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THE STRUCTURE AND PHYSIOLOGICAL ACTIVITY OF SOME W-SUBSTITUTED ADENINES

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Abstract—Some 22 N^6-substituted adenines have been prepared and their biological activity tested, for comparison with zeatin, in an aseptic carrot assay system that measures the growth promoting activity in terms of fresh weight (mg), and the number of cells in thousands [and hence their average weight (μg/cell)] produced in carrot root explants. The test medium contained all the prerequisites for growth and its induction other than the factors which specifically induce growth by cell division and enlargement. In a series of n-alkylaminopurines, where n varied from 1 to 10, the maximum activity occurred with $n = 5$ and, in this series, the activity was due to the effects upon cell division per se, relatively uncomplicated by effects upon cell enlargement. The activity of compounds with a formal similarity to zeatin varies with the functional groups in the side chain, but more because of their relative tolerance for concomitant cell enlargement than due to the effects of substituent groups on cell division *per se.* The data contribute to the understanding of the chemical induction of growth, which is essential to interpret growth regulation in plants.

INTRODUCTION

As PART of an investigation to relate the structure of plant growth regulators to their activity, a series of N^6-substituted adenines have been prepared (by G.S. and B.M.S.) for comparison with each other and with the naturally occurring and recently synthesized substance, zeatin.[1] The measure of physiological activity of the compounds is their ability to stimulate cell division in the standardized assay system long in use in the laboratory of one of us (F.C.S.). It is this type of activity which commonly classifies these substances as cytokinins.[1–8] Assays to demonstrate the activity of some of these compounds, especially n-alkylaminopurines, have of course been carried out on other test systems,[2] but it is important to note that the growth response may be stimulated by many classes of compounds in addition to those which meet the specifications of cytokinins as defined by Skoog and Armstrong.[1]

[1] G. SHAW, B. M. SMALLWOOD and D. V. WILSON, *J. Chem. Soc. 921, (1966).*
[2] G. SHAW, B. M. SMALLWOOD and D. V. WILSON, *J. Chem. Soc.* 1516, (1968).
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[8] F. SKOOG, H. Q. HAMZI, A. M. SZEYKOWSKA, N. J. LEONARD, K. L. CARRAWAY, T. FUJII, J. P. HELGESON and R. N. LOEPPKY, *Phytochem.* 6, 1169 (1967).

RESULTS

The compounds examined fall into two classes:

(1) These compounds relate to formula I and they include the substance zeatin where R is $-CH_2-CH=C(CH_3)-CH_2OH$. Some close structural analogues of zeatin contain an exocyclic group with a formal structural resemblance to that in zeatin. These compounds were prepared with varying side chain lengths within the range R = C_3 to C_7 and they are largely characterized by the presence in the chain of an unsaturated grouping either C=C or $C\equiv C$.

(2) A second series of compounds consist of n-alkylaminopurines which relate to formula II, where *n* had the values I-10.

It is known that there are many substances in such naturally occurring growth promoting fluids as those which (in coconut, in immature corn or horsechestnut fruits) constitute the liquid endosperm and nourish young plant embryos and that these also give positive results in the carrot assay system. These various substances, however, fall into different classes and some (of which zeatin and other adenines are notable examples) also require the participation of exogenous indoleacetic acid (IAA) while the activity of others is mediated by myo-inositol (Inos). Also the activity of both naturally occurring isolates and synthetic compounds may be accentuated by casein hydrolysate (CH). Therefore, the activity of the compounds was tested in the basal medium which was already supplemented with CH + IAA + Inos.

Zeatin, which together with IAA, typically represents what has been called growth promoting system I in the carrot assay[9] characteristically produces many small cells which do not enlarge. Alternatively the substances mediated by myo-inositol, which may also influence the metabolism but in distinctively different ways,[10] are more involved with cell enlargement, as well as cell division, and the new cells formed therefore tend to be larger. For these reasons, therefore, the comparisons in this paper first focus attention on the influence of the tested compounds on cell multiplication (Fig. 4). All the comparisons between compounds were made on a clone of explants removed from a single carrot root. However, because individual carrot roots yield explants which react, quantitatively, somewhat differently toward the component parts of Systems I and II, respectively" the usual procedure was to repeat the entire assay with two clones cut from arbitrarily selected roots (A and B).

