Phytochemistry, 1973, Vol. 12, pp. 2445 to 2455. Pergamon Press. Printed in England.

# CYTOKININS FROM *ZEA MAYS**

DAVID STUART LETHAM

Research School of Biological Sciences, Australian National University, Canberra, Australia

(*Received* 16 *March* 1973. *Accepted* 2 *May*, 1973)

**Key Word Index**—*Zea mays*; Gramineae; sweet corn; cytokinins; purine derivatives, cell division.

**Abstract**—A number of adenine derivatives with cytokinin activity were isolated from immature sweet corn (*Zea mays*) kernels. The following structures were assigned: 9-β-D-ribofuranosylzeatin, 9-β-D-ribofuranosylzeatin 5'-monophosphate, 6-(1-carboxy-2-hydroxypropylamino)-9-ribofuranosylpurine, 6-(2,3,4-trihydroxy-3-methylbutylamino)purine, 2-hydroxy-6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine, 6-(3,4-dihydroxy-3-methylbutylamino)purine, a 9-glycoside of zeatin (identity of sugar moiety not established), and 6-(1,2-dicarboxyethylamino)-9-β-D-ribofuranosylpurine.

## INTRODUCTION

THE ABILITY of extracts of immature sweet corn kernels to induce cell division in carrot phloem tissue cultures was established many years ago.[1,2] These extracts were later found to promote growth of cultures of other plant tissues, for example, apple fruitlet,[3] tobacco stem pith[4] and soybean callus.[5] Because the activity of corn extracts could not be accounted for by the then-known naturally-occurring growth regulators, attempts were made to purify the active compounds,[5–9] and zeatin, the first naturally occurring cytokinin to be purified, was eventually isolated in crystalline form.[10–13] Zeatin, 6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine,[14] was highly active in several cytokinin bioassays[15] but accounted for only part of the cytokinin activity of corn extract.[12,16] The other active compounds have now been identified. Some aspects of this work have been presented previously in preliminary form.[17–19]

* Part XV in the series "Regulators of Cell Division in Plant Tissues". For Part XIV see LETHAM, D. S., PARKER, C. W. and GORDON, M. E. (1972) *Physiol. Plant.* **27,** 285.

[1] NETIEN, G., BEAUCHESNE, G. and MENTZER, C. (1951) *Compt. Rend.* **233,** 92.
[2] STEWARD, F. C. and CAPLIN, S. M. (1952) *Ann. Botany* **16,** 491.
[3] LETHAM, D. S. (1958) *Nature* **182,** 473.
[4] BEAUCHESNE, G. (1962) *Rev. Bot.* **69,** 493.
[5] MILLER, C. O. (1961) *Proc. Nat. Acad. Sci. U.S.* **47,** 170.
[6] STEWARD, F. C. and SHANTZ, E. M. (1959) *Annu. Rev. Plant. Physiol.* **10,** 379.
[7] BEAUCHESNE, G. (1961) in *Plant Growth Regulation*, p. 667, Iowa State University Press, Ames, Iowa.
[8] BEAUCHESNE, G., LEBOEUF, M. and GOUTAREL, R. (1964) in *Regulateurs Naturels de la Croissance Végétale*, p. 119, C.N.R.S., Paris.
[9] SHANTZ, E. M. and STEWARD, F. C. (1964) in *Regulateurs Naturels de la Croissance Végétale*, p. 59, C.N.R.S., Paris.
[10] LETHAM, D. S. (1963) *Life Sci.* **2,** 569.
[11] LETHAM, D. S. (1964) in *Regulateurs Naturels de la Croissance Végétale*, p. 109, C.N.R.S., Paris.
[12] LETHAM, D. S. (1966) *Phytochemistry* **5,** 269.
[13] LETHAM, D. S. and MILLER, C. O. (1965) *Plant Cell Physiol.* **6,** 355.
[14] LETHAM, D. S., SHANNON, J. S. and MCDONALD, I. R. C. (1967) *Tetrahedron* **23,** 479.
[15] LETHAM, D. S. (1967) *Planta* **74,** 228.
[16] MILLER, C. O. (1965) *Proc. Nat. Acad. Sci. U.S.* **54,** 1052.
[17] LETHAM, D. S. (1966) *Life Sci.* **5,** 551.
[18] LETHAM, D. S. (1966) *Life Sci.* **5,** 1999.
[19] LETHAM, D. S. (1968) in *Biochemistry and Physiology of Plant Growth Substances*, p. 19, Runge Press, Ottawa.

## RESULTS

In addition to zeatin, sweet corn extracts are known to contain a number of other cytokinins. Certain of these compounds are not extracted by *n*-butanol from an aqueous solution (pH 7), while others, which like zeatin are extractable by *n*-butanol, exhibit $R_f$ between 0·20 and 0·50 (cf. zeatin, 0·70) on paper chromatograms with solvent *E*[12]. Methods for fractionating the complex mixture of cytokinins and the characterization of the active compounds by their chromatographic behaviour were attempted first.

Adsorption onto charcoal and elution with pyridine–water (1:9) gave a good recovery of the factors which were not extracted by butanol, but a very poor recovery of the butanol-extractable factors including zeatin. The active compounds of $R_f$ 0·20–0·50 (solvent *E*) were almost completely lost. A considerable proportion of the activity in corn extract was retained by the cation exchanger, Zeo-Karb 225, and eluted with dilute aqueous $NH_4OH$, but precipitation of the cytokinins in the eluted fraction with $AgNO_3$, a procedure of value in the isolation of zeatin,[12] was not satisfactory. Partial loss of other factors extracted by butanol occurred. At pH 11 the anion exchanger, De-Acidite FF, retained a very large proportion of the total activity including all factors not extracted by butanol, and also some active compounds extracted by butanol. Elution with 1·5 N acetic acid gave a good recovery of the latter but not of the former compounds. However these were eluted effectively by 2 N formic acid.

TABLE 1. THE DISTRIBUTION OF CYTOKININ ACTIVITY ON PAPER CHROMATOGRAMS†

| Solvent | *A* | | *E* | | *E* | |
|---|---|---|---|---|---|---|
| Fraction | *A* | | *F* | | *H* | |
| | $R_f$ | Increment % | $R_f$ | Increment % | $R_f$ | Increment % |
| | 0·08 | +100 | 0·11 | −4 | 0·08 | 0 |
| | 0·16 | +13 | 0·18 | +1 | 0·16 | +21 |
| | 0·25 | +18 | 0·27 | +50 | 0·24 | +10 |
| | 0·33 | +2 | 0·37 | 0 | 0·34 | +42 |
| | 0·45 | +1 | 0·43 | +10 | 0·40 | +84 |
| | 0·50 | +2 | 0·52 | +2 | 0·48 | +70 |
| | 0·58 | +1 | 0·59 | +1 | 0·58 | +28 |
| | 0·66 | +1 | 0·66 | +75 | 0·66 | +5 |
| | 0·78 | +1 | 0·75 | +100 | 0·74 | +100* |
| | 0·89 | +3 | 0·84 | +15 | 0·84 | 0 |
| | 1·00 | +2 | 0·93 | 0 | 0·90 | −5 |
| | | | 1·00 | +3 | 1·00 | +3 |

* Position of co-chromatographed zeatin.
† Each $R_f$ value listed defines the upper limit of a chromatographic zone.

