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CYTOKININS: STRUCTURE/ACTIVITY RELATIONSHIPS*

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Abstract—Sixty-nine compounds, mostly purine derivatives and closely related substances, were tested for promotion of growth and regulation of organ formation in the tobacco bioassay to determine relationships between chemical structure and cytokinin activity. Forty-three substances were synthesized in this study, and 13 of these were reported for the first time. N^6-Alkyladenines (I) varied in activity over a wide concentration range depending on the length of the alkyl chain. Starting with adenine, detectable at $\geqslant 200$ μM, activity increased with the chain length to an optimum for 6-pentylaminopurine detectable at *ca.* 0·001 μM, and then decreased to reach a barely detectable level for 6-decylaminopurine. The result of the incorporation of polar groups in the side chain was not necessarily reduction in activity. One hydroxyl group, as in zeatin (Id), improved the activity of 6-(γ,γ-dimethylallylamino)purine (Ib) if it affected it at all; two hydroxyl groups, as in 6-(2,3-dihydroxy-3-methylbutylamino)purine strongly reduced activity. Comparisons of 6-isoamylaminopurine with 6-(γ,γ-dimethylallylamino)purine and of other closely related pairs of compounds showed that a double bond in the side chain greatly increased cytokinin activity. Adenine derivatives with cyclic substituents in the N^6-position (benzyl-(Ic), cyclohexyl-, etc.) showed the same general range of activity, potentiation by unsaturation, and variation in activity with substituent size, etc. as did the alkyl derivatives. Heteroatoms in or on the substituent groups decreased activity (in the case of N or Cl) or had little effect (S for O in furfuryl). Of the mono-substituted adenines only the N^6-derivatives definitely possessed cytokinin activity. The 1-(III), 3-(II), or 9-substituted adenines probably are inactive but could be activated by conversion to the N^6-isomers. Except for slight activity in tests of high concentrations, which could be ascribed to contaminants, 7-substituted adenines were completely inactive. Modification in the adenine moiety lowered the cytokinin activity, often by 95 per cent or more. Substitution of N for the 8-C atom in kinetin and in 6-benzylaminopurine or S for the 6-amino N atom in 6-(γ,γ-dimethylallylamino)purine did not eliminate but drastically reduced activity in the tobacco bioassay. Elimination of the 6-amino group without substituting another group completely removed activity; thus, the purine derivatives, 1-benzylpurine and 1-(γ,γ-dimethylallyl)purine, were inactive in tests where the 1-adenine derivatives could be activated to give a positive response. Addition of a second substituent on the 1- or 3-position of N^6-substituted adenines drastically reduced or eliminated cytokinin activity. It is suggested that the 1-position and possibly also the 3-position must be free. A second substituent in the N^6-, 7-, or 9-position of N^6-substituted adenine derivatives lowered but did not eliminate activity. Also, the disubstituted 1-adenine derivatives, 1,9-dibenzyladenine and 1,7-dibenzyladenine were active, presumably after rearrangement to the corresponding N^6-substituted isomers.

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INTRODUCTION

FOLLOWING the isolation and identification of kinetin (Ia)[1] a large number of chemically related substances have been synthesized and/or tested for cytokinin activity by various procedures. This is a report of collaborative work between the laboratories of N. J. Leonard, Department of Chemistry and Chemical Engineering, University of Illinois and F. Skoog, Department of Botany, University of Wisconsin, to determine relationships between the chemical structure of cytokinins and their biological activity as measured by the promotion of growth and regulation of organ formation in tobacco callus cultures. The early work at the University of Wisconsin, summarized by Strong,[2] indicated that cytokinin activity in the tobacco test is limited to 6-substituted purines. This was confirmed in general by Okumura and co-workers, who utilized the expansion of radish leaf discs to test a large number of purine derivatives and related substances.[3,4] Assays based on the capacity to induce germination of lettuce seed, as in reports by Skinner and Shive,[5] have indicated less stringent structural requirements of the biologically active molecules, and the results obtained are not entirely in agreement with those of the above growth tests. However, it would appear that the seed germination test has such marked dependence on conditions[6,7] (high sensitivity to temperature, light and chemical agents other than cytokinins) that positive results obtained by this method perhaps may be considered only as preliminary evidence of cytokinin activity. The response of the radish leaf test also is highly dependent on light conditions and on the age of the leaves. Furthermore, both these tests are based on measurements of cell expansion regardless of whether or not the increase in size is accompanied by cell division. Growth responses, therefore, are elicited also by agents which are not cytokinins.

Okumura and Kuraishi found a significant response to kinetin at 0·1 μM and optimal activity at about 10 μM. In the tobacco assay a barely detectable growth increase ordinarily is obtainable with 5×10^{-4}–5×10^{-3} μM and maximum tissue yields with 0·1 μM of kinetin; i.e. with reference to concentration, the tobacco callus test as now used is about 100 times more sensitive than the radish leaf disc assay. For the above reasons a number of substances with known cytokinin activity as well as new synthetic substances were used in comparative tests of structure/activity relationships in the tobacco bioassay. This study almost exclusively deals with cytokinin activity of adenine derivatives as affected by the nature and location of the substituent groups.

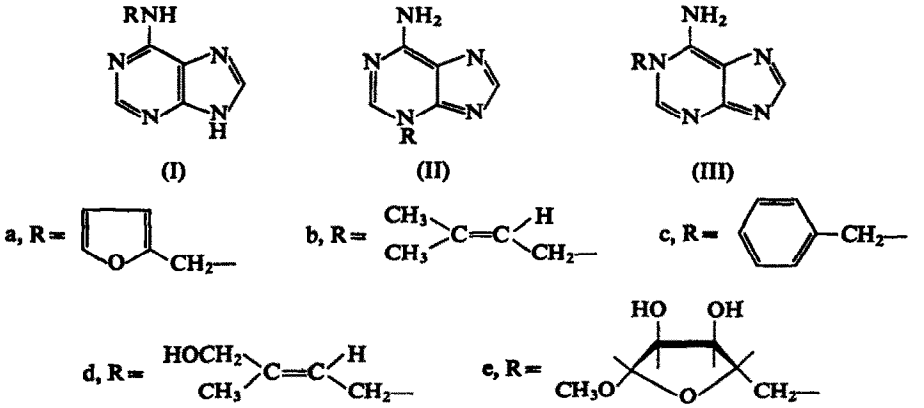
RESULTS AND DISCUSSION

Chemistry of Test Substances

In addition to a number of known compounds which were made for this investigation, several new 6-substituted-aminopurines (I) have been prepared by the general method of reaction of the appropriate amine with 6-chloropurine or with 6-chloro-9-(2-tetrahydropyranyl)purine (IV)[8] followed by an acid hydrolysis step. Compounds obtained thereby

[1] C. O. MILLER, F. SKOOG, F. S. OKUMURA, M. H. VON SALTZA and F. M. STRONG, *J. Am. Chem. Soc.* **78**, 1375 (1956).
[2] F. M. STRONG, In *Topics in Microbial Chemistry*, pp. 98–157. Wiley, New York (1958).
[3] F. S. OKUMURA, N. ENISHI, H. ITOH, M. MASUMURA and S. KURAISHI, *Bull. Chem. Soc. Japan* **32**, 886 (1959).
[4] S. KURAISHI, *Sci. Paper Coll. Gen. Educ., Univ. Tokyo* **9**, 67 (1959).
[5] C. G. SKINNER and W. SHIVE, *Plant Physiol.* **34**, 1 (1959).
[6] A. H. HABER, *Physiol. Plantarum* **12**, 456 (1959).
[7] A. H. HABER and H. J. LUIPPOLD, *Physiol. Plantarum* **13**, 298 (1960).
[8] R. K. ROBINS, E. F. GODEFROI, E. C. TAYLOR, L. R. LEWIS and A. JACKSON, *J. Am. Chem. Soc.* **83**, 2574 (1961).

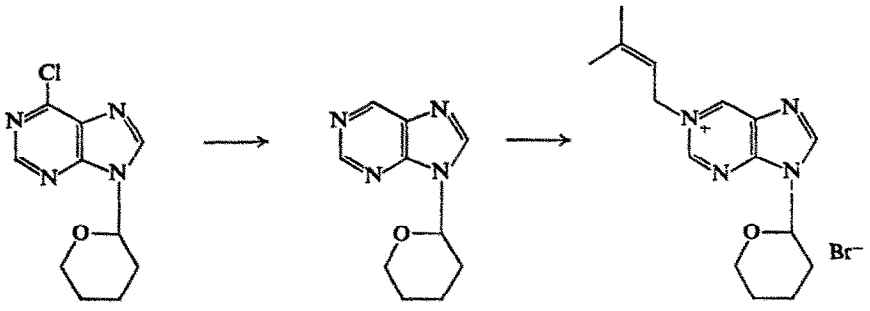
include: 6-(cyclopropylmethylamino)purine, 6-(α,α-dimethylallylamino)purine, 6-farnesylaminopurine, 6-geranylaminopurine, and methyl-5-(purin-6-yl)amino-5-deoxy-β-D-ribofuranoside (Ie),[9] a close analog of kinetin (Ia). 6-(γ,γ-Dimethylallylamino)-9-β-D-ribofuranosylpurine[10,11] was synthesized to compare the biological activity of 6-(γ,γ-dimethylallylamino)purine (Ib) with that of its 9-ribofuranoside.



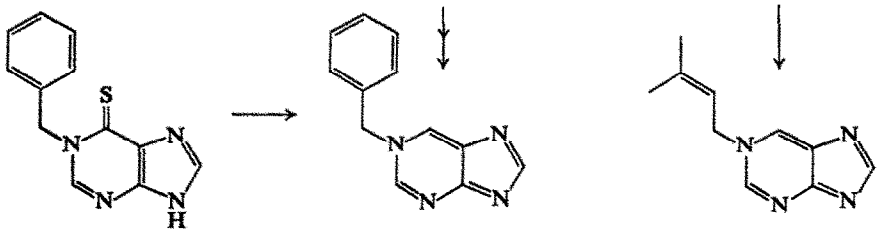
Several compounds lacking amine-substitution at the 6-position were prepared in order to delineate further the importance of the amine-substitution at this position. 9-(2-Tetrahydropyranyl)purine (V) was first prepared by the catalytic hydrogenation of the known 6-chloro-9-(2-tetrahydropyranyl)purine (IV)[8] using palladium on charcoal in the presence of magnesium oxide. Alkylation of this intermediate with γ,γ-dimethylallyl bromide and with benzyl bromide occurred at the 1-position in each case (e.g. VI), and following acid hydrolysis 1-(γ,γ-dimethylallyl)purine (VII) and 1-benzylpurine (VIII) were obtained. The u.v. spectra were similar to those reported for 1-methyl- and 1-ethylpurine of established structure. In the case of the 1-benzylpurine prepared via the alkylation of 9-(2-tetrahydropyranyl)purine, the structure assigned was confirmed by positive comparison with the product of Raney nickel desulfurization of 1-benzyl-6-thiopurine (IX).[12] This comparison validated the structural assignment of 1-(γ,γ-dimethylallyl)purine obtained in the parallel alkylation reaction. A variant of 6-substitution and a close analog of 6-(γ,γ-dimethylallylamino)purine, namely 6-(γ,γ-dimethylallylthio)purine (X), was obtained by S-alkylation of 6-thiopurine with 1-chloro-3-methyl-2-butene according to the general procedure of Johnston, Holum, and Montgomery.[13]

Representative compounds substituted on both 1- and N^6-positions were prepared for the first time: 6-benzylamino-1-methylpurine (XII) and 1-benzyl-6-methylaminopurine, the latter as the hydrobromide (XIV). The route to the former was based upon treatment of 6-benzylthio-1-methylpurine (XI)[14] with benzylamine, while the latter was obtained via reaction of 6-methylamino-9-(2-tetrahydropyranyl)purine (XIII) with benzyl bromide. The

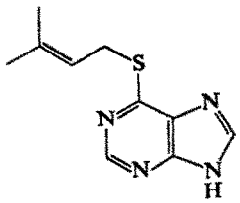
[9] N. J. LEONARD and K. L. CARRAWAY, *J. Heterocyclic Chem.* **3**, 485 (1966).
[10] N. J. LEONARD, Lecture at Fairleigh-Dickinson University, Madison, N.J., October 21, 1963; see *Trans. Morris County Res. Council* **1**, 11 (1965).
[11] N. J. LEONARD, S. ACHMATOWICZ, R. N. LOEPPKY, K. L. CARRAWAY, W. A. H. GRIMM, A. SZWEYKOWSKA, H. Q. HAMZI and F. SKOOG, *Proc. Natl Acad. Sci., U.S.* **56**, 709 (1966).
[12] J. A. MONTGOMERY and H. J. THOMAS, *J. Org. Chem.* **28**, 2304 (1963).
[13] T. P. JOHNSTON, L. B. HOLUM and J. A. MONTGOMERY, *J. Am. Chem. Soc.* **80**, 6265 (1958).
[14] L. B. TOWNSEND and R. K. ROBINS, *J. Org. Chem.* **27**, 990 (1962).