The relationship between structure (side-chain length) and cell division activity in the series of n-alkylaminopurines was independent of the differences between clones A and B. These relationships are shown in Fig. 1 for compounds tested at 0·1 ppm; in this figure increments in thousands of cells are plotted above a base line which represents the status of the explants after culture in the test medium (B + CH + IAA + Inos); in this formulation inhibitory effects appear as ***decreases*** plotted below the base line.

The compounds were all tested at a low external concentration compatible with their activity, expressed conveniently as ppm. The meaningful comparisons between closely related compounds override the small differences that might be attributable to the disparity between their weight and molar concentrations. The selection of a single concentration level (0·1 ppm) for all the compounds tested was based on the activity of selected compounds of this type at several concentrations, which ranged from 0·1 to 10 ppm. Again

[9] N. DEGANI and F. C. STEWARD, Ann. *Bot.* 33,483 (1969).
[10] F. C. STEWARD and N. DEGANI, *Ann. Bot.* 33, 615 (1968).
[11] F. C. STEWARD, *Proc. Roy. Soc. Lond. B 175, 1 (1970).*

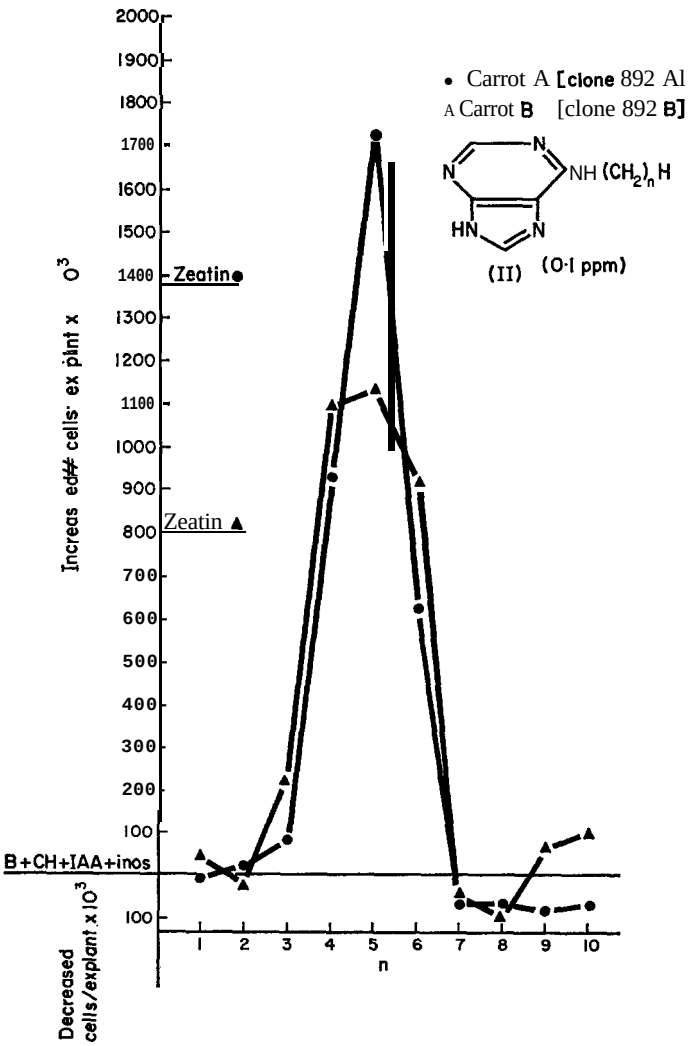


FIG. 1.

the objective was to make broad comparisons which override the concentration chosen for the test. Similarly the comparisons made override any small changes which these compounds may encounter on brief heat sterilization, even as they also necessarily neglect any changes in the tested substances brought about by the tissue itself. It may be emphasized however that all the adenines were prepared in boiling *n*-butanol (b.p. 11 8°) and may be crystallized from boiling water in which they show no signs of decomposition.

