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EFFECTS OF ADENYL CYTOKININS ON THE SOLUTES OF CULTURED CELLS*

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Abstract—When zeatin acts upon cultured carrot explants in a basal medium lacking IAA its most significant effect is to increase the Na^+/K^+ ratio. In the presence of IAA, zeatin greatly stimulates cell multiplication and the Na^+/K^+ ratio is then low. The effect of a series of N^6-substituted adenines with side chains $(-CH_2)_nH$ varying from 1 to 10 have been tested for their effect upon cell multiplication and cell size and upon the total osmotic value and the relative concentrations of Na^+, K^+ and Cl^- in the cells. Whereas the maximum response in terms of cell multiplication occurred with the compound having a $-(CH_2)_5 \cdot H$ sidechain, the maximum effect on the Na^+/K^+ ratio of the cultured cells as so grown was caused when the side chain was $-(CH_2)_4H$.

INTRODUCTION

CERTAIN *N*-substituted adenines have been classified as cytokinins; among these kinetin (or 6-furfurylaminopurine) was the prototype and zeatin [6-(4-hydroxy-3-methylbut-trans-2-enylamino)purine] is a naturally occurring representative. Several naturally occurring substances which display this physiological activity have been held to do so by virtue of their presence as minor nitrogen bases in transfer RNA molecules and their consequential ability to affect protein synthesis.[1] While this property may seem to explain the mechanism of cytokinins their other physiological effects may also be involved. This is so because (a) the modification of nucleic acid bases (as in *t*RNAs) only appears to occur at the polymer level, (b) 9-methylzeatin possesses substantial cytokinin activity although, presumably, it cannot be glycosylated, (c) there are many molecules with obvious cytokinin activity that are not adenine derivatives, and (d) cytokinin activity is generally characterized by a relatively low degree of molecular specificity but optimum (i.e. maximal) activity is apparently associated with relatively high molecular specificity.

The induction of maximum growth in tissue explants of the otherwise quiescent secondary phloem of mature carrot roots, as stimulated by such complex fluids as coconut milk or by a fluid from *Aesculus* fruits, involves the activity of substances of this kind but only in part. While the adenyl compounds interact with auxin, the total stimulus of the naturally occurring growth promoting fluids also involves other compounds, and the activity of some of these may be mediated by inositol.[2] That part of the total growth response which may be

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[1] STEWARD, F. C. and KRIKORIAN, A. D. (1971) *Plants, Chemicals and Growth*, 232 pp., Academic Press, New York.

[2] STEWARD, F. C. (1970) *Proc. Roy. Soc. Lond.* **175B**, 1.

mediated by substituted adenyl compounds and which is measurable in terms of cell multiplication, average cell size and the total weight of the tissue as cultured, has been related to structure in a series of synthetic substituted adenines.[3] The N^6-substituting groups, $(-CH_2)_nH$, have varied in length from $n = 1$ to 10, in their branching (as in zeatin), and by the introduction of other functional groups.

While the induction of growth in the cultured carrot tissue involves both cell multiplication and subsequent cell enlargement, nevertheless the composition of the cultured tissue is also changed in terms of its pigmentation, content of protein and non-protein nitrogen, water and both organic and inorganic solutes.[2] The present study therefore extends the observations already made on growth[3] and includes the effects of the compounds as tested on the solutes of the cells, (as measured in terms of their total osmotic value and, specifically, the concentrations of K^+, Na^+, Cl^-). These results are of interest inasmuch as they extend the range of the effects of the adenyl compounds and as they indicate other parameters by which these effects may be observed and measured; the results also have interest because of their relation to the problems of solute and water uptake by growing cells and to the problem of the mechanism of active transport of ions and of their accumulation in cells. Growth factors, including cytokinins and their antagonists, have been variously implicated through the regulation of solutes and water in plant cells in stomatal movements,[4] wilting and recovery[5] and drought resistance.[6]