By the procedures based on ion-exchange resins (see Experimental) sweet corn extracts were fractionated with little loss of total activity. The complete procedure yielded three active fractions (*A*, *F* and *H*) and three fractions (*D*, *G* and *I*) which were usually inactive. In some experiments, fraction *D* showed very weak activity. The relative total activities of the fractions were: $A = B > H > F \gg D$. The distributions of cytokinin activity (carrot phloem assay) on paper chromatograms of fractions *A*, *F* and *H* are presented in Table 1.

*The Active Compounds in Fraction* A

Bioassay of a chromatogram of fraction *A* (solvent *A*) revealed high activity at $R_f$ 0·05 and a zone of weak activity at $R_f$ 0·21 (see Table 1). All the activity in an aqueous solution of fraction *A* was adsorbed by charcoal and a high proportion recovered by elution with pyridine-water (1:9). No activity was extracted from an aqueous solution (pH 7) of the eluted fraction by ethyl acetate or by *n*-butanol. The active compounds in fraction *A* were purified from an aqueous solution of the water-soluble fraction of the ethanol extract by the following sequence of steps: adsorption onto and elution from charcoal, *n*-butanol extraction of an aqueous solution of the eluted fraction, anion-exchange chromatography, and preparative PC using the solvent sequence *B*, *C*, *D* and *A*. The final chromatographic separation using solvent *A* yielded a highly active fraction termed *Aa* and a weakly active fraction of higher $R_f$ termed *Ab*. Anion-exchange chromatography of *Aa* on Dowex 1 yielded a cytokinin designated C3 because, when first isolated,[18] it was the third cytokinin purified from sweet corn. Chromatography of *Ab* on Dowex 1 yielded two very weakly active compounds termed C4 and C5. C3 accounted for the activity at $R_f$ 0·05 on the chromatogram (solvent *A*) of fraction *A* (see Table 1), while C4 and C5 accounted for the weak activity at $R_f$ 0·21.

*Compound C3*. C3 was hydrolysed by a pure alkaline phosphatase to a compound termed C3a which was degraded to C3b by periodate oxidation followed by reaction with cyclohexylamine (a procedure which degrades ribonucleosides to bases). The m.p. of the picrate of C3b, UV spectra, and TLC indicated that C3b was zeatin. Confirmation was provided by oxidation of C3b with $KMnO_4$ under conditions previously used for the oxidation of zeatin.[14] TLC and UV spectroscopy indicated that C3b and zeatin yielded the same oxidation products, namely, adenine, *N*-(purin-6-yl)glycine and a compound [probably 6-(2,3,4-trihydroxy-3-methylbutylamino)purine] which is degraded by periodate. Hence C3b was identified as zeatin.

C3a when hydrolysed by heating with a sulphonic acid resin yielded a sugar which co-chromatographed with ribose during TLC on silica gel (three solvents) and yielded a colour with the anisaldehyde-$H_2SO_4$ spray for sugars[20] identical to that given by ribose. The sugar of C3a reacted with chromogenic reagents (orcinol[21] and *p*-bromophenylhydrazine[22]) to yield coloured solutions with absorption spectra ($\lambda_{max}$ 671 and 448 nm respectively) identical to those given by ribose. The ribose content of C3a determined by the orcinol method was 0·97 mol per mol of C3a (MW of C3a was taken as 351, the MW of zeatin riboside); assuming the same MW, periodate consumed per mol of C3a was 0·95 mol. The above results strongly suggested that C3a was a riboside of zeatin. C3a did not depress the m.p. of authentic, synthetic 9-β-D-ribofuranosylzeatin. Furthermore, the UV spectra of C3a (at pH 2, 7 and 11) and the MS duplicated those of the synthetic riboside. The two compounds could not be distinguished by a variety of chromatographic methods or by thin-layer electrophoresis in borate buffer. Hence C3a was 9-β-D-ribofuranosylzeatin.

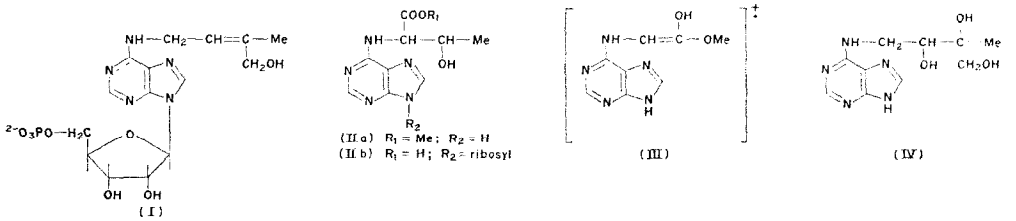
The phosphorus content (0·98 g-atom per mol of ribose) indicated that C3 was a monophosphoric ester of zeatin riboside. This was confirmed by the fact that AMP and C3 exhibited very similar mobilities during thin-layer electrophoresis [mobility of C3/mobility of AMP: at pH 3·6 (acetate buffer), 0·91; at pH 9·3 (borate buffer), 0·91]. The phosphate

[20] LEWIS, B. A. and SMITH, F. (1969) in *Thin-layer Chromatography* (Stahl, E., ed.), p. 810, Springer, Berlin.
[21] MARKHAM, R. (1955) in *Modern Methods of Plant Analysis* (PAECH, K. and TRACEY, M. V., eds.), Vol. 4, p. 246, Springer, Berlin .
[22] WEBB, J. M. (1956) *J. Biol. Chem.* **221**, 635.

group could be located at the 2′, 3′ or 5′ positions of the ribose moiety or it could engage in ester linkage with the allylic hydroxyl of the side chain. Since C3 was not dephosphorylated by base, the latter was unlikely. C3 reacted positively to a periodate spray reagent[23] which detects *vic*-glycols, indicating the 2′ and 3′ hydroxyl groups were not esterified. Hence the phosphate group appeared to be located at the 5′ position and this was confirmed by the hydrolysis of C3 to C3a with snake venom 5′-nucleotidase.

