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CYTOKININ ACTIVITY IN A HOMOLOGOUS SERIES OF ω-HYDROXYPOLYMETHYLENEAMINOPURINES

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Abstract—A series of ω-hydroxypolymethyleneaminopurines [R·NH·$(CH_2)_n$·OH with n=2, 3, 4, 5 and 6] were synthesized. Their cytokinin activities were compared with those of kinetin and zeatin in the young wheat coleoptile, the wheat leaf senescence and tobacco pith tests. The member of the series having the five carbon side-chain was the most active, but all of them were less active than kinetin or zeatin except in the tobacco pith test where some were more active than kinetin.

INTRODUCTION

SINCE the discovery of kinetin (I)[1-3] a large number of analogues have been synthesized and examined, particularly for their ability to promote plant cell division.[4,5] The first natural cytokinin to be isolated was zeatin (II) which was obtained from maize kernels (*Zea mays*) by Letham.[6] Its structure was elucidated[7] and confirmed by synthesis.[8] A cytokinin from immature seeds of *Lupinus luteus* has recently been isolated[9] and its structure established as (−)-dihydrozeatin (III). Cytokinins have also been detected in coconut milk,[10,11] and ribozeatin was subsequently isolated from this source.[12] Attempts have also been made to identify naturally occurring cytokinins in extracts of plum fruitlets,[13] barley,[14] pea seeds,[15] apple fruitlets[16] and crown-gall tumour cells.[17]

Three methods for the bioassay of cytokinins are used at present in this laboratory. These depend upon different physiological processes, as follows:

(*a*) The tobacco pith test[18] which measures a response which involves both cell division and cell enlargement.

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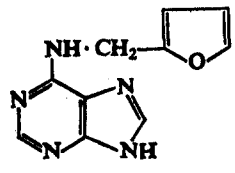
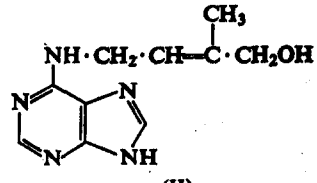
(*b*) The young wheat coleoptile test[5] depending almost entirely on a cell enlargement response.

(*c*) The wheat leaf senescence test which measures the residual chlorophyll in excised leaves after senescence has been allowed to occur for a chosen period.

This study describes the synthesis of a series of ω-hydroxypolymethyleneaminopurines (IV), which appear to be structurally more closely related to zeatin (II) and dihydrozeatin (III) than to kinetin (I). The activities of the compounds have been compared with those of kinetin and zeatin using the three methods given above.



(I)



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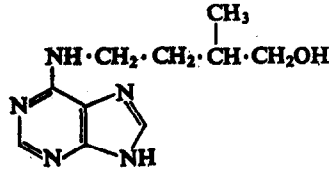

(III)

(IV)

RESULTS AND DISCUSSION

In discussing the results obtained with the ω-hydroxypolymethyleneaminopurines, individual members will be referred to according to the numbers of methylene groups in the side-chain.

Young Coleoptile Test

In this test the order of activity in the series [R·NH·$(CH_2)_n$·OH with $n=2$, 3, 4, 5 and 6] was $C_5 > C_4 > C_6 > C_3 > C_2$. The three compounds C_4 to C_6 showed similar activity, but were not as active as kinetin or zeatin. Indeed, the significant activity shown by zeatin at 10^{-8} M was not shown by any of the other compounds examined (Fig. 1).

The normal alkylaminopurine series [R·NH·$(CH_2)_n$·CH_3 with $n=0$ to 8] were examined by Kuraishi in the radish leaf disk test,[19] a method which, like the young coleoptile test, is based on cell enlargement. The order of activity was $C_6 > C_5 > C_4 > C_3 \gg C_2 > C_1 > C_0$. There was a marked decrease in activity from C_3 to C_2. Thus the normal alkyl series showed similarities to our alkanol series in that the C_4, C_5 and C_6 members were the most active.

The order of activity of the three members of the alkoxyalkyl series [R·NH·$(CH_2)_2$O $(CH_2)_n$·CH_3 with $n=1$, 2 and 3] in the young wheat coleoptile test, was $C_4 > C_5 > C_6$— the C_4 and C_5 members having activity similar to kinetin.[5] Summarizing, in the alkanol and alkoxyalkyl series maximum activity was given by the C_4 and C_5 members, whereas in the

[19] S. KURAISHI, *Sci. Paper Coll. Gen. Educ. Univ. Tokyo* 9, 67 (1959).

alkyl series C_6 was the most active,[19] followed closely by C_5 and C_4. Taking the activity of kinetin as standard, the alkoxyalkyl series were the most active in cell expansion tests, followed by the alkyl and alkanol series.