The results are clear. In this series of compounds marked activity requires a side chain length of 4-6 (a minimum chain length of 3 and a maximum of 6) and there is an evident maximum activity when the chain length is 5. For each clone the maximum activity when $n = 5$ exceeded that of the naturally occurring zeatin, a compound with different functional groups in a side chain with a total of 5 carbon atoms. It should be noted also that (a) the

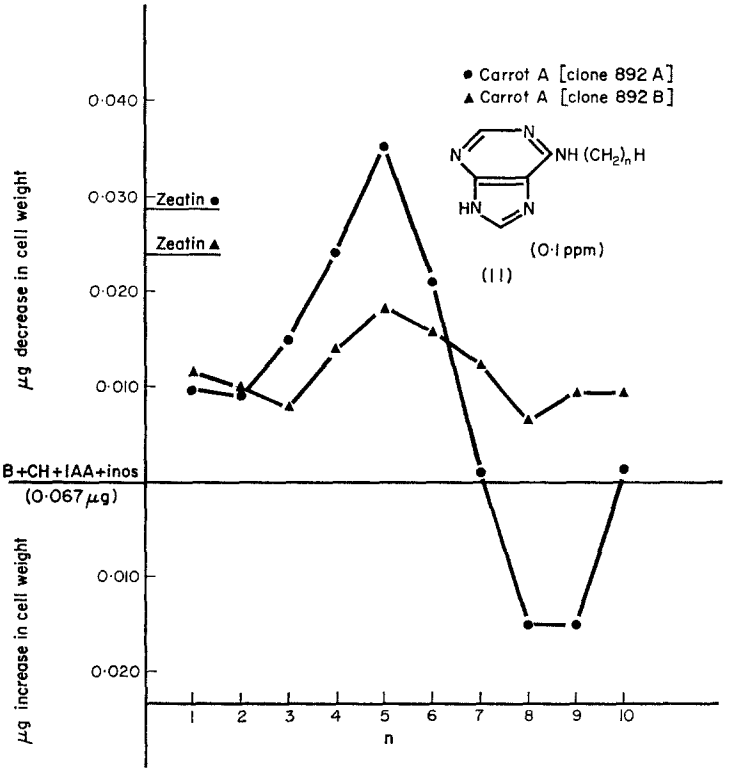


FIG. 2.

basal medium (B) alone produced very little cell division activity (order of one third of that in the test medium B + CH + IAA + Inos) and (b) the full growth due to coconut milk (CM) usually exceeded the best of the tested compounds (increases of fresh weight ×3 and 1·6 for clones A and B, respectively), although this superiority of growth due to the coconut milk stimulus also involved its effect on cell size (0·113 and 0·079 μg/cell in clones A and B, respectively; in contrast to 0·031 and 0·036, respectively, for the compound where $n = 5$.

If a test compound markedly accentuates cell division without an equivalent effect upon cell enlargement it tends to cause the average cell size of the cultured explants to decrease; if, by contrast, it affects *both* cell division and cell enlargement in approximate balance, or affects neither, it leaves the average cell size approximately unchanged or, if the compound is an inhibitor, it may even suppress the enlargement of cells that may result from endogenous activity of the explant in the test medium alone (B + CH + IAA + Inos). The data of Fig. 2, which is complementary to Fig. 1, illustrate these points.