The PMR spectrum of C3 was determined with a 0·8-mg sample of the barium salt in $D_2O$ solution using a computer averaging technique. A series of complex signals were just detectable over the range δ 3·3–5·5 ppm, but a prominent singlet occurred at δ 1·73. This indicated the presence of a methyl group attached to an olefinic linkage and not coupled to adjacent protons. Hence the view that the side chain of C3 was the same as that in zeatin was supported by the PMR spectrum. Hence C3 was assigned the structure 6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)-9-β-D-ribofuranosylpurine 5′-monophosphate (I). Synthetic I prepared by Shaw *et al.*[24] and C3 were chromatographically indistinguishable.

*Compound C4.* This compound exhibited UV spectra characteristic of $N^6$, 9-disubstituted adenines. A pentose sugar moiety was indicated by the orcinol colour reaction but the compound did not contain phosphorus. The presence of an anionic group was established by thin-layer electrophoresis (pH 7·8; ammonium bicarbonate buffer); the mobility of C4 relative to that of AMP was 0·64. The IR spectrum showed a broad band at 1726 $cm^{-1}$ suggesting the presence of a carboxyl group. The molecular ion was not revealed in the MS which consisted almost entirely of peaks (*m/e* 160, 149, 148, 136, 135, 120, 119, 108, 81) which are common in the spectra of 6-(substituted amino)purines.[25] A MS of the methyl ester of C4 also failed to reveal the molecular ion. In order to obtain a derivative more suitable for MS, the sugar moiety of C4 was cleaved. Cleavage by reacting successively with periodate and an amine yielded two products with strong UV absorption, suggesting the presence of a periodate-sensitive group in the side chain. Acid hydrolysis, however, yielded one UV-absorbing product and this was methylated with diazomethane to yield C4a. Consideration of the MS of this methyl ester suggested the compound was probably IIa. The fragment ion with *m/e* 207 was assigned structure III and appears to arise by cleavage between the α and β carbons with elimination of acetaldehyde and hydrogen transfer from the hydroxyl to the carbonyl function. The other fragment ions in the spectrum of C4a are readily rationalized in terms of structure IIa.




Compound C4 was probably the riboside of the corresponding free acid (IIb). Hence this was synthesized and found to be indistinguishable from C4 by TLC on cellulose (several solvents), by thin-layer electrophoresis at pH 7·8, and by IR and UV spectroscopy. The MS of synthetic IIa (prepared by esterifying the known acid[26] with $CH_2N_2$) was identical to

[23] CIFONELLI, J. A. and SMITH, F. (1954) *Anal. Chem.* **26,** 1132.
[24] SHAW, G., SMALLWOOD, B. M. and WILSON, D. V. (1968) *J. Chem. Soc.* C, 1516.
[25] SHANNON, J. S. and LETHAM, D. S. (1966) *New Zealand J. Sci.* **9,** 833.
[26] WARD, D. N., WADE, J., WALBORG, E. F. and OSDENE, T. S. (1961) *J. Am. Chem. Soc.* **26,** 5000.

that of C4a. Hence C4 was 6-(1-carboxy-2-hydroxypropylamino)-9-ribofuranosylpurine (IIb).

*Compound C5.* The presence of a sugar moiety, possibly ribose, in C5 was indicated by spray reactions on thin-layer chromatograms. The UV spectra of C5 were typical of an $N^6$,9-disubstituted adenine. C5 was not a phosphoric ester but it did migrate during thin-layer electrophoresis at pH 7·8 in ammonium bicarbonate buffer (mobility relative to AMP, 1·21) showing the presence of an anionic group. Two intense bands in the IR spectrum (1716 and 1420 $cm^{-1}$), which are absent in the spectra of $N^6$-alkyladenosines, indicated that C5 was a carboxylic acid. Since its electrophoretic mobility was double that of C4, C5 was probably a dicarboxylic acid. It might therefore be the known compound 6-(1,2-dicarboxyethylamino)-9-β-D-ribofuranosylpurine, the nucleoside of adenylosuccinic acid. This nucleoside was therefore synthesized and found to be identical to C5 by IR and UV spectroscopy, by TLC of both the free acid and the methyl ester, and by thin-layer electrophoresis.

*Active Compounds in Fraction* F

Fraction *F* contained cytokinins retained by a cation, but not an anion, exchanger, and yielded two active compounds on fractionation.

*Compound C2.* The cytokinin principally responsible for the activity at $R_f$ 0·70 (Table 1) was isolated in crystalline form and termed C2. Degradation of C2 with periodate oxidation followed by reaction with cyclohexylamine gave a compound indistinguishable from zeatin by UV spectroscopy, MS and PC. C2 therefore appeared to be a glycoside of zeatin. The MS and UV spectra, PC and m.m.p. determinations established that C2 was 9-β-D-ribofuranosylzeatin. Evidence has been presented previously[19] that a second compound (termed C6), possibly the *cis*-isomer of zeatin riboside, also contributes to the activity at about $R_f$ 0·70. The identity of this minor cytokinin has not been investigated further.

*Compound C8.* When fraction *F* was subjected to PC (solvent *E*), a zone of activity was centred at about $R_f$ 0·25, but this was weak relative to the zone at $R_f$ 0·70. The compound (C8) responsible for the activity at $R_f$ 0·25 was obtained in chromatographically homogeneous form but there was insufficient material for crystallization. The MS exhibited peaks at *m/e* 160, 148, 136, 135, 120, 119 and 108 which are characteristic of 6-(substituted amino)purines. A weak peak was present at *m/e* 219 (MW of zeatin) and below this the spectrum was almost identical to that of zeatin. This indicated that C8 was a 6-(substituted amino)purine and probably contained an intact zeatin moiety. Above *m/e* 219, there were a number of weak peaks (most prominent *m/e* 248, 364); some of these were probably due to impurities and the $M^+$ peak could not be located definitely. Degradation of C8 by successive treatment with periodate and cyclohexylamine yielded a compound with the properties of zeatin (TLC, MS, UV spectra). Hence C8 was a glycoside of zeatin and the UV spectra indicated it was a 9-glycoside.

*Active Compounds in Fraction* H

Fraction *H*, containing cytokinins retained by both a cation and an anion exchange resin, yielded by PC (solvent *E*) a broad zone of activity centred about $R_f$ 0·4 (see Table 1) and a second active zone of higher $R_f$ (0·70). Preliminary studies indicated that the latter activity was due to zeatin (previously termed[19] Cl) and hence this zone was not further investigated. However the active zone of lower $R_f$ contained at least four cytokinins; three

of these (C7, C9, C10) were assigned structures, while a fourth was found to be identical to compound C8 previously isolated from fraction *F*.

