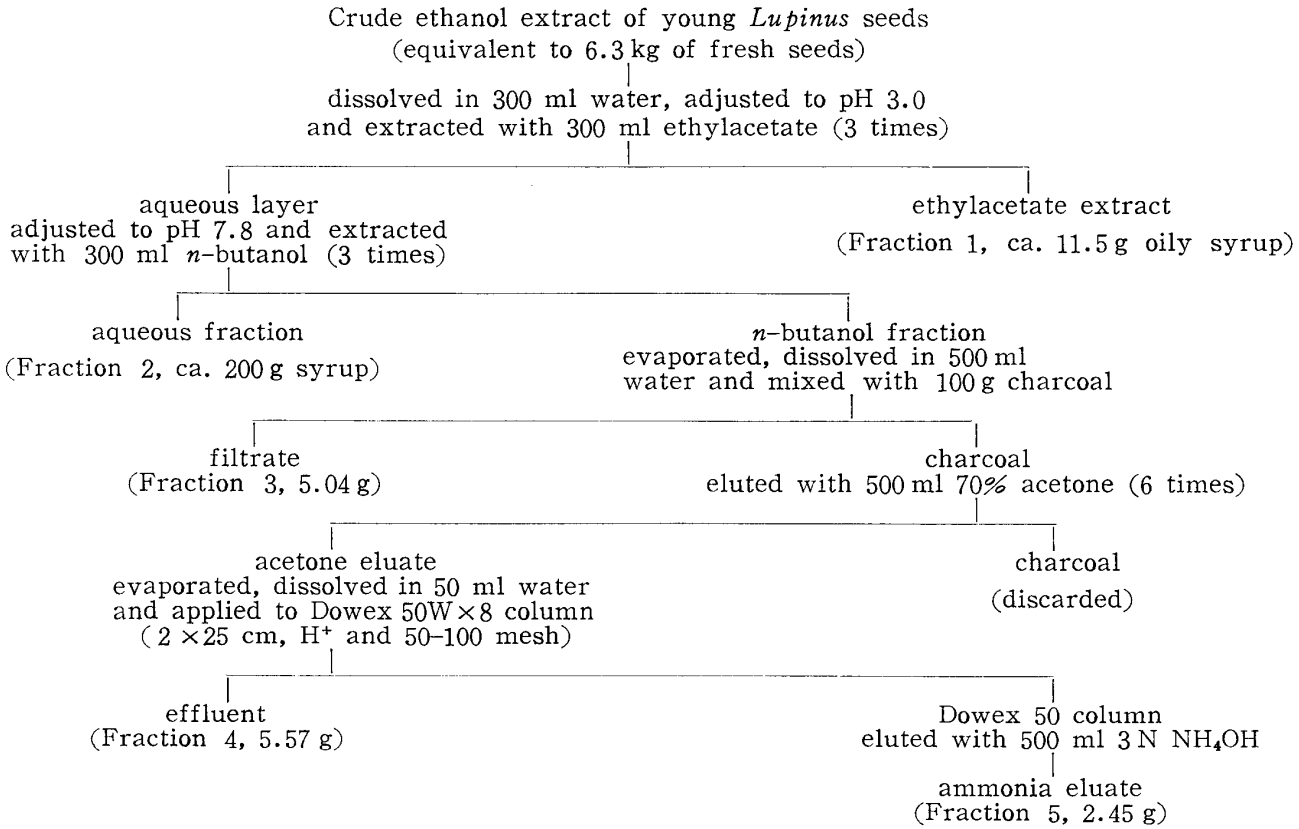

Figure 2. Fractionation of the extract of young *Lupinus* seeds.

Table 2. Effect of Fractions 1, 2, 3, 4 and 5 prepared according to Figure 2 on the growth of tobacco callus. For further details see Figure 2 and text.

