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COMPARISON OF CYTOKININ ACTIVITIES OF THE BASE, RIBONUCLEOSIDE AND 5′- AND CYCLIC-3′,5′-MONOPHOSPHATE RIBONUCLEOTIDES OF N^6-ISOPENTENYL-, N^6-BENZYL- OR 8-BROMO-ADENINE*

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Abstract—The cytokinin activities of adenosine 3′,5′-monophosphate, N^6,$O^{2'}$-dibutyryladenosine 3′,5′-monophosphate, 8-bromoadenosine 3′,5′-monophosphate, N^6-(Δ^2-isopentenyl)adenosine 3′,5′-monophosphate, and N^6-benzyladenosine 3′,5′-monophosphate were determined in the tobacco bioassay and compared with the activities of the corresponding non-cyclic nucleotides, nucleosides and bases of the N^6-isopentenyl-substituted, N^6-benzyl-substituted, 8-bromo-substituted, and unsubstituted adenine series. In each of these series the cytokinin activities in decreasing order were: bases $\gg$ nucleosides $\geq$ nucleotides $>$ cyclic nucleotides. All members of the N^6-isopentenyl-substituted and N^6-benzyl-substituted series were highly active cytokinins, reaching maximum activity at concentrations of 1 μM or less, whereas, as expected, all members of the unmodified adenine series were inactive in the tested concentration ranges of up to 180 and 200 μM for adenosine and adenine, and 40 μM for the adenine nucleotides. Members of the 8-bromo-substituted adenine series were much weaker cytokinins than the N^6-substituted adenine derivatives but showed activity in the same sequence starting at a concentration of about 5 μM. Thus, in the cases of 8-bromoadenosine 3′,5′-monophosphate and N^6,$O^{2'}$-dibutyryl-adenosine 3′,5′-monophosphate, both of which have been reported to promote cell division and growth of plant tissues, the cytokinin activity is related to the 8-bromo substituent and to the N^6-butyryl substituent, respectively, rather than to the 3′,5′-cyclic monophosphate moiety.

INTRODUCTION†

Correlations found between cell division and cAMP† levels in animal tissues [1,2] raise the question of a possible similar relationship in plant tissues. Reports of the occurrence of cAMP in plant tissues have been based on a variety of assays including bioluminescence and protein kinase assays [3], co-chromatography in a variety of solvent systems and electrophoretic buffers [4], and cAMP binding assays [5–7]. A severe critique of the methodology and doubts of the validity of the conclusions of most if not all the above reports have been presented [8]. Similar criticism applies to the interpretations of several studies

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† See Table 1 for abbreviations used in this article.

which imply a functional role for cAMP in plants comparable to its role as a second messenger in animals [9], for example, reports that exogenous IAA increases the synthesis of cAMP [10–13] and that exogenous cAMP alone and in combination with GA_3 induces dark germination of lettuce seed [14] and the formation of the enzymes α-amylase [15, 16] and isocitrate lyase [17]. It appears, therefore, that to date no unequivocal evidence for the presence and function of cAMP in higher plants has been presented.

If indeed cAMP does have a functional role in plants, a relationship between cAMP levels and cytokinin activity might be expected since native cytokinins which are N^6-substituted adenine derivatives occur also in the ribonucleoside and ribonucleotide forms [18–20]. Furthermore, one of the products of short term metabolism of the unnatural cytokinin bzl^{6}Ade is the corresponding 5′-ribonucleotide, bzl^{6}AMP [21, 22]. The ribonucleotides of zeatin [23], bzl^{6}AMP [24] and kinetin [25] have been reported to be less active than the corresponding bases, but cytokinin activities have not to our knowledge been compared for complete series of derivatives including base, ribonucleoside, and the two monophosphate nucleotides. We report here the cytokinin activities of three series, having Br^{8}Ade, bzl^{6}Ade, and the naturally-occurring i^{6}Ade, respectively, as the parent compounds. Of these, the last two series are complete, while the first lacks the non-cyclic monophosphate nucleotide (Table 1).