*Compound C7.* The MS and UV spectra of C7 were characteristic of a 6-(substituted amino)purine. The MS indicated a MW of 253 for the compound and this was confirmed by the MS of the acetates of C7. Acetylation with acetic anhydride–pyridine yielded an acetyl derivative of MW 337 (diacetate) which when reacetylated with acetic anhydride in the presence of *p*-toluene sulphonic acid gave a derivative of MW 379 (triacetate). Studies with model compounds showed that *N*-acetyl derivatives of the adenine moiety would not be produced under the conditions used; thus 6-propylaminopurine was not acetylated by either of the above methods. Hence the sites of acylation were in the C7 side chain and the results are consistent with the presence of one tertiary hydroxyl group. By the method of Weiss and Smith,[27] C7 was found to be oxidized by periodate with formation of formaldehyde. In the MS of diacetyl C7, a prominent peak occurred at *m/e* 160 and a metastable peak indicated the transition 259 [i.e. M-($H_2O$ + $CH_3COOH$)] → 160 (for probable structures for this ion, see Refs. 25 and 28). Hence the carbon atoms $\alpha$ and $\beta$ to the exocyclic nitrogen in C7 probably did not carry an alkyl group. These observations indicated that C7 probably possessed structure IV. This was therefore synthesized and found to be indistinguishable from C7 by TLC, UV spectroscopy and MS. Hence C7 was very probably 6-(2,3,4-trihydroxy-3-methylbutylamino)purine.

*Compound C9.* The MS of C9 resembled that of zeatin but differed in one very significant respect; the *m/e* values for the peaks were 16 greater than those for the corresponding peaks in the zeatin spectrum. This suggested the compound contained the zeatin side chain but in addition possessed a hydroxyl group attached to the purine ring; the UV spectrum indicated it was at position 2. The MS and UV spectra and chromatographic behaviour of C9 were identical to those of synthetic 2-hydroxy-6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine,[29] i.e. 2-hydroxyzeatin. This structure was therefore assigned to C9.

*Compound C10.* The UV spectra of C10 were characteristic of 6-(monosubstituted amino)-purines; the MS indicated that MW of C10 was 237 and that the substituent was $C_5H_9(OH)_2$. The peak at *m/e* M-31 and a metastable peak for the transition 237→206 established the presence of a $-CH_2OH$ group. Since C10 was oxidized by periodate with production of formaldehyde,[27] the $-C(OH)-CH_2OH$ grouping was indicated. The strong peak at *m/e* 162 indicated two methylene groups occurred adjacent to the exocyclic nitrogen atom. These observations indicated C10 was the known compound,[30] 6-(3,4-dihydroxy-3-methyl-butylamino)purine. The picrate of this purine was synthesized and found to be identical to C10 picrate (UV spectra, MS, m.m.p.).

## DISCUSSION

From sweet corn extracts, a number of compounds with cytokinin activity were purified. It is noteworthy that all are substituted adenines and that no evidence was obtained for the occurrence of a substituted-purin-6-one cytokinin of the type reported by Wood.[31] Compounds C2, C3, C7, C8, C9 and C10 could all be formed by enzymic modification of zeatin. Formation of C7 and C10 would involve hydroxylation of the zeatin side chain,

[27] WEISS, J. B. and SMITH, I. (1967) *Nature* **215**, 638.
[28] LETHAM, D. S., SHANNON, J. S. and MCDONALD, I. R. (1964) *Proc. Chem. Soc.* No. 230.
[29] HECHT, S. M., LEONARD, N. J., SCHMITZ, R. Y. and SKOOG, F. (1970) *Phytochemistry* **9**, 1173.
[30] LEONARD, N. J., HECHT, S. M., SKOOG, F. and SCHMITZ, R. Y. (1968) *Israel J. Chem.* **6**, 539.
[31] WOOD, H. N. (1970) *Proc. Nat. Acad. Sci. U.S.* **67**, 1283.

while hydroxylation at position 2 would yield C9. Crotonoside (2-hydroxyadenosine) is a structurally related compound previously isolated from plants. C4 and C5 are the ribosides of purin-6-yl amino acids. C5, the nucleoside of adenylosuccinic acid which is an intermediate in the biosynthesis of AMP, has not previously been isolated from a plant tissue.

In order of decreasing activity in the radish cotyledon cytokinin bioassay, the compounds purified from sweet corn were (bracketed compounds are approximately equally effective): C1 (zeatin), (C2 and C3), (C8 and C9), (C7 and C10), C4 and C5. Isolated C2, C3, C4, C5, C9 and C10 exhibited activities equal to those of the synthetic compounds; however, isolated C7 was considerably more active than synthetic 6-(2,3,4-trihydroxy-3-methylbutylamino)purine which was appreciably less active than C10. The two most probable explanations for this discrepancy are the following: firstly, stereochemical differences between the isolated and synthetic compounds; secondly, the isolated C7 could have been impure and contaminated by a more active compound. At 50 $\mu$M, C2, C3, C8 (concentration of C8 estimated from UV absorption) and C9 all induced marked growth increments (range 60–90% of weight of control cotyledons), but C10, synthetic C7, C4 and C5 evoked only small increments (range 7–20% of weight of control) and were inactive at 5 $\mu$M. Hence hydroxylation of the zeatin side chain markedly reduces activity in the radish cotyledon assay. Previously synthetic C10 was reported to be less effective than zeatin in the tobacco pith cytokinin assay.[30,32] Zeatin nucleotide (C3) has previously been compared with zeatin in the carrot phloem assay and found to be less active.[18]

Extracts of several plant tissues have been reported to contain unidentified cytokinins which are retained by cation exchange resins, are extracted from aqueous solution by *n*-butanol, and exhibit $R_f$ of about 0·5 in solvents *E* and *G*. Cytokinins with these properties have been extracted from pumpkin seeds,[33] watermelon seeds,[34] *Rhizobium japonicum*[35] and apple fruitlets.[36] These unidentified cytokinins could be C7, C8 and C9 which also possess the properties mentioned above.

## EXPERIMENTAL

*Materials and methods. Bioassays and analytical methods.* The carrot-phloem[15] and radish cotyledon[37] cytokinin bioassays were performed as described previously. The latter assay was used to give a rapid, preliminary assessment of activity of fractions which had been considerably purified. Activity was subsequently confirmed by the carrot assay. The following analytical methods were used: ribose determination, orcinol method of Markham;[21] phosphorus, method of Morrison;[38] periodate consumption by nucleosides, according to Dixon and Lipkin.[39] All MS were determined with an AEI MS9 mass spectrometer; IR spectra were obtained with micro KBr discs using a beam condenser. M.ps determined with a Kofler micro apparatus are referred to as 'micro' m.ps to distinguish them from capillary m.ps.