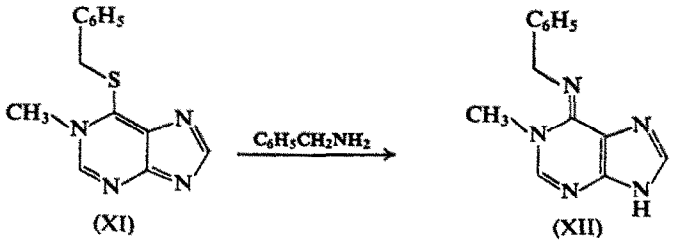
(IV) (V) (VI)



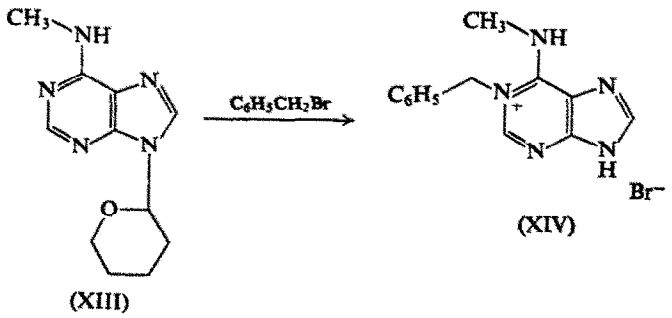
(IX) (VIII) (VII)



(X)



(XI) (XII)



(XIII) (XIV)

structures of these products were confirmed by the close correspondence of their ultraviolet absorption spectra with those reported for other 1,N^6-disubstituted adenines.[11, 15] A 3,N^6-disubstituted model was also made, namely 6-benzylamino-3-methylpurine, by the methylation of 6-benzylaminopurine.

Cytokinin Activity of the Test Substances

Purine derivatives and related compounds listed in Table 1 were tested. Forty-three of these were prepared for this study. Other sources as specified in Table 1 are gratefully acknowledged. Most substances were tested in five-fold concentration steps in the range from 0·00016 to 12·5 μM. Higher or lower concentrations were tested whenever necessary. Unless specifically stated, all reported data are from one of two or more experiments with consistent results. For accurate comparisons of activities, pairs or groups of substances were assayed in the same experiment. A basal medium control (O or BC) and kinetin control (KC) or a previously tested cytokinin of comparable activity were included as standards in each experiment. The activities of test substances [relative to kinetin (KE)] were estimated throughout the ranges of concentration required to induce from barely detectable to maximum increases in fresh and dry weight yields. Activities of compared substances were consistently in the same order in repeat experiments, but the concentrations needed for minimum detectable growth and optimal activities (maximum yields) might vary by 1 or 2 dilution steps from one experiment to the next. Also the total yield of callus obtained with a given concentration varied with the history of the stock tissue and other factors. In general, two-fold differences in activity of substances could be distinguished in tests in which five-fold dilution steps were used. No attempt has been made, therefore, to establish specific activity constants.

General growth responses. A total of 51 compounds including those activated by heating (Table 1) gave positive tests for cytokinin activity. Although the minimum concentration required for barely detectable growth promotion varied between extremes of 5×10^{-5} and 200 μM for different substances, and some of the weakly active ones failed to produce "expected maximum" yields of tissue in any concentration, the growth responses of tobacco callus followed at least superficially a common pattern typified by that of kinetin (Ia) in Fig. 1. In many tests the sensitivity was somewhat less, but in general the lowest concentrations ($5 \times 10^{-4} - 5 \times 10^{-3}$ μM KE) produced a sparse growth of translucent, watery tissue. Concentrations from 5×10^{-3} to 5×10^{-2} μM KE gave somewhat more growth, with patches of white compact tissue distributed through the mass of large loose cells. Occasionally such patches occurred in lower concentrations including the basal controls. From these patches roots formed occasionally. With *ca.* 0·1 μM KE maximum fresh weight yields of granular white parenchyma were obtained. Higher concentrations gave masses (sometimes folding layers) of parenchyma of less total volume, and with 1–5 μM KE round knobs and eventually flat shallow discs of firm, white parenchyma were formed. In old cultures areas within these knobs or discs might form shoots. Concentrations greater than 10 μM KE in most cases were strongly inhibitory. The above stages are characteristic of cultures supplied with the standard concentration of 2 mg/l 3-indoleacetic acid (IAA) in RM-1965. Lower levels of IAA would have hastened the onset of shoot formation, and higher levels, depending also on other factors, would have retarded or entirely prevented it. High levels of kinetin, on the other hand, would have prevented the formation of roots. These inhibitory effects on total yield and on root

[15] T. P. JOHNSTON, A. L. FIKES and J. A. MONTGOMERY, *J. Org. Chem.* 27, 973 (1962).

TABLE 1. LIST OF TEST CHEMICALS AND THEIR CYTOKININ ACTIVITIES

Compound	Concentration (μM)			
	Range tested		Required for	
	Min.	Max.	Detectable response	Maximum yield
Adenine	1	800	200	∞
3-Allyladenine*	10^{-1}	12.5	inactive	
6-Allylaminopurine*	$1{\cdot}6\times10^{-4}$	25	0·05	5
Benzothiazolyl-2-oxyacetic acid**	8×10^{-4}	12·5	inactive	
1-Benzyladenine (IIIc)*	8×10^{-4}	25	0·004[a]	0·1[a]
			0·1–1·0[f]	2·5[f]
2-Benzyladenine‡	1	64	inactive	
3-Benzyladenine (IIc)*	10^{-1}	12·5	0·1[f]	∞
6-Benzylamino-8-azapurine*	8×10^{-4}	62·5	0·02	0·5
6-Benzylamino-1-methylpurine (XII)*	$1{\cdot}6\times10^{-4}$	25	12·5	∞
6-Benzylamino-3-methylpurine*	$1{\cdot}6\times10^{-4}$	25	0·1–0·5[a]	15[a]
			0·1[f]	∞
6-Benzylaminopurine (Ic)*	$6{\cdot}4\times10^{-6}$	12·5	0·0008	0·07
1-Benzyl-6-benzylaminopurine*	8×10^{-4}	25	0·5[a]	12·5[a]
			>0·5[f]	12·5[f]
3-Benzyl-6-benzylaminopurine*	8×10^{-4}	25	12·5	25
7-Benzyl-6-benzylaminopurine*	8×10^{-4}	25	0·02–0·1[a]	0·5[a]
			0·5[f]	12·5[f]
9-Benzyl-6-benzylaminopurine*	$1{\cdot}6\times10^{-4}$	25	0·1	0·5–2·5
1-Benzyl-6-methylaminopurine.HBr (XIV)*	4×10^{-3}	62·5	12·5	∞
6-N-Benzylmethylaminopurine*	$1{\cdot}6\times10^{-4}$	25	0·02	2·5
1-Benzylpurine (VIII)*			inactive	
6-Butylaminopurine†	$1{\cdot}6\times10^{-4}$	25	0·004	0·1–0·5
6-(5-Chlorofurfurylamino)purine†	$1{\cdot}6\times10^{-4}$	12·5	0·02	0·5
6-Cyclohexylaminopurine†	4×10^{-3}	62·5	0·5	12·5
6-(Cyclopropylmethylamino)purine*	8×10^{-4}	12·5	0·1	2·0
6-Decylaminopurine†	$1{\cdot}6\times10^{-4}$	125	25–125	∞
1,7-Dibenzyladenine*	4×10^{-3}	25	0·1[a]	∞[a]
			0·5[f]	∞
1,9-Dibenzyladenine perchlorate*	$1{\cdot}6\times10^{-4}$	25	0·1–0·5[a]	2·5[a]
			2·5[f]	∞[f]
6-(2,3-Dihydroxy-3-methylbutylamino)purine*			0·1	2·5(?)
1-(γ,γ-Dimethylallyl)adenine (IIIb)*	$1{\cdot}6\times10^{-4}$	12·5	0·0002[a]	0·02–0·5[a]
3-(γ,γ-Dimethylallyl)adenine (IIb)*	1×10^{-3}	100	0·5–1·0[f]	2·5[f]
7-(γ,γ-Dimethylallyl)adenine*	2×10^{-1}	25	5 (trace)	∞[f, a]
9-(γ,γ-Dimethylallyl)adenine*	10^{-1}	62·5	2·5[a]	∞[a]
			2·5–12·5[f]	∞[f]
γ,γ-Dimethylallyl alcohol*	4×10^{-2}	25	inactive	
γ,γ-Dimethylallylamine.HCl*	2×10^{-1}	25	inactive	
Di-(γ,γ-dimethylallyl)amine.HCl*	2×10^{-1}	25	inactive	
Tri-(γ,γ-dimethylallyl)amine.HCl*	2×10^{-1}	25	inactive	
6-(α,α-Dimethylallylamino)purine*	$1{\cdot}6\times10^{-4}$	12·5	0·004–0·02	0·5
6-(γ,γ-Dimethylallylamino)purine (Ib)*	1×10^{-6}	60	0·001 (0·00003–0·0008)	0·02
6-(γ,γ-Dimethylallylamino)-9-β-D-ribofuranosylpurine*	$1{\cdot}6\times10^{-4}$	12·5	0·01	0·5
3-(γ,γ-Dimethylallyl)-7-methyladenine perchlorate*	2×10^{-2}	12·5	inactive	
N-(γ,γ-Dimethylallyl)phthalimide*	$1{\cdot}6\times10^{-4}$	100	inactive	
1-(γ,γ-Dimethylallyl)purine (VII)*	4×10^{-3}	12·5	inactive	
6-(γ,γ-Dimethylallylthio)purine (X)*	$6{\cdot}4\times10^{-6}$	25	0·1	2·5