Additives	Conc. (mg/l)	Fresh weight of tissue (mg)
—	0	22.5
Fraction 1	10^2	31.5
	10^3	10.0
	10^4	3.7
Fraction 2	10^2	193.7
	10^3	891.0
	10^4	332.5
Fraction 3	10	22.5
	10^2	10.5
	10^3	5.0
Fraction 4	10	17.5
	10^2	14.3
	10^3	10.0
Fraction 5	1	87.5
	10	627.0
	10^2	1422.0
Kinetin	3×10^{-2}	379.0

Studies on Fraction 2……It is necessary to know whether cytokinin activity in this fraction is due to dihydrozeatin or other unknown cytokinins. To reveal this, aliquots of Fraction 2 were paper-chromatographed with solvent systems, *n*-butanol : formic acid : water (80 : 15 : 15) (solvent B) and water-saturated *n*-butanol (solvent D), and location of the cytokinin activity was detected with bioassay. As shown in Figure 3 a and b, chromato-

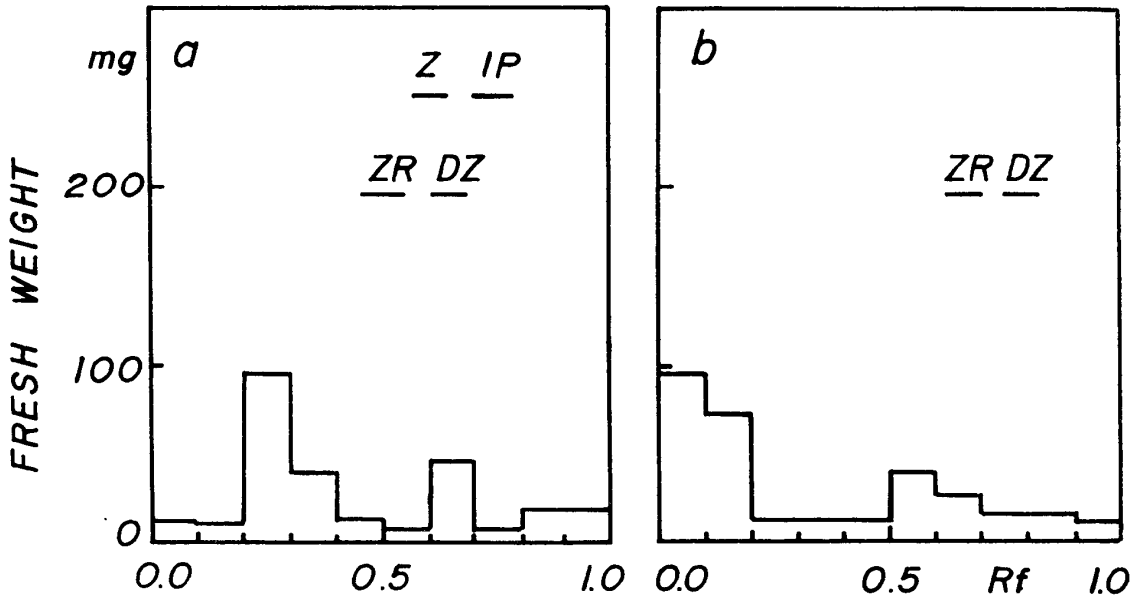

Figure 3. Location of cytokinin activity on paper chromatograms of Fraction 2 (the aqueous fraction) developed with *n*-butanol : formic acid : water (80 : 15 : 15) (a) and water-saturated *n*-butanol (b). Lines at the top of the histograms indicate the loci of zeatin (Z), dihydrozeatin (DZ), zeatin ribonucleoside (ZR) and isopentenyladenine (IP), co-chromatographed.