*Chromatographic methods.* Merck $PF_{254}$ silica gel was used for all preparative TLC. In preparative TLC the chromatographic zones were eluted with MeOH–$H_2O$–HOAc (75:25:2). For large-scale preparative PC, sheets of Whatman No. 120 (drop reaction) paper were usually used. Prior to use, these were washed exhaustively with 1% HOAc and then $H_2O$. Usually the zones of PCs were eluted with 1% HOAc; however if the eluate was to be used directly for MS, elution was effected with MeOH. Compounds which absorbed UV light were located on chromatograms with a Mineralight lamp (254 nm). On some occasions, UV printing of PCs according to Markham and Smith[40] was desirable, especially when purines were partially

[32] Leonard, N. J., Hecht, S. M., Skoog, F. and Schmitz, R. Y. (1969) *Proc. Nat. Acad. Sci. U.S.* **63,** 175.
[33] Gupta, G. R. P. and Maheshwari, S. C. (1970) *Plant Physiol.* **45,** 14.
[34] Prakash, R. and Maheshwari, S. C. (1970) *Physiol. Plant.* **23,** 792.
[35] Phillips, D. A. and Torrey, J. G. (1970) *Physiol. Plant.* **23,** 1057.
[36] Letham, D. S. and Williams, M. W. (1969) *Physiol. Plant.* **22,** 925.
[37] Letham, D. S. (1971) *Physiol. Plant.* **25,** 391.
[38] Morrison, W. R. (1964) *Anal. Biochem.* **7,** 218.
[39] Dixon, J. S. and Lipkin, D. (1954) *Anal. Chem.* **26,** 1092.
[40] Markham, R. and Smith, J. D. (1949) *Biochem. J.* **45,** 294.

masked by fluorescent compounds. The following chromatographic solvents were used: (*A*) *n*-BuOH–HCOOH–$H_2O$ (10:4:5 upper phase); (*B*) MeOH–HCOOH–$H_2O$ (16:3:1): (*C*) MeOH–*iso*-PrOH–14N $NH_4OH$–$H_2O$ (9:6:3:2); (*D*) *n*-PrOAc–HCOOH–$H_2O$ (11:5:3); (*E*) *n*-BuOH–HOAc–$H_2O$ (12:3:5); (*F*) MeCOEt–satd. $H_2O$; (*G*) *n*-BuOH satd. $H_2O$; (*H*) *n*-BuOH–14N $NH_4OH$–$H_2O$ (6:1:2 upper phase).

*Preparation of sweet corn extracts.* The kernels were stripped from immature cobs of sweet corn (*Zea mays*, cv. Golden Cross Bantum) and stored at −20°. Frozen kernels (1 kg) were homogenized with 95% EtOH (3 l.) in a large Waring blendor and this process was repeated until the required amount of corn had been processed. The bulked homogenates were stirred at room temp. for 4–5 hr and then filtered. The filtrate was concentrated in a cyclone evaporator at 30° to about one-eleventh of the original vol. After clarification by filtration, the concentrate was used for isolation of cytokinins.

*Fractionation of extracts and purification of active compounds. Fractionation with ion-exchange resins to give fractions* A-I. Concentrated extract derived from 4·5 kg of sweet corn kernels was acidified to pH 3·4, filtered and percolated through a column (5 × 30 cm) of Zeo-Karb 225 (SRC 10; $NH_4^+$ form equilibrated to pH 3·4 by washing with 0·1 M $NH_4OAc$ buffer of that pH) which was then washed with $H_2O$ (1·5 l.). Concentration of the combined effluents yielded a solution containing *fraction A*. Elution of the column with 1·5 N aq. $NH_4OH$ (2·2 l.) followed by EtOH–$H_2O$–14 N $NH_4OH$ (80:10:10, 2 l.) and evaporation of the combined eluates yielded a residue termed *fraction B* which was dissolved in $H_2O$ (500 ml). A portion of this (200 ml) was adjusted to pH 11·0 by addition of 14 N $NH_4OH$ (4 ml) and then KOH. The resulting solution was filtered and then percolated through a column (3 × 18 cm) of De-Acidite FF (SRA 66; acetate form equilibrated to pH 10·9 by washing with $NH_4OAc$ buffer of that pH) which was then washed with 0·04 N $NH_4OH$ (400 ml). Evaporation of the combined effluents yielded *fraction C*. Further washing of the column with $H_2O$ (100 ml) and then elution with 400 ml of pyridine–HOAc solution (1% aq. pyridine adjusted to pH 5·8 with HOAc) yielded effluents which were combined and evaporated to give *fraction D*. Evaporation of the eluate obtained by further elution with 1·5 N HOAc (400 ml) yielded *fraction E*. *Fractions C* and *E* were further fractionated by *n*-BuOH extraction. An aq. soln of *fraction C* (100 ml; pH 7) was extracted with four 100-ml vol. of $H_2O$-saturated *n*-BuOH. Evaporation of the extracts yielded *fraction F* while the aq. phase constituted *fraction G*. The corresponding fractions derived from *fraction E* were *fraction H* and *fraction I* respectively.

*Isolation of cytokinins in fraction* A. Concentrated extract (15 l.) equivalent to 65 kg of sweet corn was stirred with purified charcoal (1 kg) for 3 hr and then filtered. The charcoal was then stirred with $H_2O$ (10 l.) for 30 min, filtered off, washed with $H_2O$ (4 l.) and finally eluted by stirring with pyridine–$H_2O$ (1:9; 11 l.) for 14 hr. The filtered eluate was evaporated to dryness under vacuum and an aq. soln (500 ml, pH 8·2) of the resulting residue was extracted with three 500-ml vol. of $H_2O$-saturated *n*-BuOH. Evaporation of the extracted aq. soln yielded a residue (91 g dry matter) which was dissolved in $H_2O$ (200 ml). The solution (pH 8·0) was percolated through a column of De-Acidite FF (SRA 67, acetate form, 5 × 54 cm) which was washed with $H_2O$ and then eluted sequentially with 0·05 N HOAc (2 l.), 1·5 N HOAc (1·5 l.) and 2 N HCOOH (3 l.). Fractions (40 ml) were immediately evaporated under vacuum (bath temp. 28°). Activity was confined to fractions 37–40 of the HCOOH eluate which were combined, evaporated (dry matter 3·5 g) and subjected to preparative PC using solvent *B*. The active zone ($R_f$ 0·68) on each sheet was eluted with 0·1 N $NH_4OH$ and the combined eluates rechromatographed on paper with solvent *C*. The active UV-absorbing zone ($R_f$ 0·30) from each chromatogram was eluted with 0·1 N $NH_4OH$ and the combined eluates rechromatographed using solvent *D*. This resolved the UV-absorbing material into two zones with $R_f$ of 0·18 (inactive) and 0·41 (active). The paper sheets were then cut transversely just above the lower zone and No. 120 paper with a serrated edge was stapled across the top of each sheet to increase the length by 20 cm. The sheets were then developed for 7–8 hr with solvent *A* which was allowed to drip from the serrated edge. This resolved the active UV-absorbing zone into two zones; the one of lower $R_f$ was eluted yielding a highly active fraction (*Aa*), while a weakly active fraction (*Ab*) was obtained from the other.