TABLE 1.—*continued*

Compound	Concentration (μM)			
	Range tested		Required for	
	Min.	Max.	Detectable response	Maximum yield
6,6-Dimethyl-1,3,4,5a,9-pentaaza-5a,6,7,8-tetrahydro-3H-benz[e]indene*	$1{\cdot}6\times10^{-4}$	50	inactive	
6-Ethylaminopurine†	2×10^{-3}	62·5	0·5	12·5
6-Farnesylaminopurine*	4×10^{-3}	25	0·004–0·1	2·5
3-Furfuryladenine (IIa)*	1×10^{-3}	100	10[a]	100[a]
			inactive[f]	
6-Furfurylamino-8-azapurine*§ (Azakinetin)	1×10^{-6}	25	0·1	2
6-Furfurylaminopurine (kinetin) (Ia)	1×10^{-6}	50	0·001	0·1
2-N-Furfurylaminopurine‡	1	64	inactive	
6-Furfurylamino-9-β-D-ribofuranosylpurine†	8×10^{-4}	12·5	0·001	0·1
6-N-Furfurylmethylaminopurine†	$1{\cdot}6\times10^{-4}$	12·5	0·5	2·5
6-[2-(2-Furyl)ethylamino]purine††	$1{\cdot}6\times10^{-4}$	12·5	0·02	0·5
6-Geranylaminopurine*	$1{\cdot}6\times10^{-4}$	25	0·1–0·5[a]	2·5[a]
			2·5[f]	12·5[f]
Gibberellic Acid	2×10^{-2}	200	inactive	
6-Heptylaminopurine†	$1{\cdot}6\times10^{-4}$	25	0·1	2·5
6-Hexylaminopurine†	6×10^{-6}	25	0·02	0·3
6-(*trans*-γ-Hydroxymethyl-γ-methylallylamino)-purine (Zeatin, Id)*	1×10^{-6}	25	0·00003–0·0005	0·004–0·5
3-Indoleacetic acid		200	inactive	
6-Isoamylaminopurine†	6×10^{-6}	12·5	0·004	0·1
2-m-Methoxybenzyladenine‡	1	64	inactive	
1-Methyladenine*	4×10^{-3}	62·5	inactive	
6-Methylaminopurine	2×10^{-2}	200	100	∞
Methyl 5-deoxy-5-(purin-6-yl)amino-β-D-ribofuranoside (Ie)*	6×10^{-6}	12·5	0·1	5
6-Pentylaminopurine†	$1{\cdot}6\times10^{-4}$	25	0·0008	0·1
6-(β-Phenoxyethylamino)purine†	$1{\cdot}6\times10^{-4}$	12·5	0·02	0·5
6-Phenylaminopurine†	4×10^{-3}	62·5	0·004	0·5
3-Phytyladenine*	$1{\cdot}6\times10^{-2}$	12·5	inactive	
6-Propylaminopurine†	$1{\cdot}6\times10^{-4}$	25	0·02	5
6-(2-Pyridylmethylamino)purine†	$1{\cdot}6\times10^{-4}$	12·5	0·02	0·5
6-(2-Thenylamino)purine†	$1{\cdot}6\times10^{-4}$	12·5	0·004	0·1

[a] Autoclaved in medium 15 min at 120°.
[f] Filtersterilized and added to cooling medium.
* Synthesized for this study.
** Kindly furnished by Dr. J. van Overbeek, Shell Development Co., Modesto, California.
† Kindly furnished by Professor F. M. Strong, Department of Biochemistry, University of Wisconsin, from sources specified in reference 2.
†† Kindly furnished by the Upjohn Co., Kalamazoo, Michigan.
‡ Kindly furnished by Dr. F. S. Okumura, Tanabe Seiaku Co. Ltd., Osaka, Japan.
§ Kindly furnished by Professor F. C. Steward, Cornell University, Ithaca, New York.

formation were exerted by high but non-toxic kinetin concentrations which, in fact, were optimal for long survival of the cultures.

6-Alkylaminopurines. Effects of chain length of N^6-alkyladenines on the type of growth and yield of tissue in the tobacco bioassay are illustrated in Figs. 2 and 3. 6-Pentylaminopurine, with a detectable growth promoting effect starting below 1×10^{-3} μM, was about as

active as kinetin. The others had lower specific activities, decreasing as a function of shorter or longer chain length. The yield/concentration curves in Fig. 3 show that all the derivatives from C_2H_5- to C_7H_{15}- inclusive, gave about the same maximum yield of tissue as did kinetin, provided they were supplied in high enough concentrations. The tested substances with shorter side chains, regardless of concentration, never gave the expected maximum yields; nor did 6-decylaminopurine. Low solubility of the latter prevented its being tested in concentrations higher than 25 μM. Possibly the concentrations of weak compounds which would be required for "maximum yield" would exceed the tolerance of the tobacco tissue to purines in general. The more active 6-alkylaminopurines in high concentrations also were about as effective as kinetin in promoting bud formation in tobacco callus cultures (Fig. 4), but none approached the capacity of 6-(γ,γ-dimethylallylamino)purine (Ib) in this respect.

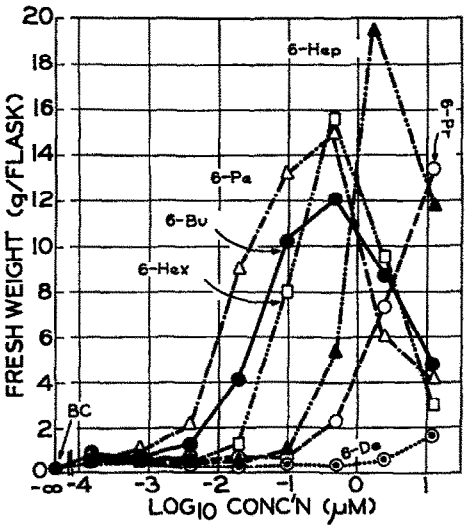


FIG. 3. EFFECTS OF CONCENTRATION OF 6-ALKYLAMINOPURINES ON FRESH WEIGHT YIELDS OF TOBACCO CALLUS.

Expt. 116, Jan. 14–Feb. 18, 1965. Labels as in Fig. 2.

As to the requirement of a substituent in the N^6-position for cytokinin activity, i.e. whether adenine itself is active, numerous tests have been done in our laboratory and elsewhere with different assay methods over the past 10 years. Opinions have differed, but our recent tobacco assays showed definite and increasing activity in the range from 200 to 800 μM. Our negative results obtained earlier were with lower concentrations. Adenine heated with furfuryl alcohol is reported to form kinetin *in vitro*,[16] and we have confirmed the presence of cytokinin activity in such heated mixtures. The nature of the reaction is being studied because similar heat treatment of equimolar mixtures of adenine and either α,α-dimethylallyl alcohol, γ,γ-dimethylallyl alcohol or benzyl alcohol produced less of the corresponding N^6-substituted adenine even though the expected products would be biologically more active than kinetin and thus detectable at lower concentrations.[11, 17] Possibly adenine also gives rise to active products on heating in nutrient media. Its activity in promoting bud formation appeared

[16] R. H. HALL and R. S. DE ROPP, *J. Am. Chem. Soc.* 77, 6400 (1955).

[17] N. J. LEONARD, S. ACHMATOWICZ, R. N. LOEPPKY, K. L. CARRAWAY, R. B. BRADBURY, W. A. H. GRIMM, A. SZWEYKOWSKA and F. SKOOG, (In preparation).

to be greater in autoclaved than in filter sterilized media.[18] But in recent attempts to separate out an active product by chromatography of heated adenine solutions, no activity was found at R_f values other than that of adenine itself. Concentrations $\geqslant 200$ μM were required for detection of activity in these experiments. No maximum yields approaching those obtained with kinetin were obtained with any concentration or treatment of adenine. The possibility still remains that the apparent activity of adenine is due to N^6-substituted derivatives formed within the tobacco tissue with high exogenous adenine supplies.

6-Methylaminopurine has been reported to have cytokinin activity in certain test systems.[19] In the present tests a 100 μM concentration was active by itself, but markedly depressed the response to optimal levels of more active cytokinins (see also 20). Lower concentrations were inactive in this and earlier studies.[20] 6-Methylaminopurine is a well known constituent of RNA and has been isolated from many sources. It may be relatively more active in test systems other than tobacco cultures, so that its role as a naturally occuring cytokinin should not be underestimated.

In general, therefore, it can be said that N^6-alkyl derivatives of adenine exhibit a wide range of cytokinin activity which clearly is dependent on the length of the alkyl group. The influence of chain length apparently relates to physical properties facilitating uptake or access to reactive sites in the cells, because it follows the usual pattern of change found for biological activities, including toxicity, of other alkyl-substituted series of substances.[21]

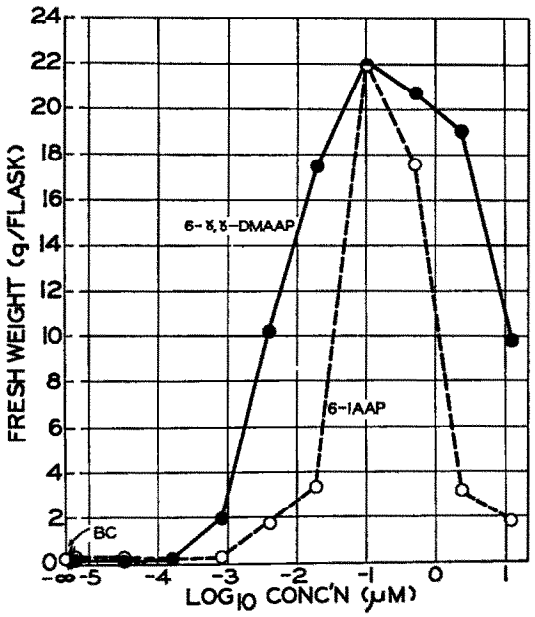


FIG. 5. INFLUENCE OF DOUBLE BOND ON CYTOKININ ACTIVITY AS ILLUSTRATED BY YIELD OF TOBACCO CALLUS OBTAINED WITH 6-(γ,γ-DIMETHYLALLYLAMINO)PURINE (6-γ,γ-DMAAP) AND 6-ISOAMYLAMINOPURINE (6-IAAP).

Expt. 54, June 17–July 22, 1964.

[18] C. O. MILLER, and others in this laboratory, unpublished.
[19] C. O. MILLER, *Ann. Rev. Plant Physiol.* **12**, 395 (1961).
[20] D. KLÄMBT, G. THIES and F. SKOOG, *Proc. Natl Acad. Sci. U.S.* **56**, 52 (1966).
[21] S. E. ALLEN and F. SKOOG, *Plant Physiol.* **26**, 611 (1951).

Although all tested N^6-adenine derivatives with 4–7 C atoms in the side chain tended to be highly active, further details of the specific structure of the substituents markedly affected their relative potencies. 6-Isoamylaminopurine and 6-*n*-pentylaminopurine both had nearly the same activity as kinetin, i.e. detectable around 10^{-3} μM and producing maximum yields starting at about 0·1 μM.

The introduction of a double bond in the chain greatly increased the cytokinin activity. Thus, 6-(γ,γ-dimethylallylamino)purine (Ib) was *ca.* 10 times more active than either 6-isoamylaminopurine (Fig. 5) or kinetin (Fig. 6).

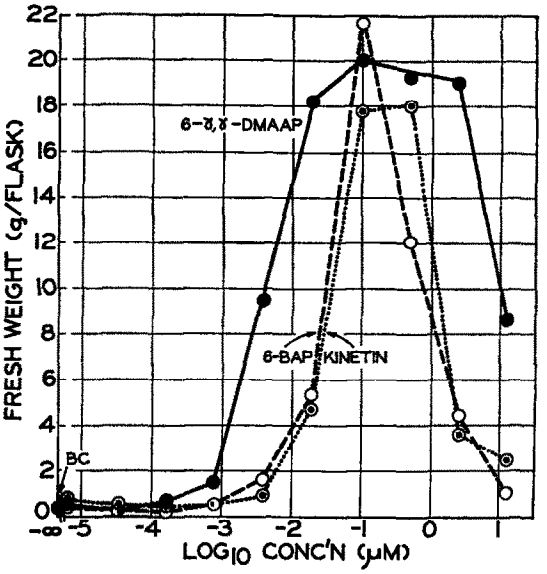


FIG. 6. EFFECTS OF 6-(γ,γ-DIMETHYLALLYLAMINO)PURINE (6-γ,γ-DMAAP), 6-BENZYLAMINOPURINE (6-BAP) AND KINETIN ON YIELD OF TOBACCO CALLUS.
Expt. 52, June 11–July 16, 1964.

The enhancement of activity by unsaturation was also observed in comparative tests of other pairs of substances. 6-Phenylaminopurine was much more active than 6-cyclohexylaminopurine (Fig. 7). The unsaturated long chain compounds, 6-farnesylaminopurine and 6-geranylaminopurine were both more active than 6-decylaminopurine (Fig. 7). Unexpectedly, 6-farnesylaminopurine was consistently more active than 6-geranylaminopurine in these tests. In direct comparisons of 6-propyl- and 6-allylaminopurine no consistent difference between the two was found. Possibly the low activity conferred by the short chain obviates any significant influence of unsaturation.

The importance of structural features other than unsaturation is strikingly demonstrated by the fact that shifting the methyl groups from the γ-carbon atom to the α-carbon atom of the allyl chain greatly reduced activity. (see Fig. 18).