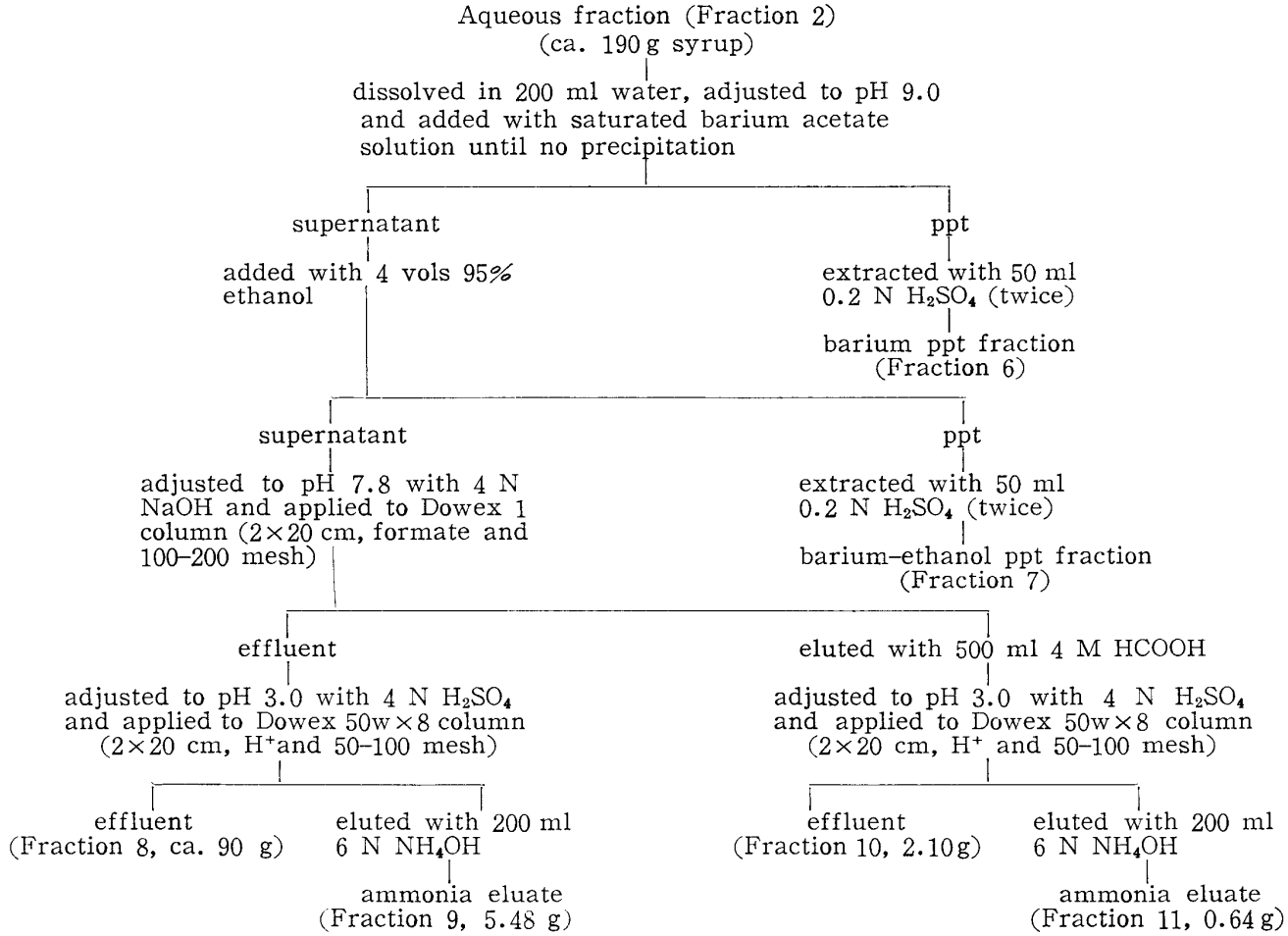

Figure 4. Fractionation of the aqueous fraction (Fraction 2).

grams showed a main active peak at lower Rf than dihydrozeatin and zeatin ribonucleoside co-chromatographed. This result indicates that this unknown cytokinin is more water-soluble than known cytokinins co-chromatographed. Unfortunately ribonucleotides of zeatin and dihydrozeatin were not available, so it is impossible to compare their location on chromatograms. The minor active peak located near dihydrozeatin or zeatin ribonucleoside.

It is possible that the unknown cytokinin in Fraction 2 is ribonucleotide of dihydrozeatin. To reveal this possibility Fraction 2 was fractionated further by precipitations with barium acetate and ethanol, and application to ion exchange resins, Dowex 1 and Dowex 50 W × 8, as shown in Figure 4. Fractions 6-11 were obtained and tested for their cytokinin activity. Result revealed that Fractions 6, 7 and 10 were nearly inactive or inhibitory (Table 3). Fractions 8 and 9 were slightly effective at 100 mg/l. Fraction 11 was the most stimulative. Inactivity of Fractions 6 and 7 suggests that the unknown cytokinin in Fraction 2 is not ribonucleotide of zeatin or

Table 3. Effect of Fractions 6, 7, 8, 9, 10 and 11 prepared as shown in Figure 4 on the growth of tobacco callus. For further details see Figure 4 and text.