An aq. soln (5 ml, pH 8·0) of *Aa* was chromatographed on Dowex 1 (200–400 mesh, formate form, 1·0 × 15 cm) which was eluted first with 0·3 N HCOOH (280 ml) and then with 1·5 N HCOOH. A plot of A (264 nm) of the effluent against vol. showed only one prominent peak which was due to the active compound. The corresponding fractions were immediately evaporated *in vacuo* (bath temp. 26–28°) and the residue dissolved in $H_2O$. The resulting soln was adjusted to pH 9 with N $NH_4OH$ and evaporated, yielding a semicrystalline residue (C3). A small portion of this was subjected to PC (2 further solvents), TLC on cellulose (5 further solvents), TLC on borate-impregnated cellulose (2 solvents containing borate), TLC on DEAE-cellulose (3 solvents differing in $H^+$ and $Cl^-$ concn) and thin-layer electrophoresis at pH 3·6 (acetate buffer) and at pH 9·3 (borate buffer). All these systems revealed only one component in C3. Except for those containing borate, all chromatograms were cut into zones which were assayed for cytokinin activity. This was located only in the zones containing C3.

C3 was recrystallized as the Ba salt. The semicrystalline product obtained above was dissolved in $H_2O$ (1·0 ml) and mixed with $BaCl_2$ solution (20% w/v; 0·2 ml). EtOH (2·4 ml) was then slowly added at 2° yielding the *Ba salt of C3* as small white crystals which were centrifuged off, washed with EtOH–$H_2O$ (3:1) and then absolute EtOH, and dried at 1 mm over $P_2O_5$ (yield 7·8 mg). UV spectra: $\lambda_{max}^{H_2O}$ 265, 268 and 268 nm at pH 2·2, 6·7 and 11·0 respectively; $\lambda_{min}^{H_2O}$ 233·5, 233 and 233 nm at pH 2·2, 6·7 and 11·0 respectively;

$A_{max}/A_{min}$ for the three pH values, 5·52, 6·54 and 6·74 respectively. To convert the Ba salt to the $NH_4$ salt a solution of the former was passed through a small column of cellulose phosphate ($NH_4^+$ form).

The active compounds in fraction *Ab* were purified by anion-exchange chromatography. An aq. soln of *Ab* (7 ml; pH 9·0) was passed through a column of Dowex 1 (formate, 200–400 mesh, 0·8 × 16 cm) which was washed with $H_2O$ and then eluted with 0·2 N HCOOH (27 ml). Fractions with UV absorption were combined and evaporated; the residue was recrystallized twice from $H_2O$–$CH_3CN$ yielding *compound C*4 (0·24 mg). UV spectra: $\lambda_{max}^{H_2O}$ 267, 269 and 269 nm at pH 1·0, 7·1 and 11·0 respectively; $\lambda_{min}^{H_2O}$ 234, 232 and 232 nm at pH 1·0, 7·1 and 11·0 respectively. IR $\nu_{max}$ 1726, 1620 $cm^{-1}$. C4 and also C5 did not possess a characteristic m.p. and decomposed on heating. Further elution of the column with N HCOOH yielded a second compound with strong UV absorption. Evaporation of the appropriate fractions yielded a residue which was crystallized from $H_2O$ to yield *compound C*5 (7·5 mg). This exhibited UV spectral characteristics identical to C4; IR $\nu_{max}$ 1716, 1636, 1420 $cm^{-1}$.

*Isolation of cytokinins in fraction* F. Fraction *F* derived from 30 kg of sweet corn was prepared by a large scale version of the procedure described above with one modification; the amount of Zeo-Karb 225 used was proportionately reduced to one half. Fraction *F* thus obtained was subject to preparative PC using solvent *E*. On each chromatogram, there were two regions with activity, the $R_f$ being *ca.* 0·30 and 0·65. The fraction eluted from the latter regions yielded three main UV-absorbing zones by PC (solvent *F*) and activity was associated with only one of these ($R_f$ 0·44). The active eluate was rechromatographed on paper using solvent *G*. The material eluted from the UV-absorbing zone was fractionally crystallized from *n*-BuOH–light petrol. to yield a crude product which was recrystallized 2× from the same solvent giving *compound C*2 (yield 0·20 mg), micro m.p. 180–181° unaltered by admixture with authentic 9-β-D-ribofuranosylzeatin. UV spectra: $\lambda_{max}^{H_2O}$ 265, 265·5, 269 and 269 nm at pH 1, 3, 7 and 11 respectively; $\lambda_{min}^{H_2O}$ 232–233 at pH 1–11; $\lambda_{max}^{EtOH}$ 269·5 nm. MS: *m/e* 351 ($M^+$, 27), 334 (47), 320 (37), 262 (25), 248 (33), 220 (11), 219 (11), 218 (11), 202 (100), 201 (65), 200 (32), 188 (83), 186 (34), 185 (15), 178 (16), 174 (10), 164 (20), 160 (35), 149 (20), 148 (50), 136 (85), 135 (55), 121 (18), 120 (25), 119 (40), and 108 (23).

The fraction eluted from the active zone at $R_f$ 0·30 on the PCs mentioned above (solvent *E*) was purified by preparative PC using first solvent *H* ($R_f$ active compound 0·41) and then solvent *G*. Eluate of the active zone ($R_f$ 0·37) was further purified by TLC on silica gel with solvent *F* ($R_f$ active compound 0·10) and then with solvent *E* ($R_f$ active compound 0·41). The resulting fraction was finally chromatographed on washed Whatman No. 40 paper (solvent *G*) yielding about 20 μg of product; TLC indicated it contained only one UV absorbing substance now termed *compound C*8. UV spectra: $\lambda_{max}^{EtOH}$, 269–270 nm; $\lambda_{max}$ in 0·2 N ethanolic $NH_4OH$, 269–270 nm (spectra determined with minimal amount of compound). MS, main peaks: *m/e* 202 (base peak), 201, 200, 199, 198, 188, 186, 185, 160, 148, 136, 135, 120, 119, 108. $R_f$ relative to adenosine, 0·58 (TLC silica gel, solvent *H*).

*Isolation of cytokinins in fraction* H. Fraction *H* derived from 90 kg of sweet corn was prepared by a large scale version of the procedure previously described. The resulting product was subjected to preparative PC on sheets of Whatman seed-test paper[12] (solvent *E*). The eluate of the broad, active region extending from $R_f$ 0·30 to $R_f$ 0·60 was purified by preparative TLC on silica gel (solvent *H*) which yielded 3 active zones, the $R_f$ being 0·17, 0·27 and 0·44; the eluates are referred to henceforth as fractions *H*1, *H*2 and *H*3 respectively.