The N^6-isoprenoid adenines are of special interest because of their natural occurrence[20, 22–26] and extreme potency.

[22] J. H. ROGOZINSKA, J. P. HELGESON and F. SKOOG, *Physiol. Plantarum* **17**, 165 (1964).
[23] J. P. HELGESON and N. J. LEONARD, *Proc. Natl Acad. Sci., U.S.* **56**, 60 (1966).
[24] H. G. ZACHAU, D. DÜTTING and H. FELDMANN, *Angew. Chem.* **78**, 392 (1966).
[25] R. H. HALL, M. J. ROBINS, L. STASIUK and R. THEDFORD, *J. Am. Chem. Soc.* **88**, 2614 (1966).
[26] D. S. LETHAM and C. O. MILLER, *Plant Cell Physiol.* **6**, 355 (1965).

The superiority of Ib over Ia in promoting organ-formation as well as growth in tobacco cultures is evident from comparisons of their effects in combinations with IAA in Fig. 8. Both substances promoted growth and bud formation, but by virtue of its much lower "toxicity" at high concentrations as well as its higher potency Ib was enormously more effective in promoting development.

It should be noted that high concentrations of kinetin (Ia) and especially of 6-(γ,γ-dimethylallylamino) purine (Ib) permitted growth and development also in callus cultures not supplied with auxin.

The relative activities of highly active cytokinins vary with the test system. Thus in tests of chlorophyll retention in leaf discs, 6-benzylaminopurine (Ic) was most active and was followed in order by Ia, Ib and Id.[27, 28]

Comparisons of the activities of Ib and Id in the tobacco bioassay showed both compounds to be active down to 5×10^{-5} μM in the most sensitive tests, but on the average Id was the more active. This is in agreement with reports[26, 29] of a minimum detectable concentration of 10^{-10}–10^{-11} M for zeatin in soybean bioassays. But 6-(γ,γ-dimethylallylamino)purine was reported as no more active than kinetin in this bioassay. The effects of serial concentrations of these two naturally occurring cytokinins on appearance and yield of tobacco cultures may be compared in Fig. 9. In this assay the response to Ib was less than optimal (as compared with Fig. 1).

Ring substituted purine derivatives. 6-Benzylaminopurine (Ic) was the most active ring-substituted cytokinin which has been tested. Its average minimum detectable concentration from 16 experiments was 8×10^{-4} μM, and the concentration required for maximum yield

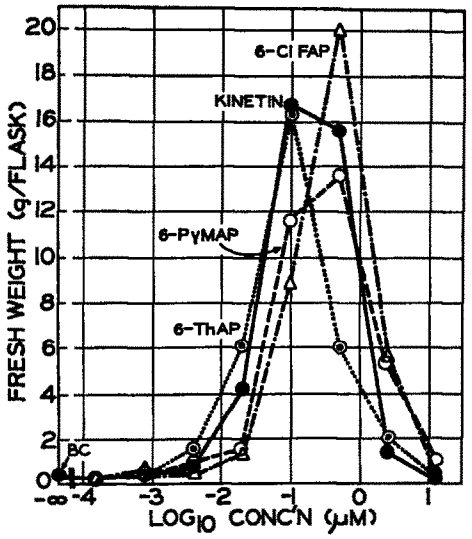


FIG. 10. EFFECT OF PURINE DERIVATIVES WITH HETERO-ATOMS IN SIDE CHAIN ON THE YIELD OF TOBACCO CALLUS. 6-(2-THENYLAMINO)PURINE (6-ThAP), 6-(2-PYRIDYLMETHYLAMINO)PURINE (6-PyMAP) AND 6-(5-CHLOROFURFURYLAMINO)PURINE (6-ClFAP).

Expt. 63, July 14–Aug. 19, 1964.

[27] H. Q. HAMZI and F. SKOOG, *Proc. Natl Acad. Sci. U.S.* **51**, 76 (1964).
[28] J. VAN OVERBEEK, Private communication.
[29] C. O. MILLER, *Plant Physiol., Suppl.* **40** LXXVIII (1965).

was 0·07 μM. The corresponding average values for kinetin from 18 experiments were $1{\cdot}7 \times 10^{-3}$ and 0·1 μM respectively. In all tests in which direct comparisons were made (as in Fig. 6) its activity was between that of 6-(γ,γ-dimethylallylamino)purine and kinetin. 6-(2-Pyridylmethylamino)purine, which differs from 6-benzylaminopurine merely in the exchange of a nitrogen atom for a carbon in the ring, had less than one tenth the activity of the latter (Fig. 10). Kinetin and 6-(2-thenylamino)purine, in which the oxygen atom in furfuryl is replaced by sulfur were about equally active (Fig. 10).

Either removal of the methylene group attaching the ring to the N^6-position or lengthening the bridge decreased the activity. Thus 6-phenylaminopurine and 6-(β-phenoxyethylamino)-purine were less active than kinetin and therefore no more than a fifth as active as 6-benzyl-aminopurine. The depressive effect of lengthening the bridge also is illustrated by 6-[2-(2-furyl)ethylamino]purine, which was one fifth as active as kinetin (Fig. 11).

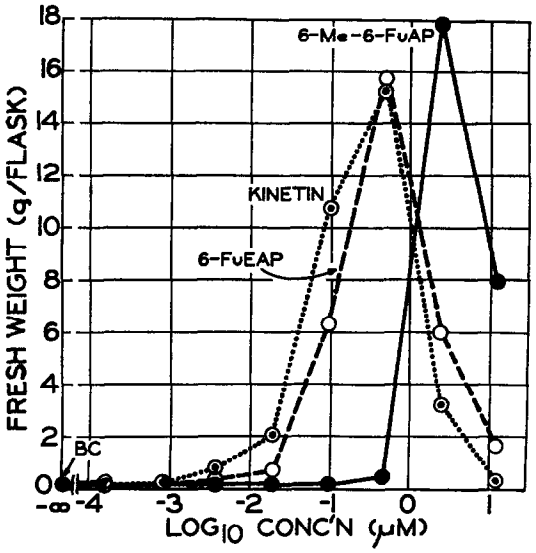


FIG. 11. EFFECTS OF 6-(2-FURYLETHYLAMINO)PURINE (6-FuEAP), 6-N-FURFURYLMETHYLAMINO-PURINE (6-ME-6-FuAP) AND KINETIN ON YIELD OF TOBACCO CALLUS.
Expt. 59, July 2–Aug. 6, 1964.

6-(Cyclopropylmethylamino)purine and 6-cyclohexylaminopurine, the two tested substances with saturated rings, were both less active than the above ring-substituted compounds. The only tested substance with a halogenated ring, 6-(5-chlorofurfurylamino)purine, was much less active than the unsubstituted kinetin control. Evidence from extensive tests with the radish leaf disc assay suggests that chlorine in the *ortho*-position in the 6-benzylamino series increases biological activity, in the *meta*-position it has little effect, and in the *para*-position it is highly inhibitory. Other substituents (—OH, —OCH_3, —NO_2, —SO_3H, —NH_2) all lowered the cytokinin activity.[4] Introduction of the highly polar deoxyribose ring in the 6-position (as in Ie) gave an active cytokinin, with a detectable effect starting between 0·02 and 0·1 μM and a broad maximum in the 2·5–25 μM range (Fig. 12).

6-(2,3-Dihydroxy-3-methylbutylamino)purine was active starting at about 0·1 μM in spite of its polar groups. The fact that these substances with two hydroxyl groups are at all active is unexpected in view of earlier evidence that 6-benzoyl-, 6-furoyl-, and 6-succinylamino-

purine were inactive, which led to the notion that the mere presence of a polar group in the side chain would eliminate cytokinin activity.[2] The extreme potency of zeatin also argues against this broad interpretation (see Fig. 9).

Effect of modifying the adenine moiety. Not only the N^6-substituent but also the N^6-atom itself influences cytokinin activity of adenine derivatives. This is shown by the *ca.* 100-fold decrease in activity resulting from the replacement of N^6 with a sulfur atom as in (X) (Fig. 13). The removal of the NH_2 group as in 1-benzylpurine and 1-(γ,γ-dimethylallyl)purine eliminated cytokinin activity; perhaps in this case by preventing the formation of a 6-substituted derivative.

The effect of eliminating the NH_2 group from 6-substituted adenines, *i.e.* attaching the alkyl group directly to the 6-position of purine has not been tested with substituents of appropriate size for cytokinin activity. It has been tried with 6-methylpurine, which was inactive.

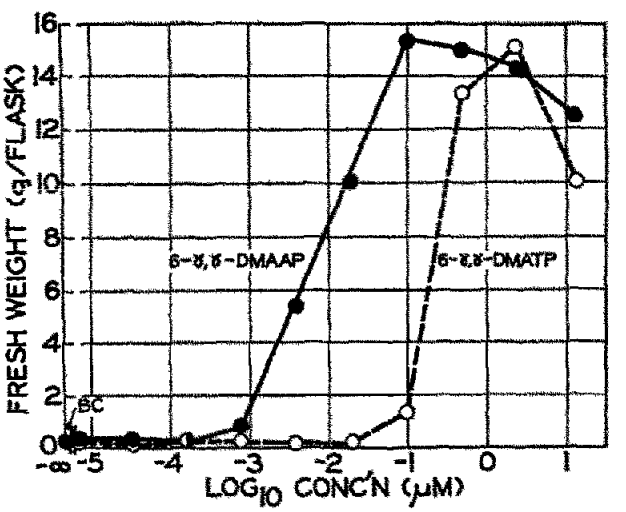


FIG. 13. EFFECTS OF 6-(γ,γ-DIMETHYLALLYLTHIO)PURINE 6-(γ,γ-DMATP) AND 6-(γ,γ-DIMETHYLALLYLAMINO)PURINE ON YIELD OF TOBACCO CALLUS.

Expt. 97, Nov. 17–Dec. 22, 1964.

Substitution of a nitrogen atom for the 8-carbon atom in the purine ring of kinetin yields 8-azakinetin, which has been claimed to be more active than kinetin in the carrot tissue bioassay.[30] The quoted optimal concentrations are 0·1–1·0 mg/l. Tests of a sample kindly furnished by Professor Steward showed that in the tobacco assay these concentrations were supraoptimal for kinetin but not for the 8-azakinetin. Further comparative tests of 8-azakinetin and 6-benzylamino-8-azapurine, prepared by methods[31] which preclude the presence of true purine derivatives, showed that these substances possess cytokinin activity. However, they were only about 5 per cent as active as the corresponding true purine derivatives (Figs. 14 and 15). The reason for the difference in quantitative results obtained with the two tissues has not been determined. As is shown in Fig. 15, the aza-derivatives gave higher yields than

[30] F. C. STEWARD with E. M. SHANTZ, J. K. POLLARD, MARION O. MAPES and J. MITRA, In *Synthesis of Molecular and Cellular Structure, 19th Growth Symposium* (Edited by DOROTHEA RUDNICK), pp. 193–246. Ronald Press, New York (1961).

[31] R. WEISS, R. K. ROBINS and C. W. NOELL, *J. Org. Chem.* **25**, 765 (1960).

the adenines in the tobacco test in the high range of concentrations referred to by Steward as optimal for carrot tissue. Perhaps, therefore, the claim for higher specific activity of aza-kinetin requires further documentation. It is of interest that the exchange of the 8-C and 7-N atoms in kinetin to give 4-furfurylpyrazolo(3,4-d)pyrimidine completely eliminates cytokinin activity.[2] Benzothiazolyl-2-oxyacetic acid, another non-purine compound reported to have strong kinetin-like activity in carrot tissue,[30] was completely without cytokinin activity in the tobacco bioassay (Fig. 16) and also in the radish leaf disc test.[28]

Recent reports on pyrimidine derivatives,[32] and extensive comparative tests of urea derivatives in various test systems including the tobacco bioassay[33] suggest that substances other than purine derivatives may be active in a similar manner as kinetin.