The new work arose out of some preliminary observations[7] made as carrot cultures were being routinely tested for their responsiveness to the component parts of growth promoting Systems I and II.[2] Zeatin, unaccompanied by its synergistic cofactor, IAA of System II, unexpectedly caused the cultured cells to reverse their normal preponderant content of K^+, to acquire an unusually high content of Na^+, thus altering the normally high K^+/Na^+ ratio (order of 10) to one (order of 0·6) which was in favor of Na^+ in cells of low halide content. Other work[7] presents the background against which the results may be interpreted and has furnished the developed techniques[8] by which the experimental observations were made upon the cultured carrot explants (clones 892 A and B) on which the growth inducing properties of the compounds were tested.[3]

The known behavior of the cells of cultured carrot explants with respect to solutes, throughout their time course of growth[7] and as stimulated by balanced complements of growth factors[2] (e.g. coconut milk, or Systems I and II) is as follows. At first the rapidly dividing small cells (order of 10 μg/cell) utilize their absorbed solutes in producing form and complex substance and therefore have relatively low osmotic value (order of 200 mOsm) in which K^+, accompanied mainly by organic anions, is the prominent ion (order of 100 μmol/g fr. wt).

The first higher osmotic values in cells, however, are built in large part of organic solutes, but, as cell division subsides and vacuoles enlarge greatly, the complement of organic solutes may be extensively replaced by alkali halides (K^+, Na^+ and Cl^-).[9] During the latter events, cation-anion balance ($K^+ + Na^+ = Cl^-$) tends to be preserved while the preference for K^+ over Na^+ is somewhat relaxed.

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RESULTS

Table 1 shows, for two clones of carrot explants, that zeatin acting alone reverses the preferential accumulation of potassium over sodium. Sodium accumulated over potassium only when zeatin acted upon the tissue in the absence of the IAA, the IAA being necessary, as in System II, to promote cell multiplication. The two clones differed somewhat in their growth responses, especially with respect to the cell size associated with the use of zeatin. Whether the zeatin-treated cells were larger (clone 886-A at 0·126 μ/cell) or smaller (clone 886-B at 0·053 μg/cell) their sodium was in excess of their potassium. Similar explants, which received the combination of IAA and zeatin, produced many more but smaller cells which consistently accumulated K^+ over Na^+ and had a low Cl^- content and relatively low total osmotic value; these properties are characteristic of small cells which divide more than they enlarge. Therefore, zeatin has an effect upon the Na^+/K^+ ratio independently of its influence upon their growth.

TABLE 1. GROWTH AND SOLUTE RESPONSES OF CULTURED CARROT EXPLANTS (CLONES 886 A AND B) TO THE COMPONENT PARTS OF GROWTH PROMOTING SYSTEM II (IAA AND ZEATIN)

Treatments	Growth			Solute content				
	Fr. wt (mg/explant)	Cell No. (thousands)	Cell wt (μg/cell)	K^+ (μmol/g fr. wt)	Na^+	Cl^-	Osmotic value (mOsm)	Na^+/K^+ ratio
Clone 886-A								
Basal Medium (B)	18	105	0·173	75	24	44	334	0·320
B + IAA	33	345	0·095	54	37	20	358	0·685
B + zeatin	30	239	0·126	61	103	16	385	1·689
B + IAA + zeatin	25	453	0·058	60	10	9	227	0·167
Clone 886-B								
Basal Medium (B)	7	59	0·126	124	15	38	389	0·121
B + IAA	22	290	0·076	47	49	23	485	1·043
B + zeatin	13	257	0·053	84	96	42	561	1·143
B + IAA + zeatin	11	483	0·023	98	35	9	314	0·357

Table 2 records the data which test whether growth and ionic content of the cells are similarly affected by a series of zeatin-like compounds. The medium used throughout for the assay of growth (i.e. the control medium of Table 2) contained the co-factors (IAA and inositol) of growth promoting Systems I and II and casein hydrolysate which increases their effectiveness.[2] In this situation the zeatin-like compounds, working with IAA and inositol, produced many cells although the stimulated responses in terms of their Na^+/K^+ ratio, though evident, were not as great as when zeatin acted alone (Table 1).