When both cell enlargement and cell division are involved as criteria of growth the clonal differences as between A and B become more apparent. The endogenous properties of clone B were such that even those compounds ($n = 1$–3; $n = 7$–10) which had little effect upon cell multiplication nevertheless arrested the enlargement of cells formed in the unsupplemented test medium. But, superimposed on this behavior, the compounds that caused marked cell division ($n = 4$–6) did so *without* comparable effects on their enlargement so that they decreased cell size still further. Notably zeatin, which was markedly

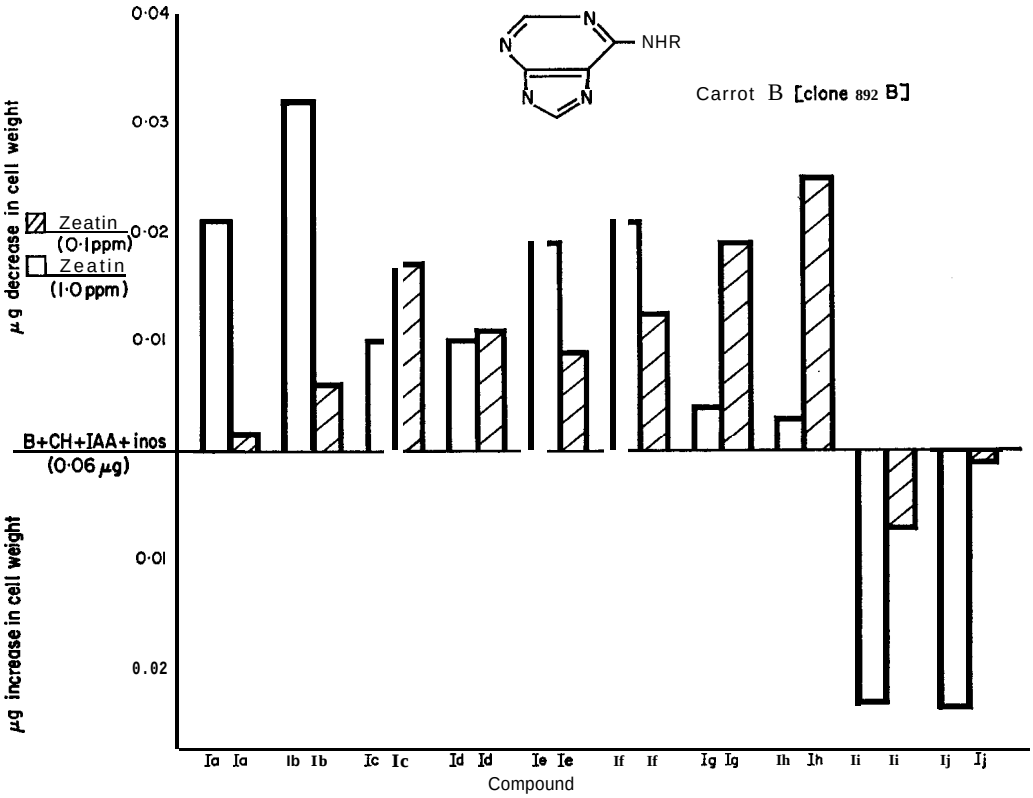


FIG. 3. COMPOUNDS DESIGNATED Ia–Ij ARE ARRANGED IN THE ORDER OF INCREASING LENGTH OF SIDE CHAIN R.

effective in cell division, was also, by virtue of its different side chain and functional groups, effective in restricting cell size. The broad pattern of behavior of clone A was similar, with the recurring feature that the peak cell division activity occurred at n = 5 without concomitant stimulus to cell enlargement while some compounds (n = 7-9) which did not stimulate cell division were either tolerant of (n = 7) or actually increased (n = 8-9) the cell enlargement fostered by the test medium alone.

In short, the side chain lengths n = 4-6, in this series of n-alkylaminopurines give molecules that stimulate cell division without concomitant effects on cell enlargement while zeatin, which has *less* cell division activity than a straight side chain of comparable size with 5 carbon atoms, is concomitantly more intolerant of cell enlargement. There may, of course, be other metabolic or morphological criteria which would distinguish further between zeatin and the unsubstituted n-alkylaminopurines.

Figures 3 and 4 record the effect on cell multiplication and average cell size respectively of those compounds in class 1 above which had various side chain lengths (n = 3-7) but which also possessed unsaturated groups (C=C or C==C) at different positions along the chain. The 10 compounds in question are identified along the axis as Ia to Ij.