Table 1. Compounds tested for cytokinin activity

Chemical	Abbreviation
Adenine	Ade
Adenosine	Ado
Adenosine 5′-monophosphate	AMP
Adenosine 3′,5′-monophosphate	cAMP
8-Bromoadenine	Br^{8}Ade
8-Bromoadenosine	Br^{8}Ado
8-Bromoadenosine 3′,5′-monophosphate	Br^{8}cAMP
N^6,$O^{2'}$-Dibutyryladenosine 3′,5′-monophosphate	diBcAMP
N^6-(Δ^2-Isopentenyl)adenine	i^{6}Ade
N^6-(Δ^2-Isopentenyl)adenosine	i^{6}Ado
N^6-(Δ^2-Isopentenyl)adenosine 5′-monophosphate	i^{6}AMP
N^6-(Δ^2-Isopentenyl)adenosine 3′,5′-monophosphate	i^{6}cAMP
N^6-Benzyladenine	bzl^{6}Ade
N^6-Benzyladenosine	bzl^{6}Ado
N^6-Benzyladenosine 5′-monophosphate	bzl^{6}AMP
N^6-Benzyladenosine 3′,5′-monophosphate	bzl^{6}cAMP

RESULTS AND DISCUSSION

Chemistry

The methods used for the synthesis of N^6-substituted analogs of cyclic AMP have included the following: (a) treatment of 6-chloro-2′,3′-*O*-isopropylidene-9-β-D-ribofuranosylpurine with an appropriate amine leads to the formation of the 6-substituted protected nucleoside, which is then phosphorylated, deblocked, and cyclized [26–28]; (b) cAMP is deaminated to cIMP, which is then treated with phosphoryl chloride to yield 6-chloro-9-β-D-ribofuranosylpurine 3′,5′-monophosphate, and this in turn is treated with the appropriate amine to give an N^6-substituted analog of cAMP [29, 30]; (c) alkylation of 5′-AMP followed by a Dimroth rearrangement to the N^6-substituted adenylic acids [31–33] can in turn be followed by phosphate cyclization [27, 28].

In following the last route we were able to effect the synthesis of N^6-isopentenyl AMP and N^6-benzyl AMP under conditions which gave improved yields (see Experimental section). We used CM-Sephadex C-25 in the 4-morpholino-*N*,*N*′-dicyclohexylcarboxamidinium form as a facile method of preparing the MDCHC$^+$ salt of the nucleotide necessary for cyclization. We also found that the direct alkylation of cAMP with the appropriate halide in dimethyl sulfoxide [34] at room temperature followed by Dimroth rearrangement gave i^{6}cAMP and bzl^{6}cAMP in yields of 31 and 51%, respectively. The various nucleotides were readily isolated and purified by chromatography on DEAE-Sephadex A-25 and were fully characterized as microanalytically pure species.

The anomeric protons of both i^{6}cAMP and bzl^{6}cAMP were apparent singlets in the 60 MHz pmr spectra as had been observed in the case of cAMP [35, 36], whereas the spectra of the corresponding 5′-nucleotides showed the anomeric proton to be a clearly resolved doublet. The cyclic phosphates had electrophoretic mobilities at pH 8·5 similar to cAMP, considerably different from those of the 5′-nucleotides, and underwent the same series of color reactions with periodate-benzidine spray as did cAMP. The modified cyclic phosphates i^{6}cAMP and bzl^{6}cAMP were resistant

to bacterial alkaline phosphatase. i^{6}cAMP has been shown to be a competitive inhibitor, as are i^{6}Ado and i^{6}Ade, of the high K_m beef heart cyclic AMP phosphodiesterase [37].

It will be appreciated that the alkylative-rearrangement procedure here described can be applied generally for the conversion of cAMP to many different N^6-substituted derivatives by the use of benzylic, allylic and propargylic halides. These N^6-substituted cyçlic 3′,5′-phosphates have assumed additional importance with the report of their inhibition of the growth of certain cancer cells [27] and their high lipolytic activity [29, 38].

Cytokinin activity

The cytokinin activities of i^{6}Ade, its riboside, 5′-monophosphate nucleotide, and 3′,5′-cyclic monophosphate nucleotide are compared in a typical test of serial concentrations in the tobacco assay in Fig. 1, as are the activities of Br^{8}Ade, Br^{8}Ado, Br^{8}cAMP and diBcAMP. Also included for comparison are the activities of Ade, Ado, 5′-AMP and cAMP. It may be seen that in the i^{6}Ade series the base is clearly more active than the ribonucleoside and ribonucleotides; the same relationships hold for the members of the bzl^{6}Ade series as shown in Fig. 2. Within these series the cyclic mononucleotides are consistently slightly less active than the 5′-mononucleotides.