Influence of the position of the substituents. Monosubstituted adenine derivatives with an alkyl or aryl group in other than the N^6-position exhibited apparent cytokinin activity to varying degrees depending on the position on the purine ring and also depending on the test procedure.

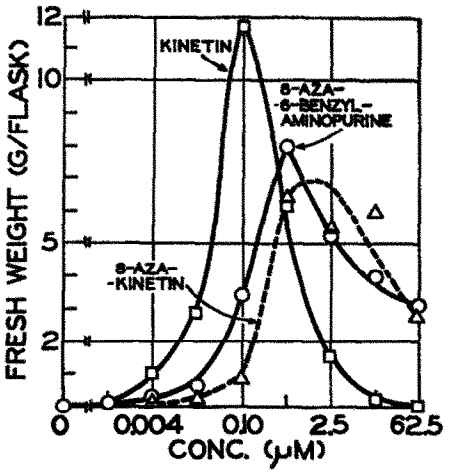


FIG. 15. EFFECTS OF CONCENTRATION OF 6-BENZYLAMINO-8-AZAPURINE AND OF 6-FURFURYLAMINO-8-AZAPURINE (8-AZAKINETIN) ON YIELD OF TOBACCO CALLUS.
Expt. J-4, Oct. 25–Nov. 30, 1965.

Earlier reports indicate apparent cytokinin activity of 1-benzyl-and 1-(γ,γ-dimethylallyl)-substituted adenines.[27] In recent tests of rigorously purified preparations by procedures which minimize exposure to heat, only the highest concentrations were active. In the example shown in Fig. 17, the filter-sterilized sample of 1-benzyladenine was active only in the 0·5–12·5 μM range, whereas the sample which was autoclaved in the nutrient medium came close to the activity of the N^6-isomer. Less than 1 per cent $1\rightarrow N^6$ conversion would account for the observed activity of the heated sample. Similarly, 1-(γ,γ-dimethylallyl)adenine can be activated by heat. In a typical experiment (Fig. 18) the autoclaved sample was almost as active as its 6-isomer and considerably more active than 6-(α,α-dimethylallylamino)purine. These results strongly suggest that a cyclization causing inversion of the side chain was not involved in the activation process. Indeed, it has been shown that the major activation process

[32] O. N. KULAYEVA, E. A. CHERNYSHEVA, L. A. KOLOTENKO, N. E. DOLGOVA, I. P. VOROBYOVA, E. A. POPOVA and M. E. KLYACHKO, *USSR Acad. Sci. Mos.* **12**, 902 (1965).
[33] M. I. BRUCE, J. A. ZWAR and N. P. KEFFORD, *Life Sci.* **4**, 461 (1965).

involves ring-opened intermediates resulting in an exchange of the N atoms in the 1- and N^6-positions.[11]

3-(γ,γ-dimethylallyl)adenine has been reported to possess cytokinin activity,[34] but in our recent tests of it and also of 3-allyl-, 3-furfuryl-, 3-phytyl-, 3-benzyl-, and 3-(γ,γ-dimethylallyl)-adenine, all these 3-substituted adenines were inactive as such. However, they may be converted to corresponding N^6-isomers all of which, except for 6-allylaminopurine and 6-phytylaminopurine, would possess high cytokinin activity. The results obtained with heated 3-furfuryladenine (Fig. 19) indicate that the yield of active N^6-product was lower than from the 1→N^6 conversion.

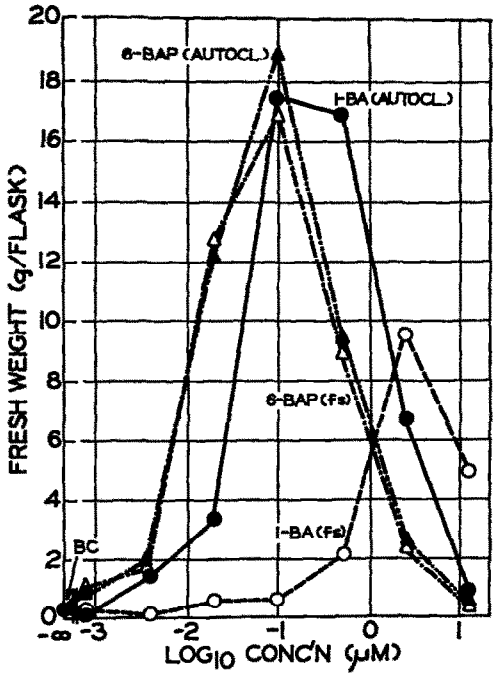


FIG. 17. EFFECTS OF AUTOCLAVED AND FILTER-STERILIZED BIOASSAY SAMPLES OF 1-BENZYLADENINE AND 6-BENZYLAMINOPURINE ON THE GROWTH OF TOBACCO CALLUS.
Expt. 83, Sep. 29–Nov. 3, 1964.

9-Substituted adenines also are activated by conversion on heating, but as shown by results of comparative tests of 1- and 9-(γ,γ-dimethylallyl)adenines (Fig. 20), the 9-derivative is activated much less readily.

The first samples of 7-benzyladenine and 7-(γ,γ-dimethylallyl)adenine, when tested in the 0·2–125 μM range, were both definitely active starting at 1·0 μM. A second, highly purified sample of 7-(γ,γ-dimethylallyl)adenine gave only a trace of activity at 25 μM. Autoclaving the medium after addition of the chemical did not increase the activity. Furthermore, the original 7-(γ,γ-dimethylallyl)adenine preparation after thin layer chromatography in a $CHCl_3$:CH_3OH (9:1) solvent system gave much less activity at the R_f corresponding to 7-(γ,γ-dimethylallyl)adenine than the starting material, and also gave evidence of a highly active substance at the R_f corresponding to 6-(γ,γ-dimethylallylamino)purine in this system.

[34] G. BEAUCHESNE and R. GOUTAREL, *Physiol. Plantarum* **16**, 630 (1963).

In a chromatogram of a mixture of 4 parts 6-(γ,γ-dimethylallylamino)purine added to 100 parts 7-(γ,γ-dimethylallyl)adenine in which the two substances appeared as two distinct UV absorbing spots > 3 cm apart, enough 6-isomer was retained with the 7-isomer to increase the cytokinin activity above that of the original 7-isomer preparation. Enough 6-isomer also had spread to be detectable by bioassays in other parts of the chromatogram.

The 2-substituted derivatives, 2-benzyladenine and 2-*m*-methoxybenzyladenine[35] were inactive in the 1–64 μM range. Also 2-(2-furfurylamino)purine[35] was inactive, but of course this compound lacks an NH_2-group in the 6-position. 8-(γ,γ-dimethylallyl)adenine (up to 1·0 μM) also has been reported to be inactive.[34]

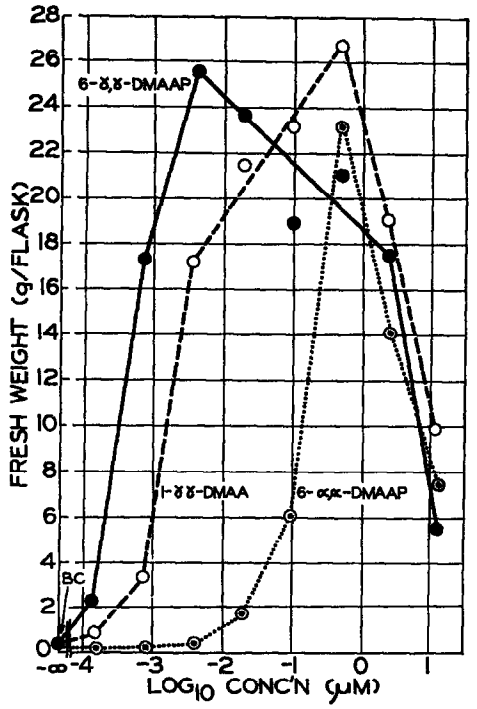


FIG. 18. EFFECTS OF 6-(γ,γ-DIMETHYLALLYLAMINO)PURINE, 1-(γ,γ-DIMETHYLALLYL)ADENINE AND 6-(α,α-DIMETHYLALLYLAMINO)PURINE ON YIELD OF TOBACCO CALLUS.
Test samples autoclaved with the nutrient medium. Expt. 43, May 8–June 12, 1964.

On the whole, tests of monosubstituted derivatives point to the conclusion that only N^6-substituents confer cytokinin activity on adenine and that other derivatives with apparent activity are converted to the N^6-isomers. The ease of this conversion decreases in the order: $1 > 3 \geqslant 9$.

The tests of 7-substituted adenines suggest that the observed activity was due to contamination with traces of the N^6-isomers which are difficult to remove completely by chromatography. They indicate that the 7-substituted adenines themselves are inactive, in agreement with an earlier report,[34] and furthermore, that they are not readily convertible to active products by heating. This behavior is in agreement with expectations from the postulated N^6-isomer

[35] Kindly furnished by F. S. Okumura.

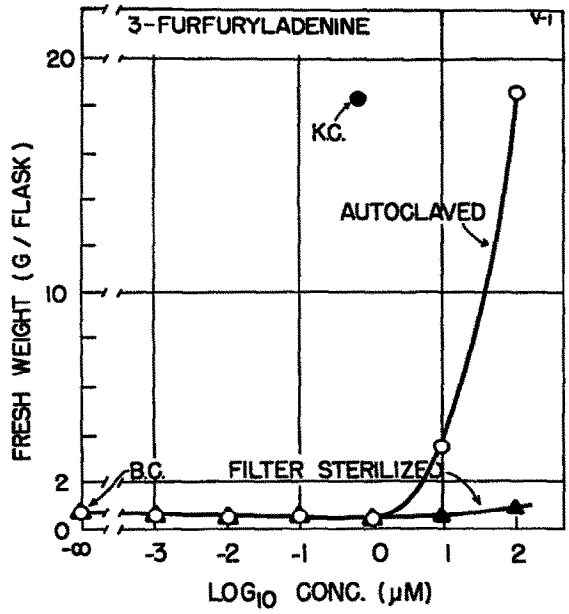


FIG. 19. EFFECT OF AUTOCLAVED AND FILTER STERILIZED TEST SAMPLES OF 3-FURFURYLADENINE ON GROWTH OF TOBACCO CALLUS.

B.C.=Basal control; K.C.=Kinetin control. Expt. V-1, May 28–July 2, 1965.

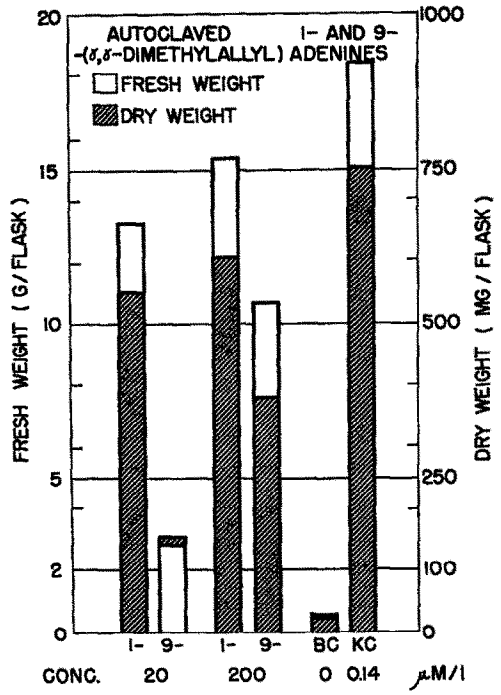


FIG. 20. COMPARATIVE YIELDS FROM AUTOCLAVED 1- AND 9-(γ,γ-DIMETHYLALLYL)-SUBSTITUTED ADENINES.

Expt. V-2, July 15–Aug. 19, 1965.

formation by rupture and reclosure of the purine ring[17] which pathway would exclude the 7-substituted adenines as possible sources of N^6-substituted adenines. Degradative removal of 7-substituents followed by N-substitution to produce trace amounts of cytokinins would of course be possible, expecially in heated, concentrated preparations. Although 2- and 8-substituted adenines apparently are inactive, further investigation is required to determine whether high concentrations may be active or whether these substances are subject to activation by chemical conversion.