The comparable effects of compound Ia to Ij on cell multiplication are shown in Fig. 3 at two concentrations (0·1 and 1·0 ppm) and, for comparison, zeatin was assayed at the

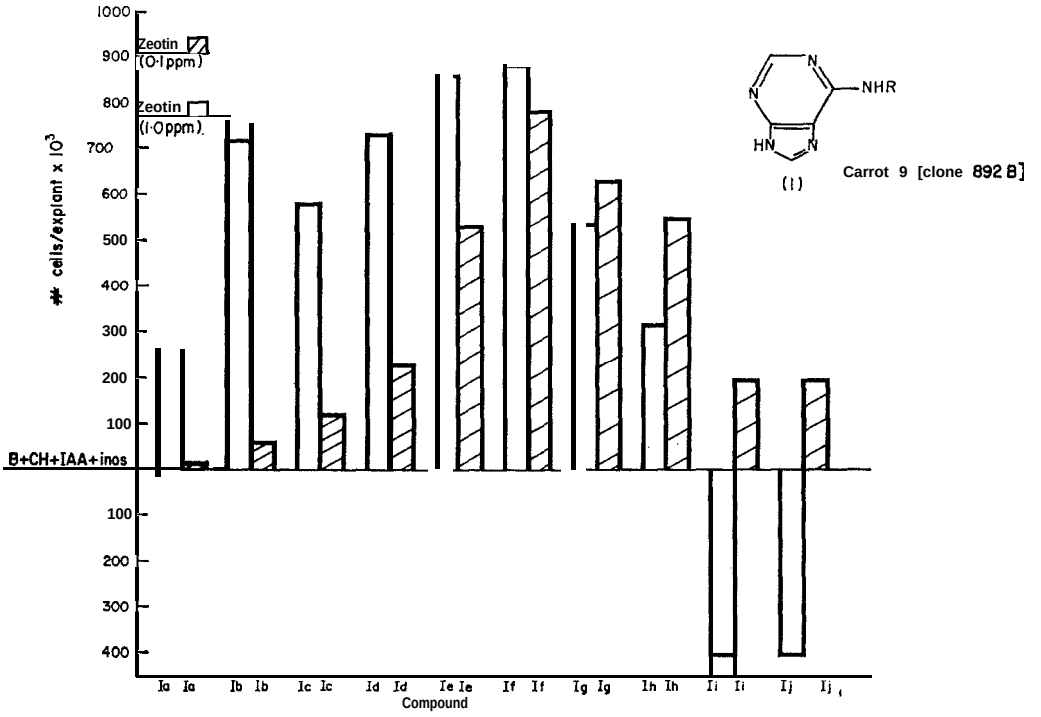


FIG. 4. COMPOUNDS DESIGNATED Ia–Ij ARE ARRANGED IN THE ORDER OF INCREASING LENGTH OF SIDE CHAIN R.

same concentrations. At 0·1 ppm none of these compounds equalled the activity of zeatin though compound If (where R was $-CH_2-CH_2-CH(CH_3)_2$) most nearly approached the cell division activity of zeatin (where *R* is $-CH_2-CH=C(CH_3)-CH_2$ OH). Neither the presence of an unsaturated group in a side chain of 5 carbon atoms (Ie) or in a side chain which is also substituted by $-CH_3$ (Ib and Ic) seems to permit the compounds to approach the maximum activity of zeatin in stimulating cell division even though they were tested at higher concentrations (1·0 ppm) which, for zeatin, were supraoptimal. The phenyl substituted side chains, whether saturated (Ij) or unsaturated (Ii) produced compounds of weak activity at low concentration (0·1 ppm) and even toxicity at high (1·0 ppm) levels.