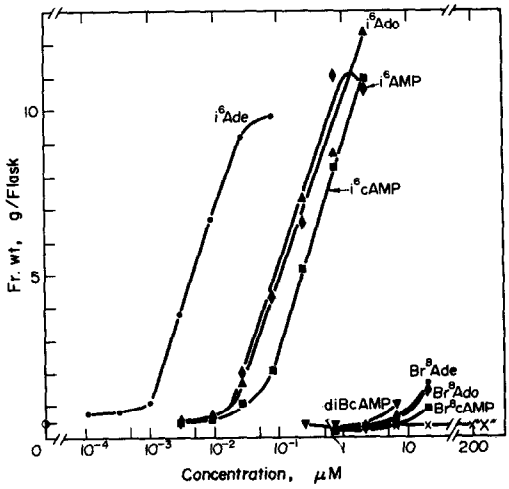


Fig. 1. Comparison of the cytokinin activities of Ade, i^{6}Ade, their respective ribosides, ribotides, and cyclic ribotides and of Br^{8}Ade, Br^{8}Ado, Br^{8}cAMP, and diBcAMP in the tobacco bioassay. Ade, Ado, 5′-AMP and 3′,5′-cAMP, which were inactive in concentrations up to 200, 180, 40 and 40 μM, respectively, are represented by curve "X". Data from Experiment C223, 19 April to 25 May 1973, except for diBcAMP data which are from Experiment C141, 29 April to 2 June 1971.

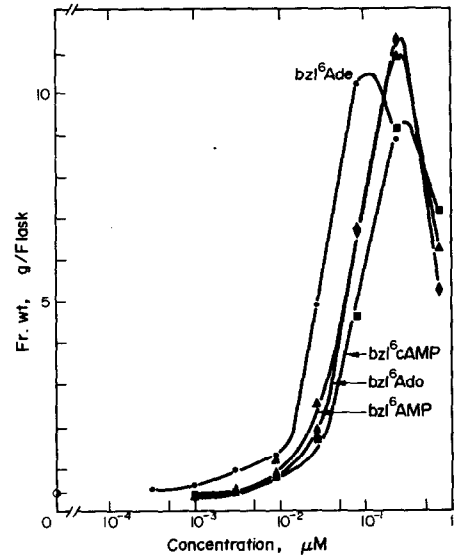
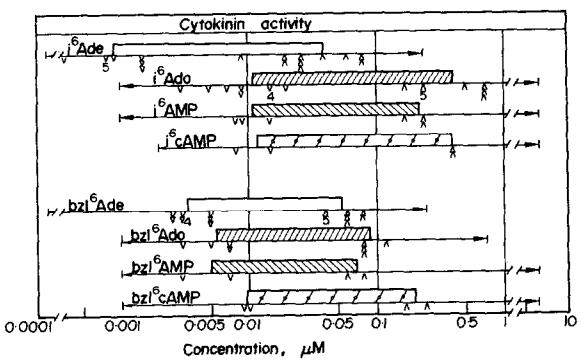


Fig. 2. Cytokinin activities of bzl^{6}Ade, its riboside, ribotide, and cyclic ribotide in the tobacco assay. Data from Experiment C228, 7 June to 12 July 1973.

The same sequence of relative activities as indicated in the individual tests is also evident in the summary of all tests of these compounds (Figs. 3 and 4).

Comparisons of the active concentration ranges show that all members of the i^{6}Ade and bzl^{6}Ade series reached maximum activity at concentrations from 0·1 to 1 μM, while diBcAMP and members of the Br^{8}Ade series only started



□ Base; ▨ nucleoside; ▧ nucleotide; ▩ Cyclic nucleotide.

Fig. 3. Summary of the cytokinin activities of i^{6}Ade, bzl^6 Ade, and their respective ribosides, ribotides, and cyclic ribotides. The base lines represent the tested concentration range for each compound and the bars represent the mean range over which growth increased as a nearly linear function of the log of concentration. Arrows indicate the start and end points of the linear ranges for individual experiments.