Relative activities of disubstituted adenines. In each of 10 tested cases, the addition of a second substituent to a highly active 6-substituted adenine reduced its activity. The degree of this reduction varied with the position and nature of the substituent.

The addition of a methyl group in the N^6-position of kinetin or 6-benzylaminopurine caused at least a 100-fold loss in activity. Results for N-methylated 6-furfurylamino- and 6-benzylaminopurine are included in Figs. 11 and 21, respectively. N^6,7-Dibenzyladenine and

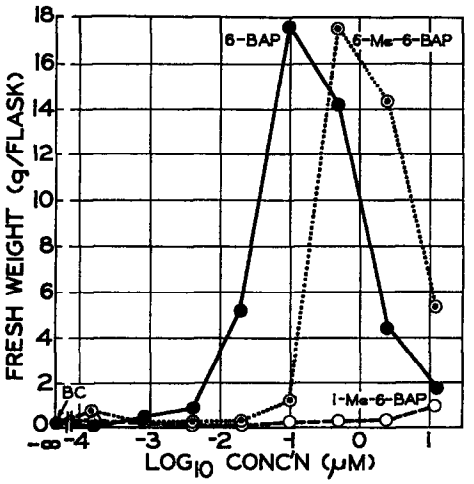


FIG. 21. EFFECTS OF 6-BENZYLAMINOPURINE (6-BAP), 6-BENZYLAMINO-1-METHYLPURINE (1-ME-6-BAP AND 6-N-BENZYLMETHYLAMINOPURINE (6-ME-6-BAP) ON YIELD OF TOBACCO CALLUS. Expt. 109, Dec. 24, 1964–Jan. 28, 1965.

N^6,9-dibenzyladenine, when autoclaved with the nutrient medium, were active starting at 0·02–0·1 μM, and reached optimal activity at 0·5 μM. When they were not exposed to heat in the testing, only higher concentrations were active. The corresponding 1-substituted isomers, 1,7-dibenzyladenine and 1,9-dibenzyladenine were almost as active as the N^6,7- and N^6,9-isomers, respectively, when they were autoclaved in the testing, but when they were filter sterilized only high concentrations had appreciable activity.

In general, results obtained in tests of disubstituted adenines were much more variable than those of N^6-monosubstituted adenines. They suggest that even if the disubstituted adenines are active as such, increased activity often results from activation in the testing process, presumably from removal of substituents in other than the N^6-position. In the case of disubstituted adenines with a substituent in the 1- but not in the N^6-position, conversion to the N^6-isomer is probably a prerequisite for biological activity, and increased activation might result from loss of the other substituent. The numerical data for disubstituted compounds given in Table 1, therefore, merely indicate the presence and relative order of activities of these substances under the test conditions.

The effect of substitution in the 1-position has been further tested with 6-benzylamino purines. A 1-methyl group practically removed cytokinin activity (see Fig. 21). A 1-benzyl group was somewhat less depressive. The difference in effectiveness between the two may be explained by easier removal of the 1-benzyl group and the assumption that both 1-substituted derivatives are themselves inactive. This interpretation is supported by the complete removal of activity from the extremely active 6-(γ,γ-dimethylallylamino)purine when its γ-carbon atom was bound to the 1-position, thus forming the tri-cyclic compound 6,6-dimethyl-1,3,4,5a,9-pentaaza-5a,6,7,8-tetrahydro-3H-benz[e]indene. On the basis of the above reasoning 1-benzyl-6-methylaminopurine (XIV, as the base) would require both the $1\rightarrow N^6$ rearrangement and loss of the methyl group to become active. As expected, this compound had practically no cytokinin activity. In our tests with tobacco callus the 9-ribosides of kinetin and 6-(γ,γ-dimethylallylamino)purine (see Fig. 12 for the latter) were 2–5 times less active than their respective free bases. The apparent "growth vigor" (texture) of callus grown in the presence of high riboside concentrations was striking. A related substance, 6-benzylamino-9-(2-tetrahydropyranyl)purine, reported to be exceptionally potent in promoting chlorophyll retention and keeping quality in plant tissues,[36] was almost as active as 6-benzylaminopurine itself in promoting growth of tobacco callus. The high activity may be due to the lability of the 9-substituent.

Substitution of a methyl or benzyl group in the 3-position of 6-benzylaminopurine also drastically reduced, or perhaps removed, cytokinin activity. 3(γ,γ-Dimethylallyl)-7-methyladenine, as expected, was completely inactive.

We conclude from the above tests of adenine derivatives and closely related substances that high cytokinin activity is limited to N^6-monosubstituted compounds. The 9-ribosyl substituent, however, still allowed for relatively high activity. Whether or not adenine derivatives with substituents in other than the N^6-position may be active as such, high potency is only obtained by rearrangement to the N^6-isomers. Additional substitution on the purine ring of N^6-substituted derivatives lowered the activity in all tested cases, Active substances which are not adenine derivatives, such as 6-(γ,γ-dimethylallylthio)purine and the 8-aza-derivatives of kinetin and 6-benzylaminopurine, are exceptions which tend to emphasize rather than minimize the stringent structural requirements for activity. Essentially an adenine moiety seems to be required but the detailed structure, size, shape, composition, saturation, and charge of the substituent groups strongly influence activity.

Certain urea derivatives seem to be exceptions to this rule.[33] Although these undoubtedly promote growth in the same bioassay systems as do adenine derivatives, it still remains to be determined whether they function as such in the same or in related growth controlling mechanisms.

In any case the cytokinins are a broad generic group which includes some of the most active known biochemicals. Their natural occurrence in RNA,[24, 25, 37] and recent localization immediately adjacent to the anticodon in serine tRNA's I and II suggests a specific role in the control of amino acid incorporation in macromolecular syntheses. The exact manner in which cytokinin molecules may function in this process, either as free bases or as constituents of RNA, is a new field open for systematic investigation with interesting prospects of increased understanding of cytokinin action. Hopefully, some new light also may be shed on the general problem of hormonal regulation of growth and development.

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EXPERIMENTAL

Synthesis of Test Substances

Syntheses of the following compounds have been reported elsewhere:

6-allylaminopurine,[3,4,38]
1-benzyladenine,[11,39]
1-benzyl-6-benzylaminopurine,[40]
3-benzyl-6-benzylaminopurine,[40,41]
6-benzylaminopurine,[42–45]
7-benzyl-6-benzylaminopurine,[40]
9-benzyl-6-benzylaminopurine,[40,46]
1,7-dibenzyladenine,[40]
1,9-dibenzyladenine,[40]
6-dibenzylaminopurine,[47]
1-(γ,γ-dimethylallyl)adenine,[11,39]
7-(γ,γ-dimethylallyl)adenine,[48,49]
9-(γ,γ-dimethylallyl)adenine,[48,49]
γ,γ-dimethylallyl alcohol,[50]
γ,γ-dimethylallylamine hydrochloride,[51,52]
6-(γ,γ-dimethylallylamino)purine,[39,53,55]
N-(γ,γ-dimethylallyl)phthalimide,[51]
1-methyladenine,[54]
6-methylaminopurine,[54]
6-N-benzylmethylaminopurine,[44]
7-methyltriacanthine perchlorate (3-(γ,γ-dimethylallyl)-7-methyladenine perchlorate),[56]
3-phytyladenine,[10]
6-(γ,γ-dimethylallylamino)-9-β-D-ribofuranosylpurine,[11]
6,6-dimethyl-5a,6,7,8-tetrahydro-1,3,4,5a,9-pentaaza-3H-benz[e]indene,[11]
6-furfurylamino-8-azapurine (azakinetin)[31]

6-(*Cyclopropylmethylamino)purine* (*I*,*R*=$(CH_2)_2CHCH_2$). Cyclopropylmethylamine, obtained from the commercially available hydrochloride, was purified by distillation, b.p. 83–84°, n_D^{24} 1·4298, NMR τ values (CCl_4, TMS) 9·71 and 9·15 (5 ring protons); 7·52 (doublet, J = 6 c/s, 2 exocyclic protons); 8·59 (NH_2 protons). A solution of 4·2 g in 50 ml of *n*-butanol was added to 3·0 g of 6-chloropurine and heated under reflux for 4 hr. The solution was evaporated to dryness *in vacuo* leaving a slightly yellowish solid which was triturated with water, collected on a filter, washed with water, and dried. The crude product (3·51 g, 96% yield) was recrystallized from 50% aqueous ethanol as colorless prisms, m.p. 231–233°, which gave a single spot in two paper chromatographic systems; λ_{max}^{EtOH} 268 nm (ϵ 17,600), λ_{min} 228 (1,900); $\lambda_{max}^{H_2O}$ 268 (17,800), λ_{min} 231 (2,000); $\lambda_{max}^{0.1N\ HCl}$ 271 (16,700), λ_{min} 233·5 (2,600); $\lambda_{max}^{H_2O}$ (0·1*N* NaOH) 274 (17,400), 281 (13,700)(sh), λ_{min} 240·5 (3,200).

Anal. Calcd. for $C_9H_{11}N_5$: C, 57·12; H, 5·86; N, 37·02. Found: C, 57·41; H, 5·91; N, 36·88.

The *picrate* crystallized as yellow needles, m.p. 240–242° dec., from 70% aqueous ethanol.

Anal. Calcd. for $C_{15}H_{14}N_8O_7$: C, 43·06; H, 3·37; N, 26·79. Found: C, 42·95; H, 3·30; N, 26·66.

The *hydrochloride* separated as colourless shafts from absolute ethanol, m.p. 225–227° dec.; NMR τ values (D_2O, external TMS): 1·20 and 1·15 (2 purine H's); 6·07 (2H, doublet, J = 8, c/s, N—CH_2); 8·38 (1H, multiplet) and 9·09 (4H, multiplet, cyclopropyl protons).

Anal. Calcd. for $C_9H_{12}ClN_5$: C, 47·89; H, 5·36; N, 31·04. Found: C, 48·13; H, 5·39; N, 30·74.

1,9-*Dibenzyladenine Perchlorate.* The salt was prepared from 1,9-dibenzyladenine[40] and perchloric acid in ethanol and was recrystallized from the same solvent, m.p. 180–181°.

Anal. Calcd. for $C_{19}H_{18}ClN_5O_4$: C, 54·87; H, 4·36; N, 16·85. Found: C, 54·55; H, 4·27; N, 16·77.

Di- and *tri-*(γ,γ-*dimethylallyl)amine hydrochlorides.* To 11·3 g (0·13 mole) of γ,γ-dimethylallylamine[51,52]

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was added dropwise 5·96 g (0·04 mole) of γ,γ-dimethylallyl bromide.[57,58] When the addition was complete the mixture was heated at 95–100° with stirring for 4 hr. The cooled mixture was rendered basic (NaOH) and extracted with ether. The dried ethereal extracts were evaporated under a Vigreux column and then fractionally distilled *in vacuo*. The fraction (2·52 g) boiling at 91–92° (15 mm), n_D^{25} 1·4623, ν_{max}^{film} 3300 cm^{-1} (N—H), 1675 (C=C), was mainly di-(γ,γ-dimethylallyl)amine and that (0·93 g) boiling at 130–133° (14 mm), n_D^{25} 1·4752 ν_{max}^{film} 1675 cm^{-1} (C=C), was mainly tri-(γ,γ-dimethylallyl)amine.

Di-(γ,γ-dimethylallyl)amine hydrochloride was formed from the secondary amine fraction and HCl in ether-ethanol and was recrystallized from the same solvent as leaflets, m.p. 202–204 dec. (preheated at either 100° or 180°), ν_{max}^{Nujol} 2800–2700, 2430 cm^{-1} ($\overset{+}{N}\overset{H}{<}_{H}$ Cl$^-$), 1676 (C=C), 1575 ($\overset{+}{N}H_2$); single spot on paper chromatogram.

Anal. Calcd. for $C_{10}H_{20}ClN$: C, 63·30; H, 10·63; N, 7·38. Found: C, 63·45; H, 10·61; N, 7·59.