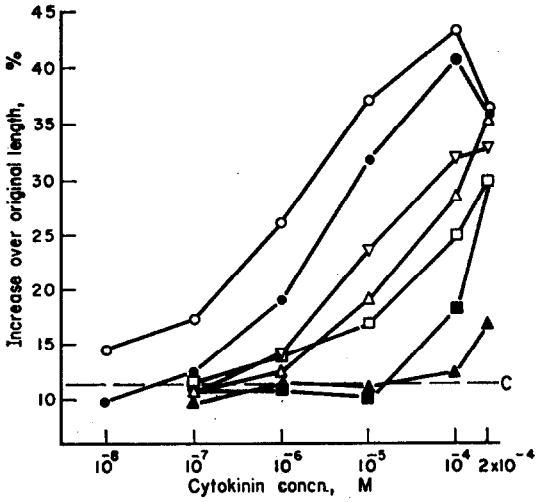

FIG. 1. YOUNG COLEOPTILE TEST. GROWTH RESPONSE OF WHEAT COLEOPTILES TO EXOGENOUS SOLUTIONS OF: ○, ZEATIN; ●, KINETIN; ▲, C_2; ■, C_3; △, C_4; ▽, C_5 AND □, C_6.

Wheat Leaf Senescence Test

In this test the order of activity of the alkanol series was $C_5 > C_4 = C_6 > C_3 > C_2$ (Fig. 2). If one compares this result with that of the same series in the young coleoptile test we see that the order of activity is almost identical. This is surprising when one considers the fundamental difference in the physiological processes involved in the two tests. The young coleoptile test is based essentially on cell expansion, whereas the wheat leaf senescence test

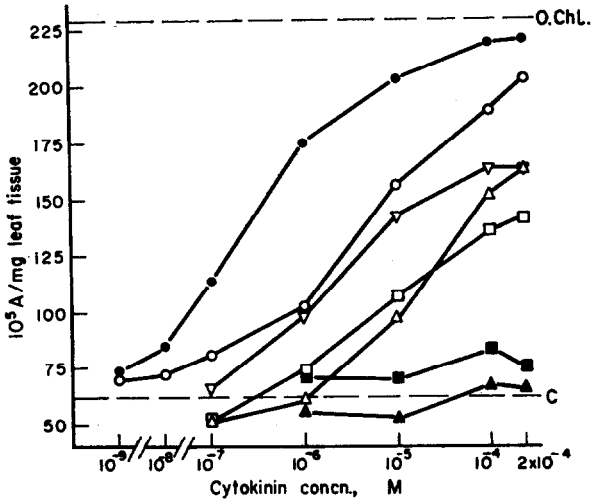

FIG. 2. WHEAT LEAF SENESCENCE TEST. RETENTION OF CHLOROPHYLL INDUCED BY SOLUTIONS OF: ○, ZEATIN; ●, KINETIN; ▲, C_2; ■, C_3; △, C_4; ▽, C_5 AND □, C_6. Original chlorophyll content of leaves = O.Chl.

depends on the ability of cytokinins to restrain the degradation of chlorophyll in detached leaves. As in the tobacco pith test to be discussed later the C_3 and C_2 members of the series had negligible activity (cf. Figs. 2 and 3).

In the wheat leaf senescence test zeatin was noticeably less active than kinetin. This difference may arise from the greater lipophilic property of kinetin facilitating its penetration into the waxy leaf surface of the leaf as it floats on the test solution.

Of the other two series discussed in the young coleoptile test only the alkoxyalkyl series have been tested in leaf senescence. The order of activity of these three members was the same as the alkanol series described above (i.e. $C_5 > C_4 > C_6$). If we again take kinetin as a standard then the alkoxyalkyl series was more active than the alkanol series.

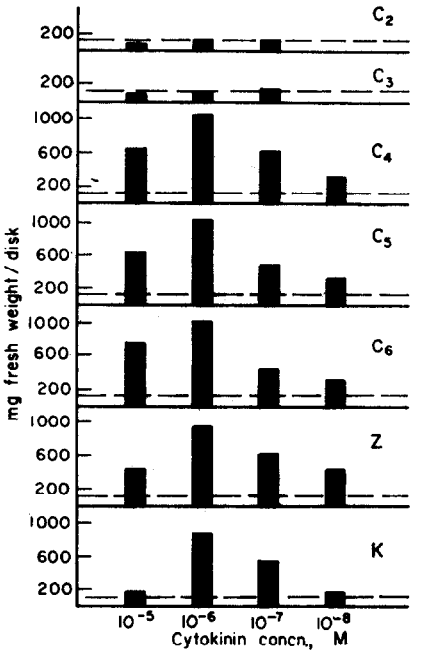

FIG. 3. ACTIVITY OF CYTOKININS IN THE TOBACCO PITH TEST. THE DOTTED LINES REPRESENT THE GROWTH OF THE CONTROL DISKS (Z = ZEATIN, K = KINETIN).