Additives	Conc. (mg/l)	Fresh weight of tissue (mg)
—	0	13.5
Fraction 6	10	5.0
	100	7.5
	1000	2.5
Fraction 7	10	8.8
	100	11.3
	1000	7.5
Fraction 8	1	17.1
	10	26.5
	100	60.0
Fraction 9	1	14.0
	10	30.1
	100	160.0
Fraction 10	1	15.2
	10	22.7
	100	25.2
Fraction 11	1	22.6
	10	96.1
	100	699.3

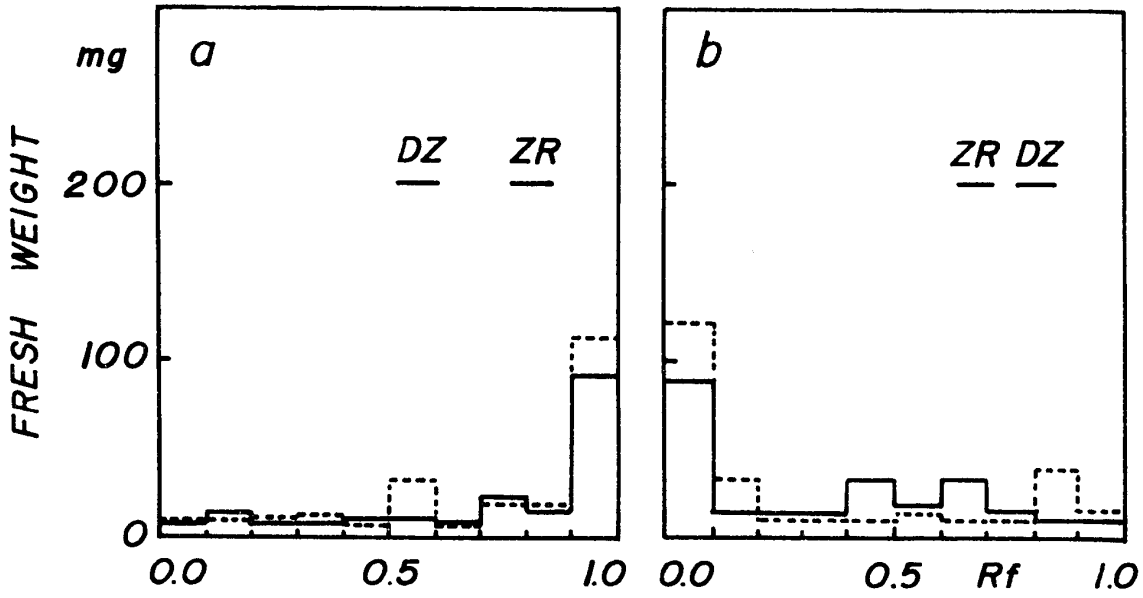

Figure 5. Location of cytokinin activity on paper chromatograms of Fraction 11 (solid line) and its hydrolysate (dotted line). The chromatograms were developed with water (a) and water-saturated *n*-butanol (b). Lines at the top of the histograms indicate the loci of dihydrozeatin (DZ) and zeatin ribonucleoside (ZR), co-chromatographed.

dihydrozeatin, because zeatin ribonucleotide was precipitated by the addition of barium acetate and 4 vols of 95 % ethanol[9].

The most active fraction, Fraction 11, was studied further with paper chromatography. Fraction 11 was streaked on filter paper and developed with *n*-butanol : formic acid : water (80 : 15 : 15). Cytokinin activity was again detected between Rf 0.2 and 0.3, so this zone was cut out and eluted with 60 ml water. Then, the eluate was divided into two parts, one half of which was hydrolyzed in 0.1 N HCl at 100°C for 15 minutes and the other half was kept unhydrolyzed. Both hydrolyzed and unhydrolyzed cytokinins were paper-chromatographed with water (solvent C) and water-saturated *n*-butanol (solvent D) (Figure 5). In chromatograms developed with solvent C, one main active peak was observed at Rf 0.9-1.0, which was different zone from dihydrozeatin and zeatin ribonucleoside. Hydrolysis of this cytokinin did not change the location but increased cytokinin activity. In the case of solvent D too, hydrolysis did not change the Rf and enhanced cytokinin activity. These results support that the unknown cytokinin in Fraction 11 is not dihydrozeatin ribonucleotide, but may be a novel water-soluble cytokinin. Too small amount of Fraction 11 prevented from further study. Larger scale of experiment is necessary.