The maximum effect of the members of a series of N^6-substituted adenines occurred when the substituent carbon side chain, $(-CH_2)_nH$, was 5 carbons long,[3] although zeatin, with its different functional groups in the side chain, produced less than this maximum effect. But the maximum sodium concentration and Na^+/K^+ ratio occurred when the side chain length $(-CH_2)_n$ was at $n = 4$ (Table 2). This result distinguished between the effects of the compounds upon the growth (cell multiplication) and upon the solute (ionic) content of the tissue as cultured.

TABLE 2. GROWTH AND SOLUTE RESPONSES OF CULTURED CARROT EXPLANTS (CLONES 892 A AND B) TO ZEATIN AND A SERIES OF N^6-SUBSTITUTED ADENINES* TO A BASAL MEDIUM (B) WITH THE COFACTORS OF GROWTH SYSTEMS I AND II

Treatments	Growth			Solute Content				
	Fr. wt (mg/explant)	Cell No. (thousands)	Cell wt (μg/cell)	K^+ (μmol/g fr. wt)	Na^+	Cl^-	Osmotic value (mOsm)	Na^+/K^+ ratio
Clone 892-A								
Control medium (B + CH + Inos + IAA)	24	407	0·067	49	30	18	220	0·612
Control medium + zeatin	76	1784	0·039	64	34	17	302	0·531
+ N^6-adenine$(-CH_2)H$	25	402	0·057	43	21	11	190	0·488
+ N^6-adenine$(-CH_2)_2H$	26	427	0·058	46	22	10	164	0·478
+ N^6-adenine$(-CH_2)_3H$	21	490	0·052	45	31	12	218	0·689
+ N^6-adenine$(-CH_2)_4H$	70	1338	0·043	50	46	18	390	0·920
+ N^6-adenine$(-CH_2)_5H$	60	2140	0·031	56	37	19	426	0·661
+ N^6-adenine$(-CH_2)_6H$	47	1035	0·046	57	32	12	380	0·561
+ N^6-adenine$(-CH_2)_7H$	24	342	0·065	44	23	10	348	0·523
+ N^6-adenine$(-CH_2)_8H$	22	304	0·082	52	30	15	265	0·577
+ N^6-adenine$(-CH_2)_9H$	31	324	0·082	46	25	9	179	0·543
+ N^6-adenine$(-CH_2)_{10}H$	28	344	0·063	47	25	11	240	0·532
Clone 892-B								
Control medium (B + CH + Inos + IAA)	28	504	0·054	66	37	7	224	0·561
Control medium + zeatin	56	1301	0·030	74	34	10	287	0·459
+ N^6-adenine$(-CH_2)H$	36	1039	0·042	74	32	10	275	0·432
+ N^6-adenine$(-CH_2)_2H$	42	479	0·044	73	15	9	191	0·205
+ N^6-adenine$(-CH_2)_3H$	50	724	0·046	64	46	7	271	0·719
+ N^6-adenine$(-CH_2)_4H$	66	1603	0·040	54	50	6	251	0·944
+ N^6-adenine$(-CH_2)_5H$	57	1631	0·036	71	38	11	252	0·535
+ N^6-adenine$(-CH_2)_6H$	54	1422	0·038	69	37	8	248	0·536
+ N^6-adenine$(-CH_2)_7H$	19	452	0·041	60	20	6	188	0·333
+ N^6-adenine$(-CH_2)_8H$	24	406	0·047	54	34	8	207	0·630
+ N^6-adenine$(-CH_2)_9H$	25	574	0·044	70	30	6	174	0·429
+ N^6-adenine$(-CH_2)_{10}H$	23	603	0·045	63	24	6	201	0·381

* Each substituted adenine is designated by its side chain $(-CH_2)_nH$. The compounds were conveniently tested at the same weight concentration (0·1 mg/l.). This represents a molar concentration of $4{\cdot}9 \times 10^{-7}$ M. for the compound with a 5 carbon side chain and the small spread in molar concentrations between adjacent members of the series [e.g. $(CH_2)_{4-6}$] would not be a significant factor in their effect on the tissue.