Tri-(γ,γ-dimethylallyl)amine hydrochloride was obtained from the tertiary amine fraction in a similar manner, m.p. 211–212° dec. (bath preheated at either 100° or 180°), ν_{max}^{Nujol} 2465 cm^{-1} ($\overset{+}{N}$—H Cl$^-$); 1677 (C=C); single spot on paper chromatogram.

Anal. Calcd. for $C_{15}H_{28}ClN$: C, 69·87; H, 10·95; N, 5·43. Found: C, 69·70; H, 10·98; N, 5·52.

6-(*α,α-Dimethylallylamino)purine* (*I*, *R*=CH_2=CH—$C(CH_3)_2$—). A mixture of 1 g (3·7 m/mole) of 6-chloro-9-(2-tetrahydropyranyl)purine[8] and 2·62 g of α,α-dimethylallylamine[59,60] was heated under reflux with stirring for 24 hr. in a nitrogen atmosphere. It was poured into 5 ml of ethyl acetate and cooled overnight at −15°. The solid which separated (0·48 g, m.p. 230°) was identified as α,α-dimethylallylamine hydrochloride. The filtrate was concentrated in N_2, and further cooling produced a solid, which was recrystallized from ether to give 0·68 g of 6-α,α-dimethylallylamino-9-tetrahydropyranylpurine, m.p. 121°. The hydrolysis of the blocking group was accomplished by stirring this compound in 25 ml of ethanol and 2·5 ml of *N* HCl for 4 hr. The pH of the solution was adjusted to 7·5 with 4*N* NaOH, and the solvent was removed in vacuum. The solid was digested with absolute ethanol, the ethanol solution was filtered, and the filtrate was evaporated to dryness. Recrystallization from ethyl acetate afforded pure 6-(α,α-dimethylallylamino)purine, m.p. 197°, $\lambda_{max}^{H_2O}$ (pH 1) 275 nm (ε 16,200), λ_{min} 234 (2,400); $\lambda_{max}^{H_2O}$ (pH 7) 269 (16,700), λ_{min} 230 (2,100); $\lambda_{max}^{H_2O}$ (pH 12) 283 (sh), 275 (16,400), λ_{min} 240 (3,300); NMR τ values (DMSO-d_6, TMS): 8·38 (6H, two CH_3 groups), in

, (A)=3·65, (B)=4·87, (X)=5·10 (3H, J_{AB}=17·8, J_{BX}=1·0, J_{AX}=10·8 c/s, ABX pattern), 1·86 (1H, purine proton), 1·46 (N^6—H), 1·72 (1H, purine proton).

Anal. Calcd. for $C_{10}H_{13}N_5$: C, 59·09; H, 6·45; N, 34·46. Found: C, 58·88; H, 6·44; N, 33·88.

9-(*2-Tetrahydropyranyl)purine* (*V*). A mixture of 0·5 g of 5% Pd/C, 0·5 g of MgO and 60 ml of 50% aqueous ethanol was stirred for several minutes at 25° in H_2 (1 at.), and a solution of 1 g (4·2 mmole) of 6-chloro-9-(2-tetrahydropyranyl)purine[8] (IV) in 5 ml of ethanol was added. After 10 min the theoretical amount of hydrogen had been absorbed. The catalyst was removed by filtration and washed with ethanol. The combined filtrates were adjusted to pH 8 with dil Na_2CO_3 and were taken to dryness on a rotary evaporator. The residue was digested with 50 ml of ethyl acetate and filtered. The filtrate was evaporated to dryness in N_2 and taken up in 30% pentane in ether. The resulting solution was cooled to give 0·70 g (82%) of 9-(2-tetrahydropyranyl)purine (V), m.p. 62–63°. The m.p. of a mixture of this product with the starting material, m.p. 67–69°, was depressed to 42–58°. Thin-layer chromatography showed a single spot, and the NMR spectrum was consistent with the structure assigned, with the purine ring protons appearing at τ values (CCl_4, TMS) 1·72, 1·22 and 1·00; $\lambda_{max}^{H_2O}$ (pH 1) 260 nm (ε 6,350) $\lambda_{max}^{H_2O}$ (pH 13) 261 (8,000).

Anal. Calcd. for $C_{10}H_{12}N_4O$: C, 58·81; H, 5·92; N, 27·44. Found: C, 58·59; H, 5·90; N, 27·19.

1-(*γ,γ-Dimethylallyl)purine* (*VII*). A mixture of 2·04 g (0·01 mole) of 9-(2-tetrahydropyranyl)purine (V), 25 ml of dry dimethylformamide and 3·0 g (0·02 mole) of γ,γ-dimethylallyl bromide was heated at 80° for 4 hr with stirring and then poured into 250 ml of ether. The ether was decanted from the brown residue (VI). A solution of the residue in 50 ml of water was heated at 100° for 2 hr (pH 1), treated with charcoal, and filtered. The filtrate was brought to pH 7·5 with dil NaOH and extracted with $CHCl_3$. The extracts were dried, partially evaporated, and ether was added. Purification of the solid (600 mg) was effected by recrystallization from toluene, sublimation, and recrystallization from ethyl acetate as colourless needles, m.p. 149°; $\lambda_{max}^{H_2O}$ (pH 1) 270 nm (ε 7,500), λ_{min} 237; $\lambda_{max}^{H_2O}$ (pH 7) 276 (7,450), λ_{min} 247; $\lambda_{max}^{H_2O}$ (pH 11) 276 (7,450), λ_{min} 247; NMR τ values ($CDCl_3$, TMS) 8·12 (6H, singlet, CH_3—C—CH_3), 5·01 (2H, doublet, CH_2—N), 4·55 (1H, multiplet, CH=), 1·42 (2H) and 1·30 (1H).

Anal. Calcd. for $C_{10}H_{12}N_4$: C, 63·38; H, 6·43; N, 29·80. Found: C, 63·64; H, 6·78; N, 29·58.

1-*Benzylpurine* (*VIII*). This compound was obtained by benzylation of 9-(2-tetrahydropyranyl)purine (V)

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with benzyl bromide followed by hydrolysis in the same manner as described above. Purification was effected by recrystallization from ethyl acetate as light yellowish needles, m.p. 212–213°; $\lambda_{max}^{H_2O}$ (pH 1) 268 nm, (pH 7) 276, (pH 11) 276 and corresponding $\lambda_{min}^{H_2O}$ 238, 247 and 247, similar to the spectra of 1-methyl- and 1-ethylpurine;[14] NMR τ values ($CDCl_3$, TMS) 4·53 (2H, N—CH_2), 2·71 (5H, phenyl protons), 1·55, 1·42, 1·35 (purine protons).

Anal. Calcd. for $C_{12}H_{10}N_4$: C, 68·63; H, 4·80; N, 26·68. Found: C, 68·38; H, 4·61; N, 26·52.

This compound was identical with an authentic specimen made *via* the desulfurization of 1-benzyl-6-thiopurine (IX)[12] with W-2 Raney nickel in refluxing ethanol. The conversion was essentially complete in 8 hr. The catalyst and solvent were removed, and the residue was recrystallized from ethyl acetate, needles, m.p. 210–211°, undepressed when mixed with the sample prepared *via* benzylation of 9-(2-tetrahydropyranyl)purine. This comparison validates the structure of 1-(γ,γ-dimethylallyl)purine prepared by the latter route alone.

6-(*γ,γ-Dimethylallylthio)purine* (*X*). To a stirred suspension of 1·0 g (6·6 mmoles) of 6-thiopurine and 0·9 g (6·6 mmoles) K_2CO_3 in 5 ml of dimethylformamide was added 0·9 g (8·6 mmoles) of 1-chloro-3-methyl-2-butene (Eastman Practical Grade), and stirring was continued at 25° for 45 min.[13] The resulting clear solution was poured into 50 ml of water, brought to pH 5 with 2*N* HCl, and filtered. The solid (0·82 g, 57%) was recrystallized from ethyl acetate as colourless, fluffy needles, m.p. 165·5–167°; $\lambda_{max}^{95\%\ EtOH}$ 291 nm (ϵ 20,400), 215 (14,400), λ_{min} 245 (4,000); $\lambda_{max}^{95\%\ EtOH}$ (0·1*N* HCl) 292 (18,500), 215 (13,300), λ_{min} 245 (3,900); $\lambda_{max}^{95\%\ EtOH}$ (0·1*N* NaOH), 292·5 (17,800) λ_{min} 252 (5,400); NMR τ values (DMSO-d_6, TMS) 8·26 and 8·24 (6H, CH_3—C—CH_3), 5·93 (2H, doublet, J = 8 c/s, CH_2—S), 4·54 (1H, triplet, J = 8 c/s, CH=), 1·49 and 1·24 (2H, singlets, purine protons), −3·3 to −3·8 (1H, broad, NH).

Anal. Calcd. for $C_{10}H_{12}N_4S$; C, 54·52; H, 5·49; N, 25·43. Found: C, 54·43; H, 5·49; N, 25·45.

6-*Farnesylaminopurine* (*T.R.* = $(CH_3)_2C{=}CH(CH_2)_2C(CH_3){=}CH(CH_2)_2C(CH_3){=}CHCH_2$—). A solution of 2·9 g (12 mmoles) of 6-chloro-9-(2-tetrahydropyranyl)purine (IV) and 5·8 g (26 mmoles) of farnesylamine[61] in 20 ml of dioxane was stirred overnight at 25° under N_2. The dioxane was removed *in vacuo* and the residue was dissolved in ether and extracted with water. The ethereal solution was dried and evaporated. The resulting oil was dissolved in methanol and the solution was brought to pH 1 with 2*N* HCl. After stirring the solution for 3 hr, it was neutralized with Dowex-1 (HCO_3^-), filtered, and evaporated to about 20 ml. Addition of water to incipient cloudiness followed by cooling caused the precipitation of a brown solid which formed a gum on filtration. Recrystallization of the gum from aqueous methanol and then from ethyl acetate yielded 0·67 g (16%) of solid which was homogeneous by TLC (1 : 1 ethyl acetate N: methanol). Two further recrystallizations from aqueous methanol gave microcrystals, m.p. 118–126°; $\lambda_{max}^{95\%\ EtOH}$ 270 nm (ϵ 19,100), λ_{min} 230 (2,200); $\lambda_{max}^{95\%\ EtOH}$ (0·1 *N* HCl) 280 (18,200), λ_{min} 237·5 (3,950); $\lambda_{max}^{95\%\ EtOH}$ (0·1 *N* NaOH) 276·5 (19,600), 285 (14,500), λ_{min} 243 (5,250); NMR τ values ($CDCl_3$, TMS) 8·1–8·5 (12H, CH_3's), 7·7–8·1 (8H, CH_2's), 5·5–5·8 (2H, CH_2—N), 4·3–5·1 (3H, multiplet, CH=), 3·1–3·4 (1H, multiplet, 6-NH), 1·99 and 1·54 (2H, singlets, purine protons), −3·3 to −2·2 (1H, multiplet, 9-NH).

Anal. Calcd. for $C_{20}H_{29}N_5$: C, 70·75; H, 8·61; N, 20·63. Found: C, 70·59; H, 8·64; N, 20·60.

6-*Geranylaminopurine* (*I.R.* = $(CH_3)_2C{=}CH(CH_2)_2C(CH_3){=}CHCH_2$—). The compound was prepared from geranylamine[61] and 6-chloro-9-(2-tetrahydropyranyl) purine (IV) as described above for 6-farnesylaminopurine. Recrystallization from aqueous methanol yielded blunt needles, m.p. 147–151° (reported[3] 146–148°), yield 66%; $\lambda_{max}^{95\%\ EtOH}$ 269 nm (ϵ 20,900), λ_{min} 229 (1,900); $\lambda_{max}^{95\%\ EtOH}$ (0·1 *N* HCl) 279·5 (19,100), λ_{min} 237 (3,750); $\lambda_{max}^{95\%\ EtOH}$ (0·1 *N* NaOH) 276 (22,800), 283·5 (sh) (16,800), λ_{min} 242·5 (5,400); NMR τ values ($CDCl_3$, TMS) 8·40, 8·32 and 8·25 (3H each, singlets, CH_3), 7·7–8·0 (4H, multiplet, CH_2CH_2), 5·63 (2H, multiplet, CH_2—N), 4·4–5·3 (2H, multiplet, CH=), 3·0–3·3 (1H, multiplet, 6-NH), 1·87 and 1·43 (2H, singlets, purine protons), −3·7 to −4·1 (1H, broad, 9-NH).