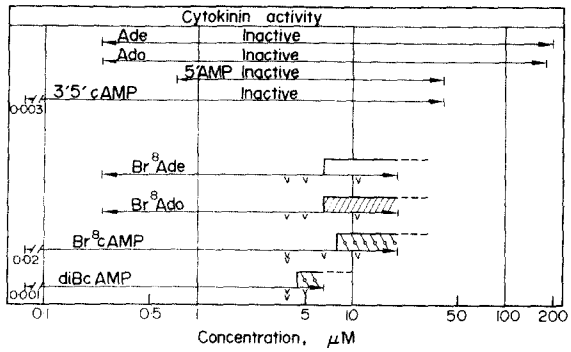


Fig. 4. Summary of cytokinin activities found for Ade, Ado, 5′-AMP and 3′,5′-cAMP; for the 8-bromo derivatives of Ade, Ado and cAMP; and for diBcAMP. Explanation of the bars as given with Fig. 3. Open-ended bars indicate that the maximum growth was not obtained at the highest tested concentration.

to become active at *ca* 5 μM and thus presumably required at least a 50 μM concentration for maximum activity. Compounds Br^8Ade and Br^8Ado are indistinguishable in activity for concentrations up to 20 μM, the low end of their activity ranges, but their low solubility precluded testing at higher concentrations. The compounds substituted at neither N^6 nor C-8, namely, Ade, Ado, 5′-AMP and cAMP, were inactive when tested in concentrations up to 200, 180, 40 and 40 μM, respectively. It should be noted that adenine was known from earlier studies to be slightly active in the 200–800 μM range and that adenosine showed barely detectable activity at these concentrations.

The cytokinin activity of the Br^8Ade series is of particular interest since the cytokinin activity of certain N^6-substituted adenines is enhanced by a methyl group in the 8-position. Specifically, 8-methylkinetin and 8-Me-bzl^{6}Ade were found to be more active than unsubstituted kinetin and bzl^{6}Ade in a senescence test [39]. 8-Me-i^{6}Ade was more active than i^{6}Ade in the tobacco bioassay, and an 8-Cl substituent did not significantly alter the activity of i^{6}Ade [40]. These results are in conflict with the proposal by Wood and Braun [41] that the cell division activity of 8-bromoadenosine 5′-monophosphate may be due to the ability of tobacco pith cells to convert it to 8-bromoadenosine 3′,5′-monophosphate. In fact, the cyclic ribose phosphate moiety depresses rather than enhances cytokinin activity in the Br^8Ade series (Figs. 1 and 4) as in the other series of compounds we have tested. It would be helpful to know whether Br^8Ade, which has the higher cytokinin activity, also has an equally high or higher activity than Br^8cAMP in stimulating protein kinases and inhibiting phosphodiesterases [42]. In this connection, it is of interest that N^6-substituted derivatives of adenine have marked effects on cultured animal cells [43]. In tests with fibroblasts, zeatin was found to induce elongation, decrease cell motion, and increase adhesion to the substratum. It should be noted that while these effects were similar to those brought about by an inhibitor of cyclic nucleotide phosphodiesterase and while the N^6-substituted adenine derivatives did inhibit phosphodiesterase in cell homogenates, the adenine derivatives did not increase cAMP levels in intact cells, and their action appeared to be independent of intracellular levels of cAMP. It has also been shown that in the case of diBcAMP, a derivative of cAMP frequently used in animal studies, it is the N^6-monobutyryl group which confers activity in the promotion of protein kinase and inhibition of phosphodiesterase [44]. With regard to plant tissues, we have confirmed reports that an acyl group on N^6-substituent of adenine derivatives does not in all cases destroy cytokinin activity [45–47].

Regardless of whether or not cAMP may exert growth regulatory functions in plants, our results confirm and extend earlier evidence that cytokinins do not act as substitutes for cAMP, nor is it likely that they function in growth merely by influencing the level of cAMP. This study provides further evidence that the biological activity of substituted adenine nucleotides may involve both cytokinin functions associated particularly with the N^6-substituents and/or the 8-substituents and other biological functions associated more specifically with the ribose monophosphate moiety. These two functions must be considered as distinct from one another both in plant and in animal systems.