Preparative TLC of *H*1 on silica gel (solvent *E*) separated the active component ($R_f$ 0·45) from a number of inactive UV-absorbing compounds. The active zone was eluted and rechromatographed on silica gel (layer developed three times with solvent *F*); the active compound ($R_f$ about 0·11) was now revealed as a weak UV-absorbing zone. It was eluted and further purified by chromatography on cellulose phosphate (method used for C9) and by PC on Whatman No. 40 paper (solvents *B* and *G*, $R_f$ 0·75 and 0·38); the yield was about 50 μg. UV spectra: $\lambda_{max}^{EtOH}$ 269–270 nm; $\lambda_{max}$ 0·2 N ethanolic $NH_4OH$, 269 nm. MS: identical to C8 except that some of the weak (? impurity) peaks above *m/e* 219 were absent. The factor co-chromatographed with C8 during TLC on silica gel and borate-impregnated silica gel.

Fraction *H*2 was subjected to preparative TLC on silica gel (solvent *E*) and the active UV-absorbing zone ($R_f$ 0·48) eluted. The eluate was then further purified by TLC on silica gel (each plate was developed 5 × with solvent *F*) which resolved the activity into 2 zones with UV absorption, the $R_f$ being about 0·07 and 0·10. The former zone was much more active than the latter. A component with higher $R_f$ (0·20) which gave a very strong UV-absorbing zone was identified as adenosine. A solution of the component of $R_f$ 0·07 in 0·1 N HOAc was passed through a small column (5 × 40 mm) of cellulose phosphate (Whatman P1, $NH_4^+$ form, equilibrated to pH 3) which was washed with $H_2O$ and then eluted with 0·1 N $NH_4OH$. The residue obtained by evaporation of the eluate fractions containing the active compound was finally chromatographed on washed Whatman No. 40 paper (solvent *G*) yielding about 80 μg of *compound C*9 ($R_f$ solvent *G*, 0·48). UV spectra: $\lambda_{max}^{EtOH}$ 245, 284 nm; $\lambda_{max}^{H_2O}$ 288 (pH 3). MS: *m/e* 235, 218, 217, 204, 202, 200, 176, 164, 152, 151, 135 (base peak), 108; $M^+$ 235·107, Calc. for $C_{10}H_{13}N_5O_2$: 235·107. $R_f$ relative to adenosine: 0·93 (TLC silica gel, solvent *H*), 0·89 (PC, solvent *H*).

The active fraction of $R_f$ 0·10 (solvent *F*) obtained above was also purified by chromatography on cellulose

phosphate and then by PC using solvent *G*. Purification was completed by the following sequential steps: PC with solvent *F* ($R_f$ 0·13), precipitation as the 3-iodo-2,4,6-trinitrophenolate ($\lambda_{max}^{H_2O}$ pH 7: 269, 369 nm), conversion to the free base with Dowex 1 ($HCO_3^-$ form), and crystallization from $H_2O$ to yield *compound C*7 (150 $\mu$g), micro m.p. 224–227° (decomp.). UV spectra: $\lambda_{max}^{H_2O}$ 273 (pH 1), 268 (pH 6), 219 and 274 with shoulder at 283 nm (pH 13). MS (principal peaks): *m/e* 222, 178, 149, 148 (base peak), 136, 135, 121, 120, 119, 108: $M^+$ probably 253 (< 2% of base) but other very weak peaks at higher *m/e* were also present. $R_f$ relative to adenosine, 0·87 (TLC silica gel, solvent *H*). C7 was acetylated with pyridine–acetic anhydride at 25° for 18 hr and the *diacetate* was isolated as a picrate, micro m.p. 170–171°. UV spectrum: $\lambda_{max}^{H_2O}$ pH 7, 265 and 358 nm. MS: *m/e* 337, 319, 294, 264, 259, 229 (picric acid), 204, 200, 199, 178, 162, 160, 149, 148 (base peak), 136, 135, 121, 120, 119. The foregoing diacetate was acetylated with $Ac_2O$ in the presence of *p*-toluene sulphonic acid at 25° for 18 hr to give the *triacetate* of C7 which was crystallized from EtOAc–light petrol., micro m.p. 180–182°. UV spectrum: $\lambda_{max}^{EtOH}$ 268 nm. MS principal peaks: *m/e* 379, 260, 246, 204, 160, 148 (base peak), 136, 135, 119.

Fraction *H*3 was purified by preparative TLC on silica gel using solvent *E* and the eluate of the active zone ($R_f$ 0·46) rechromatographed on silica gel with solvent *F* (plates developed three times). The active compound was now evident as a faint UV-absorbing zone ($R_f$ 0·16) and was eluted and further purified by chromatography on a column of cellulose phosphate (method as for compound C9). Finally the compound was chromatographed on paper (solvent *G*, $R_f$ 0·54) and then precipitated from the concentrated eluate by addition of a saturated aq. soln of picric acid yielding the picrate of *compound C*10 (200 $\mu$g), micro m.p. 161–162°. UV spectrum: $\lambda_{max}^{H_2O}$ pH 7, 266 and 358 nm; the free base exhibited $\lambda_{max}^{H_2O}$ 268 (pH 7), 273 (pH 1), 219 and 274 with shoulder at 283 nm (pH 13). MS: *m/e* 237 (6), 229 (picric acid, 100), 206 (15), 199 (14), 162 (35), 149 (15), 148 (47), 136 (23), 135 (16), 120 (7), 119 (13), 108 (6). $R_f$ relative to adenosine, 1·5 (TLC silica gel, solvent *H*).

*Degradation studies. Hydrolysis of C3 with phosphatase.* A solution of C3 ($NH_4$ salt, 2·0 mg) in $H_2O$ (1·0 ml) containing purified *E. coli* alkaline phosphatase (0·2 mg) was held at 35° for 2 hr, the pH being maintained at 9·5 by additions of dil. aq. $NH_4OH$. The soln was then extracted with five 1-ml vol. of *n*-BuOH and the extracts were evaporated. The residue was crystallized from *n*-BuOH–light petrol. (b.p. 80–100°) yielding compound C3a (9-$\beta$-D-ribofuranosylzeatin), micro m.p. and m.m.p. 180–181°. UV spectra and MS were identical to spectra recorded above for zeatin riboside (C2). C3a and 9-$\beta$-D-ribofuranosylzeatin could not be distinguished by thin-layer electrophoresis in borate buffer (pH 9·3), by PC (3 solvents, two of which separate zeatin riboside from the *cis* isomer), or by TLC on silica gel, borate-impregnated silica gel (several solvents saturated with $Na_2B_4O_7$), alumina and cellulose. C3 was also hydrolysed to C3a with snake venom (pH 9·5). The venom hydrolysed AMP rapidly but did not dephosphorylate adenosine 2′- or 3′-phosphate.