Tobacco Pith Test

In this test, which measures predominately the ability of a cytokinin to stimulate cell division, there was a marked contrast between the activity of C_2 and C_3 members of the alkanol series on the one hand and the C_4, C_5 and C_6 members on the other (Fig. 3). Both C_2 and C_3 were virtually inactive, whereas C_4, C_5 and C_6 showed closely similar high activity which was superior to that of kinetin. In fact in this test zeatin was only marginally more active than the C_4 to C_6 members.

The marked fall off in activity caused by shortening the chain length from C_4 to C_3, suggests that chain length might be more critical in this test. In the young coleoptile test (Fig. 1) there was a gradual fall off in activity with decreasing chain length from C_5 to C_2.

The normal alkyl series (C_nH_{2n} where $n = 3$ to 7) was studied in the tobacco callus test.[4] This test would be expected to give similar results to the tobacco pith test used in this study.

The most active compounds were found to be C_4, C_5 and C_6, although C_3 and C_7 were as active at higher concentrations. The activity of the C_4 to C_6 members was of the same order as that of kinetin. The C_4 and C_5 members like the C_4 to C_6 members in our series were active over a wide concentration range—in this characteristic they resembled zeatin.

Similarly, in the alkoxyalkyl series the C_4 and C_5 were more active than C_6 but all these were less active than kinetin.[5] Thus in these cell division tests if kinetin is taken as a standard then the C_4 to C_6 members of the alkanol series were more active than their corresponding members in the alkyl and alkoxyalkyl series. In all the three series the optimum concentration was in the region of 10^{-6} M.

In this present series of ω-purinealkanols it is of interest that optimum chain length is shown by the member with five methylene groups [—$(CH_2)_5$—] in the side-chain. It is noteworthy that zeatin also has a side-chain with a five carbon skeleton. The zeatin molecule, however, has two further features in its side-chain; (i) a double bond and (ii) a chain that is branched. A series of branched-chain purinealkanols which would hence include dihydrozeatin (III) are obviously worthy of examination.

EXPERIMENTAL

Synthesis of Cytokinins

ω-Hydroxypolymethyleneaminopurines [R·NH·$(CH_2)_n$·OH] with n=2, 3, 4, 5 and 6 were synthesized by condensing 6-chloropurine with the corresponding α-aminoalkan-ω-ol. This procedure was used earlier for preparing kinetin and several of its analogues.[20,21] A detailed description of the preparation of 6-(4′-hydroxytetramethylene)aminopurine is given below, and characteristics of all the homologues are listed in Table 1.

6-(4′-Hydroxytetramethylene)aminopurine

1·0 g 6-chloropurine, 1·8 g 1-aminobutan-4-ol and 7 ml ethylene glycol monoethyl ether were heated at 120° for 3 hr under N_2. After standing at room temperature for 1 hr, the mixture was stored at 0° for 24 hr. The product was filtered, and after washing with ethylene glycol monoethyl ether and dry ether, was dried *in vacuo*. Yield: 1·02 g (75 per cent). Recrystallization from aqueous ethanol gave white crystals (0·81 g), m.p. 191 to 192·5° (sealed tube).

Biological Assay Methods

(i) *Young coleoptile test.* Wheat grains (*Triticum vulgare* var. Eclipse) were soaked for 2 hr in distilled water prior to sowing. Evenly sized grains were sown with embryos uppermost on moist filter paper (Whatman No. 1) in 9-cm Petri dishes and allowed to germinate in darkness for 24 hr in a humidity box (90 per cent relative humidity) at 23·5°. Using a low-power binocular microscope, coleoptiles of uniform size were excised squarely just above the epiblast[5] and floated in a dish of shallow distilled water until a sufficient number had been prepared for the experiment. Most of the water was decanted and, with the aid of a "camel-hair" brush, the coleoptiles were then transferred to a microscope slide in groups of five and their lengths measured under a binocular microscope. Each group was placed on a 5 cm × 2·5 cm strip of Whatman No. 1 filter paper which was inserted into a 7·5 cm × 1·2 cm test-tube containing 0·6 ml of the test solution. Five replicates were used per treatment. The tubes sealed with "Parafilm" were rotated at 1 rev/min on a klinostat in darkness at 26° for 24 hr and the length of the five coleoptiles remeasured. The percentage increase in mean length of the coleoptiles was calculated for each replicate (i.e. percentage increase over original length). The cytokinins were tested within the concentration range 10^{-3} M to 10^{-8} M, and the results are given in Fig. 1.