At 0·10 ppm the compounds (Ic to Ih), which were the most active in cell division (see Fig. 3) in this series did not merely suppress cell enlargement to cause smaller cells. In fact, the most active cell division compounds (Ie and f), in contrast to zeatin, produced larger cells, while Ic (where R is $-CH(CH_3)-CH=CH_2$) and Ih (where R is $-CH_2-CH_2-CH=CH_2$), like zeatin, produced smaller ones. It is therefore, on these data, difficult to attribute direct significance to the functional groups that are in the zeatin side chain (CH=CH; CH_3; OH) in their effect upon either cell division or cell enlargement. One should recall here the comparison with the unbranched saturated side chains of the n-alkylaminopurines, for zeatin displayed *less* cell division activity than its 5 carbon counterparts in that series although its specific action appeared as a suppression of cell enlargement to produce smaller cells. Again a 5 carbon side chain (If) produced maximum cell division (at 0·10 ppm) although, being saturated and free of the other functional groups (OH) of zeatin, it failed, to keep the cells as small as did zeatin.

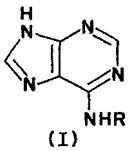
Thus, one may identify the cell division activity of P-substituted purines in this test system and with these co-factors, with a side chain of critical length and size (order of 5 carbons) and the effects of other functional groups (unsaturation; —CH_3; OH) are more evident in terms of their ability simultaneously, to limit cell enlargement.

DISCUSSION

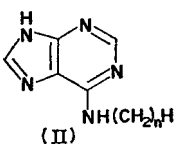
Interpretations of the structure-activity relations here disclosed are beset with difficulties. The site of action in the cells is not known; the reason why IAA is an essential co-factor or why casein hydrolysate accentuates their role is equally unknown. Where long alkyl side chains are involved they may confer properties which render the molecules more or less compatible with membranes and effect permeability and penetrability, not only into cells but to active sites in organelles, and these properties may be markedly modified by other functional groups. Any speculations upon the role of these growth regulating compounds should include in addition to effects on cell division their often contrasted effect on cell enlargement. Meanwhile the size and shape of the most active adenines may permit them to occupy with maximum efficiency some area or cavity at an active site, and the activity-concentration relationships may also reflect the ability of less active or inhibitory analogues to use less efficiently or to block and make inaccessible otherwise vacant active sites.

Many similar compounds may play a regulatory role comparable to that of zeatin. A concept of the activities of these compounds must of course refer to the range of molecular structures that are compatible with their biological activity which in the growth induction of carrot tissue, also presupposes an interaction with IAA. Others have invoked what may be termed the 'adenyl-cytokinins' very specifically as bases in *t*RNA complexes. The present study has not reached a stage where such a specific mode of action can be postulated for it is also quite clear that all the substances that trigger cell divisions in carrot tissue do not act in the same way. Indeed some systems that induce growth and cell division do so by routes which are independent of exogenous adenyl compounds.

EXPERIMENTAL



(I)



(II)

Ia R is – CH_2–C≡CH;
Ic – $CH(CH_3)$–CH=CH_2;
Ie – CH_2–CH=$C(CH_3)_2$;
Ig – CH_2–CH=CH(CH_3);
Ii – CH_2–CH=CH–C_6H_5;

Ib – CH_2–$C(CH_3)$=CH_2
Id – CH_2–CH=CH_2
If – CH_2–CH_2–$CH(CH_3)_2$
Ih – CH_2–CH_2–CH=CH_2
Ij – CH_2–CH_2–CH_2–C_6H_5

All the compounds were prepared by reaction of the corresponding amines with 6-chloropurine using the general methods which have been described elsewhere for syntheses of zeatin and its derivatives.[1–4] Unsaturated amines, required for the synthesis of many of the adenines, were prepared by reaction of the corresponding alkenyl or alkynyl halides with potassium phthalimide and hydrolysis or hydrazinolysis of the resulting phthaloyl derivatives, and were isolated generally as crystalline sulphates or hydrochlorides. Several of the substituted adenines so prepared were new compounds and their elemental analyses and other properties are recorded in Table 1 and their UV absorption spectra in Table 2.