Studies on Fraction 5……As mentioned in foregoing paragraphs, the crude extract gave two active peaks on paper chromatogram, and the slower-moving cytokinin migrated with synthetic dihydrozeatin. It is, therefore, of interest to investigate whether or not the fast-moving unknown cytokinins are fractionated in Fraction 5. When this fraction (10 mg) was developed with solvent A, only one peak of cytokinin activity was found at Rf 0.7-0.9 (Figure 6), i.e. at the position of the fast-moving cytokinin (cf. Figure 1). Dihydrozeatin ribonucleoside co-chromatographed migrated similarly to this cytokinin. This results indicated that one of the fast-moving cytokinins was contained in Fraction 5, and that dihydrozeatin was removed by adsorption on charcoal treatment (see Figure 2).

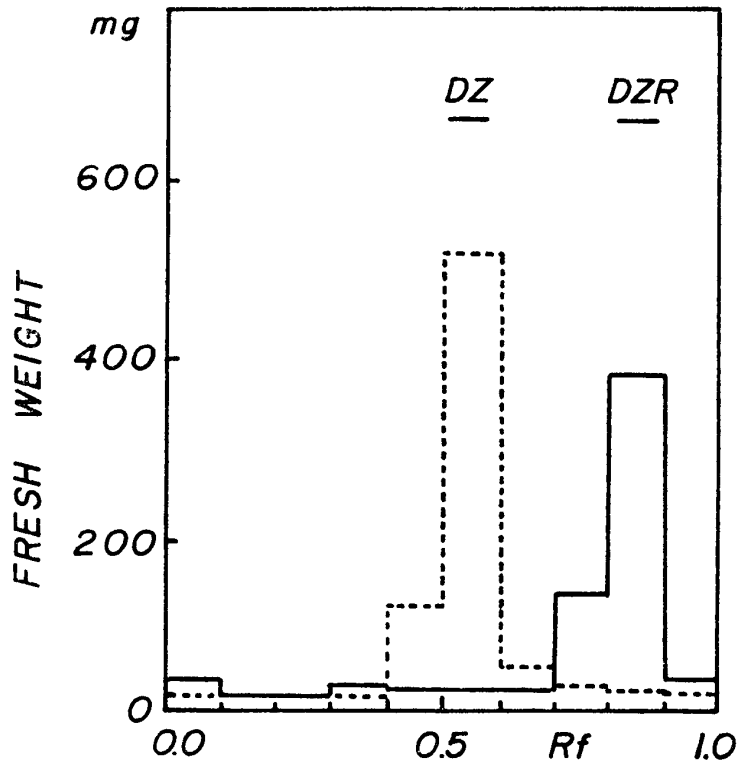

Figure 6. Location of cytokinin activity on paper chromatograms of Fraction 11 (solid line) and hydrolyzed one by heating with Dowex 50 H^+ (dotted line). The chromatograms were developed with 0.02 M boric acid (pH 8.4). Lines at the top of the histograms indicate the loci of dihydrozeatin (DZ) and dihydrozeatin ribonucleoside (DZR), co-chromatographed.

The unknown cytokinin in Fraction 5 could be a riboside of dihydrozeatin or zeatin. To investi-

gate this possibility hydrolysis of the unknown cytokinin was attempted. Fraction 5 (10 mg) was developed first with solvent A, and the active zone between Rf 0.7 and 0.9 was eluted with 80 % ethanol. The eluate was evaporated, dissolved in 3 ml water, and then heated with 2 ml Dowex 50 W×8 ion exchange resin (H^+) in a boiling water for 150 seconds as described by Miller (1965)[9]. After hydrolysis the resin was eluted with 6 N NH_4OH, and the ammonia eluate was rechromatographed with solvent A. Bioassay of the eluates from the chromatogram showed a peak of cytokinin activity at Rf 0.5–0.6, indicating a shift from Rf 0.7–0.9 (Figure 6). Hydrolyzed cytokinin moved closely to dihydrozeatin. when the cytokinin was hydrolyzed in 0.1 N HCl for 15 minutes in a boiling water, essentially identical result was obtained. However, this cytokinin was not hydrolyzed, when incubated with bacterial alkaline phosphatase in 0.01 M $MgCl_2$ and 0.1 M trishydroxymethylaminomethane (pH 8.2) for 8 hours at 32°C as described by Miller (1965)[9]. No shift of the Rf was observed. Thus the unknown cytokinin of the active fraction seems to be a derivative of dihydrozeatin. Too small quantity of the sample used gave no UV absorbing spot on chromatograms. It is, therefore, not clear whether hydrolyzed cytokinin is identical to dihydrozeatin or zeatin, because they generally gave very similar Rf value.