(ii) *Wheat leaf senescence test.* Grains of Eclipse wheat previously soaked for 2 hr in distilled water were sown with their embryos uppermost on moist vermiculite (No. 4 grade) contained in plastic trays. The grains were sown in rows 1·5 cm × 1·5 cm and then covered with a 1 cm layer of vermiculite which was lightly compressed. After watering, the trays were placed in a growth room. The plants were grown under a 15 hr photoperiod (500 lumens/ft² from fluorescent tubes giving "natural daylight") at 23° for 7 days. The shoots, approximately 11 cm tall, were excised at soil level and lined up in groups of fifteen on a cutting board with their tips against a wooden guide, and the shoots severed 7·5 cm from their tips. This portion of the shoot comprised mainly the first leaf and portions of other leaves clasped within it were discarded.

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[21] M. W. BULLOCK, J. J. HAND and E. L. R. STOKSTAD, *J. Am. Chem. Soc.* **78**, 3693 (1956).

Table 1

Compound	Code name	Yield (%)	M.P.° (sealed tube)	Analyses: Found (%) C	H	N	Required (%) C	H	N
6-(2′-Hydroxyethylene)aminopurine	2	86	254–255	46·81	5·23	39·17	46·91	5·06	39·08
6-(3′-Hydroxytrimethylene)aminopurine	3	80	223–224	49·95	5·87	36·39	49·73	5·74	36·25
6-(4′-Hydroxytetramethylene)aminopurine	4	75	191–192·5	52·47	6·50	33·67	52·17	6·32	33·80
6-(5′-Hydroxypentamethylene)aminopurine	5	77	178–179	54·41	6·92	31·53	54·27	6·83	31·65
6-(6′-Hydroxyhexamethylene)aminopurine	6	78	170–172	56·16	7·13	29·71	56·14	7·28	29·77
6-Furfurylaminopurine (Kinetin)	K	—	267–268						
6-(4′Hydroxy-3′-methylbut-2′-enylene)aminopurine (Zeatin)	Z	—	207–208						

The leaves were weighed in groups of three, and each group was floated, with the adaxial surfaces of the leaves uppermost, on 10 ml of test solution contained in a 10 cm Petri dish. The Petri dishes were maintained at 25° in darkness for 4 days. The leaves from each dish were dried by blotting them with filter paper and extracted with 10 ml of 80 per cent ethanol in test-tubes immersed in a water bath at 80° for 10 min. During this process the tubes were sealed with glass marbles to restrict evaporation. The tubes were removed from the water bath, cooled in the dark, and the absorptivity of each solution measured at 645 nm. Three replicates of each treatment were carried out and the results (Fig. 2) expressed as absorptivity A per mg of leaf. The cytokinins were tested within the concentration range 10^{-3} M to 10^{-9} M.

(iii) *Tobacco pith test.* Fresh pith from tobacco stems (*Nicotiana tabacum*, var. Wisconsin 38) was used throughout this study. The stems were cut from tobacco plants grown in a greenhouse when the first flowers were beginning to open. Only the central 40 cm section of the stem was retained; this was stripped of leaves and scrubbed with soap and water. The washed stems were immersed in NaOCl solution (10–14 per cent, w/v, available Cl_2) and transferred to a sterile room. After 5 min the stems were removed and rinsed in sterile distilled water. Cylinders of pith were obtained from internodal pieces (*ca.* 5 cm long) with a cork borer (6 mm dia.). Each pith cylinder was immediately sliced with a sterilized scalpel into disks approximately 2 mm thick and three disks were placed in each Petri dish with one of their flat surfaces in contact with the agar medium, which included 2 mg/l IAA and was made as described by Murashige and Skoog (1962).[18] The Petri dishes were transferred to a glass-fronted incubator at 25° where they were exposed to indirect light during the day-time. The humidity inside the incubator was kept high by a Pyrex dish full of water. The position of each Petri dish was changed every other day when the dishes were taken out to examine them for contamination. After 3 weeks the pieces of pith were weighed. Cytokinins giving the greatest weight increase at the lowest concentrations were considered to be the most active. The results are given in Fig. 3.

The medium was prepared with all the ingredients except IAA, and brought to the boil to dissolve the agar. While still hot, IAA crystals were stirred in and when these had dissolved the agar medium was filtered through a Green's "Hyduro" 904, 32 cm filter paper. Any loss in volume due to evaporation was corrected at this stage and the agar dispensed in 20-ml aliquots into test tubes. The cytokinins to be tested were pipetted (1 ml) on top of the agar—the control tubes receiving distilled water. After plugging with cotton wool the test tubes were autoclaved at 120° for 15 min. The nutrient agar was poured into sterile plastic Petri dishes (9 cm dia.) and left to solidify. There were six replicates for each treatment and usually four concentrations of each cytokinin, within the concentration range 10^{-5} to 10^{-8} M.

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