TABLE 1

Compound	M.p. (°)	Found C	Found H	Found N	Formula	Required (%) C	Required (%) H	Required (%) N
Ia*	285-7	55.7	4.75	39.95	$C_8H_7N_5$	55.5	4·05	40.5
Ib†	239–40	57·1	6.05	36.85	$C_9H_{11}N_5$	57.15	5.8	37.05
Ic‡	179-82	54.2	6.25	35.5	$C_9H_{11}N_5, H_2O$	54.5	6·05	35.3
Ig*	236-9	56.75	6.15	36.85	$C_9H_{11}N_5$	57.15	5·8	37.05
Ih§	225-7	56.8	6·0	37·45	$C_9H_{11}N_5$	57.15	5.8	37·05
Ii‖	240–42	66.95	5.35	27.65	$C_{14}H_{13}N_5$	66.9	5.15	27·9
Ij†	222-4	66.2	5·7	28.0	$C_{14}H_{15}N_5$	66.35	5·9	27.65

* Needles from ethanol.
† Prisms from ethanol.
‡ Needles from water.
§ Prisms from methanol.
‖ Needles from aqueous ethanol.

The standard assay procedure is based on the use of small (2-3 mg) explants from selected carrot roots. These are removed aseptically from the carrot roots (in which the cells are quiescent and would not normally grow again) in such a manner that their growth even on a basal nutrient medium is minimal. However, when the explants are given a well balanced complement of growth factors (conveniently furnished by coconut milk, with or without casein hydrolysate) they grow very rapidly. The procedures of this growth assay have been described[1,2] and the growth may be recorded in terms of the fresh weight (mg) per explant, the number of cells in thousands obtained after maceration and, as necessary, by the average size of the cells (μg/cell) calculated from the data so obtained.

TABLE 2. UV ABSORPTION SPECTRA OF SOME SUBSTITUTED ADENINES

Compound	λ_{max} (nm) (ϵ) at pH 1	λ_{max} (nm) (ϵ) at pH 7·2	λ_{max} (nm) (ϵ) at pH 12	λ_{min} (nm) 1	λ_{min} (nm) 7.2	λ_{min} (nm) 12
Ia	209(12,000), 275(16,600)	211(17,000), 266(16,450)	225(12,700), 273(15,400)	234	229	240
Ib	208(11,650), 274(16,150)	214(9950), 268(15,300)	226(8600), 275(15,700)	243	242	253
Ic	212(9750), 276(16,100)	215(7950), 270(14,600)	228(8700), 276(15,500)	235	234	244
Id	208(9600), 274(13,200)	213(13,500), 268(14,200)	226(10,650), 275(13,800)	234	230	241
Ie	207(13,250), 276(16,200)	213(10,700), 269(15,400)	226(8200), 277(16,000)	237	234	246
If	206(12,100), 274(14,750)	213(10,600), 269(13,850)	225(9300), 275(14,500)	237	234	245
Ig	216(16,600), 275(17,200)	218(13,450), 270(16,400)	229(14,600), 275(16,750)	247	247	251
Ih	210(10,100), 275(15,650)	215(7150), 269(14,200)	227(7700), 276(15,100)	234	231	242
Ii	209(22,400), 254(15,750) 276(19,550)	210(27,150), 254(sh), 270(2400)	224(11,200), 253(15,700) 276(18,650)	222 259	226	234 261
Ij	208(28,500), 254(19,950) 275(24,200)	210(32,600), 256(sh), 270(26,700)	224(17,700), 254(18,300) 276(23,900)	226 259	226	234 261
Ik*	207(10,750), 274(15,500)	212(16,700), 268(16,500)	225(11,750), 275(16,350)	234	229	241
Il†	209(11,100), 274(16,200)	214(9200), 269(15,700)	227(8550), 274(15,950)	235	232	242

* **1**; R = $-CH_2\triangledown$. † I; R = $-CH_2$  .

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[1,2] F. C. STEWARD, S. M. CAPLIN and F. K. MILLAR, Ann. *Bot.* 16, 577 (1952).