In order to resolve the question raised above, larger amount of Fraction 5 was used in following paper-chromatographic study. Fraction 5 (2 g) was extracted three times with 2 ml *n*-butanol. The *n*-butanol extract was purified twice by paper chromatography with solvent A as described above. The final eluate from UV absorbing spot with cytokinin activity was divided into two parts, one half of which was hydrolyzed by heating with Dowex 50 as described previously and the other half was kept unhydrolyzed. Both unhydrolyzed and hydrolyzed cytokinins were applied to filter papers which were developed with solvent systems A, B, C and D.

In all chromatograms of the unhydrolyzed cytokinin a peak of cytokinin activity accompanied with UV absorbing spot was detected (Figure 7). This spot invariably showed positive color reaction, when water eluate from it was treated with orcine reagent which was used for detection of pentose (Brawn, 1946)[1]. This cytokinin may contain ribose in its molecule. In these chromatograms this cytokinin migrated similarly with dihydrozeatin ribonucleoside, but exhibited a little different Rf from zeatin ribonucleoside in solvent A and C (Figure 7 a and c). The unhydrolyzed cytokinin moved quite differently from N^6-(Δ^2-isopentenyl)adenine and its ribonucleoside (Figure 7 b).

In chromatograms of the hydrolyzed cytokinin, UV absorbing spot was always observed at the peak of cytokinin activity and accompanied with purine-specific color reaction by the treatment with silver nitrate-bromophenol blue reagent (Wood 1955)[12]. Moreover, this hydrolyzed cytokinin was observed at different region from the unhydrolyzed cytokinin (Figure 7). The Rf of the hydrolyzed cytokinin invariably accorded with dihydrozeatin in all solvent systems, but showed a little difference from that of zeatin in solvents A and B. The hydrolyzed cytokinin moved differently from N^6-(Δ^2-isoentenyl)adenine (Figure 7 b) and N^6-(3-hydroxy-3-methylbutylamino)purine which was reported to occur by acid hydrolysis of N^6-(Δ^2-isopentenyl)adenosine (Robins et al. 1967)[10](Figure 7 a and c). Thus