*Acidic hydrolysis of C3a.* Zeo-Karb 225 (exhaustively washed, $H^+$ form, 100–200 mesh, 8 mg) was refluxed with an aq. soln (0·5 ml) of C3a (0·3 mg) for 1 hr and then centrifuged off. The supernatant was evaporated *in vacuo* for chromatography of sugars.

*Degradation of C3a with periodate.* C3a was successively oxidized with $KIO_4$ and reacted with cyclohexylamine (method essentially that of Yu and Zamecnik[41] for converting ribonucleosides to bases). The reaction mixture was evaporated to dryness and an aq. soln of the residue extracted with *n*-BuOH. The extracted product was purified by PC (solvent *G*). To an aq. soln of the eluted compound, saturated picric acid soln was added. The ppt. was washed with $H_2O$ and recrystallized from $H_2O$ to yield the picrate of compound C3b (zeatin picrate), micro m.p. and m.m.p. 191–193°. UV spectra of base: $\lambda_{max}^{H_2O}$ 274 nm (pH 1), 219 and 275 with shoulder at 283 nm (pH 13); $\lambda_{max}^{EtOH}$ 269 nm. C3b and zeatin could not be distinguished by TLC on silica gel, cellulose phosphate or alumina.

*Degradation of C2 and C8 with periodate.* C2 or C8 (10 $\mu$g) was dissolved in $NaIO_4$ (5·0 mM, 50 $\mu$l) and the soln was left at 35° for 18 hr when cyclohexylamine (10 $\mu$l) was added. After 4 hr at 35°, the soln was evaporated under vacuum and the residue chromatographed (TLC silica gel, solvent *H*). The principal UV-absorbing product ($R_f$ 0·60) co-chromatographed with zeatin. MS after PC: identical to that of zeatin. UV spectra: $\lambda_{max}^{EtOH}$ 269 nm; $\lambda_{max}$ 0·5 N ethanolic $NH_4OH$, 275 with shoulder at 284 nm.

*Acidic hydrolysis of C4.* C4 (160 $\mu$g) was heated with N HCl (0·2 ml) at 100° for 1·5 hr. The residue obtained by evaporation (*in vacuo*, 20°) of the hydrolysate was dissolved in MeOH (0·3 ml) and esterified by addition of an $Et_2O$ solution of $CH_2N_2$. As soon as an excess was present, the mixture was evaporated *in vacuo* and the product purified by TLC (silica gel, solvent *F*). Elution with MeOH and crystallization from MeOH–*n*–PrOAc yielded compound C4a, 6-(2-*hydroxy*-1-*methoxycarbonylpropylamino*)*purine* (IIa, 60 $\mu$g). UV spectra: $\lambda_{max}^{H_2O}$ 276 nm (pH 1), 219 and 274·5 with shoulder at 282 nm (pH 13); $\lambda_{max}^{EtOH}$ 266 nm. MS: main peaks *m/e* 207 (45), 192 (40), 189 (49), 175 (100), 148 (43), 134 (65), 120 (90) and 119 (55); $M^+$ 251 (< 1); M-$H_2O$ 233 (4); high resolution 233·090 (Calc. for $C_{10}H_{11}N_5O_2$: 233·091), 207·076 (Calc. for $C_8H_9N_5O_2$: 207·076).

*Synthesis of compounds. Synthesis of* 6-(1-*carboxy*-2-*hydroxypropylamino*)-9-$\beta$-D-*ribofuranosylpurine.* To a solution of L-threonine (90 mg) in water (7 ml, pH to 9·5 with NaOH), $Na_2CO_3$ (20 mg) and 6-chloro-9-$\beta$-D-

[41] YU, C. and ZAMECNIK, P. C. (1960) *Biochim. Biophys. Acta* **45**, 148.

ribofuranosylpurine (140 mg) were added. The soln was then refluxed for 5 hr, adjusted to pH 7 and percolated through a column of Dowex 1 (formate) which was eluted successively with $H_2O$, 0·1 N HCOOH and 0·2 N HCOOH. The fractions of the 0·2 N HCOOH eluate containing the desired product were evaporated and the residue crystallized from $Me_2CO$–$H_2O$ to yield 6-(1-*carboxy*-2-*hydroxypropylamino*)-9-β-D-*ribofuranosylpurine* (60 mg), m.p. 145–149° with decomp. (Found: C, 45·8; H, 5·4; N, 18·7. $C_{14}H_{19}N_5O_7$ requires: C, 45·6; H, 5·2; N, 19·0%. IR spectrum $\nu_{max}$ 1726, 1620 $cm^{-1}$; UV spectra identical to those of compound C4. By a method similar to the above, the known compound 6-(1,2-dicarboxyethylamino)-9-β-D-ribofuranosylpurine was prepared (see Ref. 42).

*Synthesis of* 6-(2,3,4-*trihydroxy*-3-*methylbutylamino*)*purine*. A mixture of zeatin (44 mg), *t*-BuOH (8 ml), $H_2O$ (8 ml) and 14 N $NH_4OH$ (2 ml) was stirred at 0° and $KMnO_4$ solution (10 mg/ml; 3·5 ml) added. After 7 min, an excess of allyl alcohol (8 drops) followed by EtOH (4 ml) was added and the mixture was left at −15° for 18 hr. The precipitate of $MnO_2$ was centrifuged down and washed with EtOH. The residue obtained by evaporation of the soln was purified by preparative TLC (solvent *F*) on silica gel and the main UV-absorbing zone eluted. The eluate was rechromatographed on silica gel (solvent *E*) and the eluted product ($R_f$ 0·42) crystallized from $H_2O$ to yield 6-(2,3,4-*trihydroxy*-3-*methylbutylamino*)*purine* (14 mg), micro m.p. 224–227° decomp. ($M^+$ Found 253.1182; $C_{10}H_{15}N_5O_3$ requires: 253.1175); $\lambda_{max}^{H_2O}$ 273 (pH 1), 219 and 274 with shoulder at 283 nm (pH 12).

*Synthesis of* 6-(3,4-*dihydroxy*-3-*methylbutylamino*)*purine*. This known compound[30] was synthesized from 6-(3-methylbut-3-enylamino)purine by a procedure similar to the above. The product was isolated as the *picrate* which was recrystallized from EtOH yielding needles, micro m.p. 161–162° (Found: C, 41·2; H, 3·9; N, 24·0. $C_{16}H_{18}N_8O_9$ requires: C, 41·2; H, 3·9; N, 24·0%).

*Acknowledgement*—Thanks are due to Professor N. J. Leonard for a sample of synthetic 2-hydroxy-6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine.

[42] HAMPTON, A. (1957) *J. Am. Chem. Soc.* **79**, 503.