EXPERIMENTAL

Bioassay procedure. Cytokinin activities of the test compounds were determined by the tobacco bioassay as described by Linsmaier and Skoog [48]. The media contained the mineral salts (specified in Table 6, part A, of Ref. [48]). The following organic substances were added: sucrose, 30 g/l.; Difco agar, 10 g/l; *myo*-inositol, 560 μM; indole-3-acetic acid, 11·4 μM; and thiamine HCl-ide, 1·2 μM. For reasons of solubility and to avoid possible degradation by heat, the com-

pounds were dissolved in dimethylsulfoxide, a series of 3-fold dilutions made, and aliquots added to the cooling, autoclaved agar media. The final concn of dimethylsulfoxide did not exceed 0·05% by vol., a concn which does not affect biological activity in this assay [49].

Synthesis of N^6*-substituted adenine nucleotides and cyclic nucleotides.* The lithium or triethylammonium salt of the nucleotide (3 mmol) was stirred with a 2- to 3-fold excess of the appropriate bromide in 10 ml of $(Me)_2SO$ at room temp., and the course of the reaction was followed by cellulose TLC in EtOH/1 M NH_4OAc (7:3) or 1-propanol–NH_3–H_2O (11:2:7). In the alkylation of cAMP with isopentenyl bromide it was necessary to add 0·5 ml of triethylamine. After 1 to 5 days the N^1-substituted nucleotide was precipitated in Me_2CO and was dissolved in dil. NH_4OH (unless the N^1-substituted compound was desired). After heating on the H_2O bath for 3 hr the N^6-substituted ribonucleotide was isolated by chromatography on DEAE-Sephadex A-25 with a linear gradient of triethylammonium-HCO_3 buffer.

Cyclization of N^6*-substituted AMP's.* The N^6-substituted AMP was converted to the 4-morpholino-*N*,*N*′-dicyclohexylcarboxamidinium ($MDCHC^+$) (28) salt by passage through a 5-fold excess of CM-Sephadex C-25, $MDCHC^+$ form, that had been prepared by treatment of the H^+ form of the ion exchanger with MDCHC in MeOH. The compounds were then cyclized by the method of Smith *et al.* [28].

N^6*-Isopentenyladenosine 5′-phosphate, mono MDCHC*$^+$ *salt*: Yield, ~50%; UV (H_2O) λ_{max} (pH 7) 267 nm, λ_{min} 231 nm (Found C, 53·85; H, 7·73; N, 15·45. $C_{15}H_{21}N_5PO_7 . C_{17}H_{32}N_3O$ (mono $MDCHC^+$ salt) requires: C, 54·23; H, 7·54; N, 15·81%). N^6*-Benzyladenosine 5′-phosphate, dilithium salt*: Yield, 46%, UV (H_2O) λ_{max} (pH 7) 269 nm, λ_{min} 231 nm (Found: C, 45·19; H, 4·56; N, 15·36. $C_{17}H_{18}N_5PO_7Li_2 . H_2O$ requires C, 45·24; H, 4·47; N, 15·25%). N^6*-Isopentenyladenosine 3′,5′-monophosphate* (*i*^{6}*cAMP*): Yield by alkylation of cAMP, 31%; yield from N^6-isopentenyl AMP by cyclization, 40%; UV (H_2O) λ_{max} (pH 7) 267 nm, λ_{min} 231 nm (Found: C, 48·62; H, 7·54; N, 16·26. $C_{17}H_{19}N_5PO_6 . C_6H_{16}N . H_2O$ (triethylammonium salt mono H_2O) requires. C, 48·83; H, 7·22; N, 16·27%). N^6*-Benzyladenosine 3′,5′-monophosphate* (*bzl*^{6}*cAMP*): Yield by alkylation of cAMP, 51%; yield from N^6-benzyl AMP by cyclization, 68%; UV (H_2O) λ_{max} (pH 7) 269 nm, λ_{min} 231 nm (Found: C, 43·42; H, 4·47; N, 14·37. $C_{17}H_{17}N_5PO_6Li . 2·5H_2O$ requires C, 43·41; H, 4·72; N, 14·89%). Neither cyclic phosphate was affected by *E. coli* alkaline phosphatase.

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