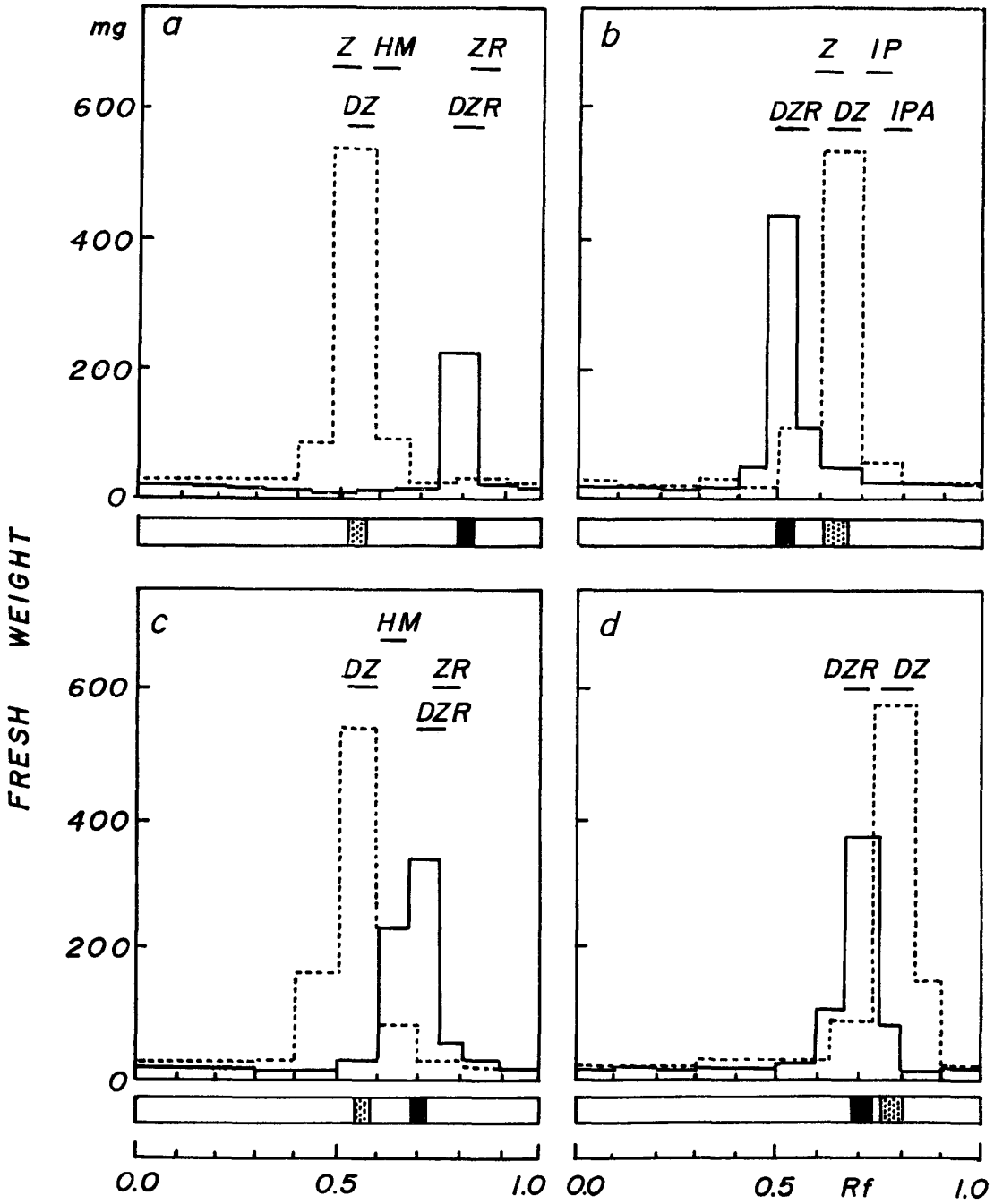

Figure 7. Location of cytokinin activity on paper chromatngrams of unhydrolyzed cytokinin (solid line) and hydrolyzed one (dotted line) purified from Fraction 11. The chromatograms were developed with 0.02 M boric acid (pH 8.4) (a), *n*-butanol : formic acid : water (80 : 15 : 15) (b), water (c) and water-saturated *n*-butanol (d). Lines at the top of the histograms indicate the loci of dihydrozeatin (DZ), dihydrozeatin ribonucleoside (DZR), zeatin (Z), zeatin ribonucleoside (ZR), N^6-(Δ^2-isopentenyl)adenine (IP), N^6-(Δ^2-isopentenyl)adenosine (IPA) and N^6-(3-hydroxy-3-methylbutylamino)purine (HM), co-chromatographed. Locations of UV absorbing spots of unhydrolyzed and hydrolyzed cytokinins are shown below the histograms.

all the data presented above support a conclusion that the unhydrolyzed cytokinin in Fraction 5 is dihydrozeatin ribonucleoside, although chemical identification is not complete.

Discussion

The present study revealed occurrence of dihydrozeatin and some unknown cytokinins in the young *Lupinus* seeds. One of unknown cytokinins was water-soluble, but it was apparently different from ribonucleotide of dihydrozeatin, because this unknown cytokinin was not precipitated by addition of barium acetate and ethanol. Moreover, hydrolysis of this cytokinin in 0.1 N HCl at 100°C for 15 minutes did not produce any dihydrozeatin, zeatin and their ribonucleosides, but water-soluble cytokinin with higher activity. In such hydrolysis condition ribonucleoside and ribonucleotide of dihydrozeatin should be degraded to dihydrozeatin. Therefore, this result suggests the presence of quite different type of cytokinin in young *Lupinus* seeds. Another unknown cytokinin in Fraction 5 was related to dihydrozeatin. This cytokinin contains pentose in its molecule and hydrolysis of it produced an active factor indistinguishable from dihydrozeatin on paper chromatograms. Therefore, it is highly possible that dihydrozeatin ribonucleoside occurs in young *Lupinus* seeds. However, it is still unknown how such native cytokinins occur and how they participate in the processes of the growth of the seed. Matsubara et al. (1966)[6] reported that the young *Lupinus* seeds contained a growth-promoting factor for young embryos besides cytokinins and that cytokinins were rather not essential for the growth of heart-shaped embryos. Cytokinins may be effective to different stages of embryos such as proembryo or different parts in the seed such as endosperm. *In vivo* roles of these cytokinins still remain to be investigated.

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