Anal. Calcd. for $C_{15}H_{21}N_5$: C, 66·38; H, 7·80; N, 25·81. Found: C, 66·57; H, 7·78; N, 25·77.

6-*Benzylamino*-1-*methylpurine* (*XII, one tautomeric form shown*). A solution of 1·3 g (5·1 mmoles) of 6-benzylthio-1-methylpurine (XI),[14] 25 ml of benzylamine, and 100 ml of abs. ethanol was stirred for 9 hr at 35°. The solvent was removed under vacuum, and the residual syrup was poured into 600 ml of ether. The precipitate was recrystallized from absolute ethanol as colorless microcrystals, m.p. 254–255°, yield 0·60 g (49%); $\lambda_{max}^{95\%\ EtOH}$ 280 nm (ϵ 14,900), 229 (20,100), λ_{min} 249 (4,900); $\lambda_{max}^{95\%\ EtOH}$ (0·1 *N* HCl) 265, 269 (dual) (14,700), λ_{min} 234 (3,800); $\lambda_{max}^{95\%\ EtOH}$ (0·1 *N* NaOH) 274, 278 (dual) (18,700); λ_{min} 244 (6,400); NMR τ values (DMSO-d_6, TMS) 6·33 (3H, singlet, CH_3, 4·53 (2H, singlet, CH_2), 2·65 (5 + H, multiplet, phenyl protons), 2·15 and 1·85 (1H each, singlets, purine protons).

Anal. Calcd. for $C_{13}H_{13}N_5$: C, 65·25; H, 5·48; N, 29·27. Found: C, 65·53; H, 5·35; N, 29·51.

6-*Benzylamino*-3-*methylpurine*. A solution of 1·0 g (4·4 mmoles) of 6-benzylaminopurine,[42–45] 0·4 ml of methyl iodide, and 25 ml of anhydrous dimethylformamide was stirred at 35° for 48 hr. The solvent was removed on a rotary evaporator using an oil pump. The gummy residue was heated with 5 ml of ethanol, and 1·45 g of solid was collected on cooling. The solid was taken up in ethanol, and 4*N* NaOH was added to pH 9. Water (30 ml) was then added, and the ethanol was partially removed. After cooling, 0·83 g (78%) of colorless needles was collected and recrystallized again from water, m.p. 203–204°; $\lambda_{max}^{95\%\ EtOH}$ 291 nm (ϵ 17,500), 218

[61] The amine was generously donated by Hoffmann-La Roche, Inc., through the courtesy of Drs. John Lee and W. E. SCOTT.

(19,000), λ_{min} 248 (3,700); $\lambda_{max}^{95\%\ EtOH}$ (0·1 *N* HCl) 286 (23,500), λ_{min} 242 (4,100); $\lambda_{max}^{95\%\ EtOH}$ (0·1 *N* NaOH) 291 (18,300), λ_{min} 248 (4,900).

Anal. Calcd. for $C_{13}H_{13}N_5$: C, 65·25; H, 5·48; N, 29·27. Found: C, 65·53; H, 5·61; N, 29·54.

1-Benzyl-6-methylaminopurine Hydrobromide (XIV). A solution of 4·0 g (16·4 mmoles) of 6-methylamino-9-(2-tetrahydropyranyl)purine (XIII),[8] 2·2 ml. of benzyl bromide, 50 ml of 2,6-lutidine, and 50 ml of acetonitrile was heated at 75° for 12 hr. The solvents were removed *in vacuo* and the residue was taken up in ethanol, partially evaporated, and cooled. The recovered starting material was removed by filtration, and the filtrate was evaporated to dryness *in vacuo*. This residue was dissolved in water, and the aqueous solution was extracted with ethyl acetate. The water layer was concentrated under vacuum, cooled and filtered. The filtrate was evaporated *in vacuo*. The resulting residue was triturated several times with benzene to yield *ca.* 65 mg (0·12%) of white solid, m.p. 215–218°; NMR τ values (DMSO-d_6, TMS) 6·46 (3H, singlet, CH_3), 4·25 (2H, singlet, CH_2), 2·63 (5H, singlet, phenyl protons), 1·4 and 1·1 (1H each, singlets, purine protons).

Qualitative u.v. data were obtained on the sample recovered from the NMR run after neutralization; λ_{max}^{EtOH} 278 nm λ_{min} 251; $\lambda_{max}^{acidic\ EtOH}$ 263, λ_{min} 240; $\lambda_{max}^{basic\ EtOH}$ 271, 279 (shoulder), λ_{min} 246. These maxima and minima are clearly similar to those reported for other 1,N^6-disubstituted adenines.[40]

Anal. Calcd. for $C_{13}H_{14}BrN_5$: C, 48·76; H, 4·41; N, 21·56. Found: C, 48·88; H, 4·67; N, 19·94. (Inconsistent, low values for nitrogen were obtained, whereas all other properties were satisfactory).

Methyl 5-Deoxy-5-(purin-6-yl)amino-β-D-ribofuranoside (Ie). This kinetin analog has been synthesized by the reaction of 6-chloro-9-(2-tetrahydropyranyl)purine with methyl 5-amino-5-deoxy-2,3-*O*-isopropylidene-β-D-ribofuranoside to give methyl 5-deoxy-2,3-*O*-isopropylidene-5-[9-(2-tetrahydropyranyl)purin-6-yl]amino-β-D-ribofuranoside, followed by treatment with methanol and HCl.[62]

6-(γ,γ-Dimethylallylamino)-9-β-D-ribofuranosylpurine. The experimental procedure was patterned after the method which Jones and Robins[54] used to make the corresponding methyl derivative. A mixture of 5·34 g (0·02 mole) of adenosine, 5·96 g (0·04 mole) of γ,γ-dimethylallyl bromide and 80 ml. of dimethylacetamide was stirred at 20° for 27 hr., followed by evaporation and treatment with ether. The crude salt (50% yield) was separated by decantation and dissolved in 100 ml of 0·45 *N* NaOH. After heating at 90–95° for 75 min, the mixture was cooled with ice-water, the pH of the mixture was adjusted to 7·5 with 10% HCl, and the oil which had separated was extracted with ethyl acetate. The ethyl acetate solution was dried and evaporated *in vacuo*, leaving a reddish brown oil which solidified when triturated with *ca.* 30 ml of absolute ether. After four recrystallizations from absolute ethanol, the product was obtained as small prisms, m.p. 142–143°; λ_{max}^{EtOH} 271 nm (ε 19,200), λ_{min} 232 (2,000); $\lambda_{max}^{H_2O}$ 269 (20,000), λ_{min} 232 (2,300); $\lambda_{max}^{0·1N\ HCl}$ 265 (20,300), λ_{min} 233 (3,900); $\lambda_{max}^{0·1N\ NaOH}$ 269 (19,800), λ_{min} 234 (3,200).

Anal. Calcd. for $C_{15}H_{21}N_5O_4$: C, 53·72; H, 6·31; N, 20·89. Found: C, 53·48; H, 6·33; N, 20·73.

The synthesis has been improved by Dr. W. A. H. Grimm at Illinois.[11] Since the original synthesis by Dr. T. Fujii,[10] another method of preparation has been reported by Hall, Robins, Stasiuk, and Thedford,[25] and 6-(γ,γ-dimethylallylamino)-9-β-D-ribofuranosylpurine has been isolated from serine t-RNA[24] and from yeast s-RNA[25] and calf liver s-RNA.[25]

6-(2,3-Dihydroxy-3-methylbutylamino)purine. This compound was prepared by reaction of 6-(γ,γ-dimethylallylamino)purine with basic potassium permanganate in *t*-butanol:water (2:5). Crystallization from water yielded a white solid, m.p. 258–259°; $\lambda_{max}^{95\%\ EtOH}$ (0·1 *N* HCl) 275nm (ε 15,600), λ_{min} 237 (2,200); $\lambda_{max}^{95\%\ EtOH}$ 269 (16,600), λ_{min} 230 (870); $\lambda_{max}^{95\%\ EtOH}$ (0·1 *N* NaOH) 276 (18,200), 285 (shoulder, 13,500), λ_{min} 242 (4,100); NMR τ values (DMSO-d_6, TMS) 8·88, 8·85 (3H each, CH_3—C—CH_3), 6·60 (center of m, 4H), 1·85, 1·77 (2H, singlets, purine protons).

Anal. Calcd. for $C_{10}H_{15}N_5O_2$: C, 50·62; H, 6·37; N, 29·52. Found: C, 50·86; H, 6·49; N, 29·60.

Another sample, identical in u.v. spectral characteristics and chromatographic behavior was prepared by the following improved procedure. A mixture of 2·1 g (10 mmoles) of 6-(γ,γ-dimethyllylamino)purine, 1 g (25 mmoles) of sodium hydroxide, 300 ml of water, and 500 ml of *t*-butanol was chilled in an ice bath. A chilled mixture of 1·9 g (12 mmoles) of $KMnO_4$ and 1 g (25 mmoles) NaOH in 200 ml H_2O was added quickly in one batch. After 15 min of vigorous stirring, 2·75 g (26 mmoles) of $NaHSO_3$ was added and stirring was continued for 15 min. The mixture was then allowed to stand for 2 hr. The MnO_2 was filtered off and the filtrate was adjusted to pH 7 (4 *N* HCl). After concentration to about 100 ml *in vacuo* the filtrate deposited 1·7 g (69%) of 6-(2,3-dihydroxy-3-methylbutylamino)purine, m.p. 258–260°.

Bioassay Procedures

General test procedures have been reported.[22, 63, 64]

The test substances were dissolved in double distilled water of slightly less than the desired final volume by heating for 10–20 min in an Arnold steamer. However, no heat was used in the preparation of the solutions which were filter sterilized. Instead the weight of chemical required to make 100 ml of a 50–100 μM solution

[62] N. J. Leonard and K. L. Carraway, *J. Heterocyclic Chem.* **3**, 485 (1966).
[63] T. Murashige and F. Skoog, *Physiol. Plantarum* **15**, 473 (1962).
[64] E. M. Linsmaier and F. Skoog, *Physiol. Plantarum* **18**, 100 (1965).

was suspended in a slightly smaller volume, and the solution was shaken by hand until the chemical was dissolved. 3–5 drops of 1 *N* HCl were added if necessary to dissolve the substances. The volume was then adjusted to 100 ml and appropriate volumes and serial dilutions were prepared by mixing water and double strength basal medium. At least one test of each chemical was done with filter sterilized solutions which were added to the medium after it had been autoclaved and cooled to near the gelation point. Fresh solutions were prepared for each experiment to minimize effects of possible chemical breakdown. Callus originally derived from the pith of tobacco plants (*Nicotiana tabacum* (var. Wisconsin No. 38) and maintained in subcultures for different periods up to 2 yr were used exclusively. Prior to March 26, 1964, the stock was kept on Murashige and Skoog medium and thereafter on the further revised medium (RM-1965)[64] with 0·20 mg/l kinetin, 2·0 mg/l IAA, 0·4 mg/l thiamine hydrochloride and 100 mg/l *myo*-inositol. To assure stock tissues of comparable age and quality at all times, a separate set of cultures was transferred each week. Before use in the bioassays, the stock tissue was subcultured for two 4-week passages on medium with only 30 μg/l of kinetin. Loose friable tissue suitable for the bioassay were obtained by this procedure.

Also in experiments the revised medium of Murashige and Skoog was used prior to March 26, 1964 and RM-1965 thereafter. [Four replicate cultures, with three pieces of callus (each piece weighing 25–40 mg) planted on 50 ml of agar medium were used for each treatment]. The cultures were kept at *ca.* 28° in continuous diffuse light. After a 5-week growth period, representative cultures were photographed, and the total fresh and dry weight yields were determined.