DISCUSSION

The N^6-substituted adenines, which affect the growth of the cultured cells in terms of cell multiplication and cell size, also affect the solute (ionic) composition of the cells produced and this is especially evident in the proportions of sodium to potassium. But it is also known that zeatin has other effects for it increases the contribution of non-protein nitrogen compounds (especially the amide glutamine) to the total osmotic value of the cells.[10] In fact, the disparity in Table 2 between the total osmotic value as measured, and the total due to alkali halides, is evidence of the preponderance of organic solutes in these heterotrophically cultured cells. Within this complement of organic solutes (which includes the non-protein nitrogen-compounds, the sugars, and the organic acids that contribute to the osmotic value) wide variations in the detail of the response to the N^6-substituted adenyl growth regulating compounds must also occur. These results therefore direct attention to the scope and range of the effects of the N^6-substituted adenines.

A series of N^6-substituted adenines affect growth and solute composition differentially. The member of the series with a 5-carbon substituent side chain $(-CH_2)_5H$ produced the maximum multiplication of cells of minimum size and maximum osmotic value; the compound that produced the maximum Na^+/K^+ ratio in the cells had a 4-carbon substituent side chain. In this homologous series, those with chain lengths of 4–6 carbons affected the growth in a manner comparable to zeatin whereas others, with different chain lengths, gave the higher Na^+, lowered K^+ response in varying degrees.

[10] STEWARD, F. C. and RAO, K. V. N. (1971) *Planta (Berlin)* **99**, 240.

Interpretations of the role of N^6-substituted adenines should not, therefore, be confined to their possible presence in transfer RNA molecules and to their consequential effects upon protein synthesis;[1] nor should they merely imply their direct involvement with transport of specific solutes.[4,11] The data indicate the importance of these compounds in balanced interactions with other substances, e.g. IAA, and to their effects upon metabolism, water and solute relations as part of their ultimate effects upon growth and cell multiplication.

EXPERIMENTAL

In the first experiment two clones of carrot explants (886-A and B) were exposed to a basal medium (B)[8] which was enriched with IAA (0·5 mg/l.), with zeatin (0·1 mg/l.) and with the two in combination (Table 1). After 18 days under standard conditions (21°, constant diffuse light, in slowly rotated culture tubes each of which contained three explants) the cultures harvested from each of three replicated tubes were separately weighed, macerated for determinations of the number and average size of cells[12], or so extracted that the osmotic value (mOsm) and ionic (K^+, Na^+, Cl^-) content (μmol/g fr. wt) could be determined by methods that have been described.[8] Having, in this way, again noted the effect of zeatin upon the growth and ionic composition of carrot explants further tests were made, by similar procedures, to demonstrate the effect of chemical structure in a series of zeatin-like compounds upon the response in question. This was done by analyzing cultures which had been harvested from the growth assays[3] and which had been routinely and rapidly deep frozen for storage in sealed containers.

The methods which were used for the determinations of K^+, Na^+, Cl^- and the total osmotic value of the solutes of the cells, have been described[8] and, since all determinations were replicated on two clones of explants, the data are adequate to support the broad conclusions to be drawn. Tests showed that determinations made on replicated tissue extracts, when calculated to the content in the tissue, gave low coefficients of variation in the range 0·03–0·10 for the alkali metals and 0·10 for chloride. When K^+ and Na^+ determinations were made on replicated tissue samples which had received the same treatments they did not deviate by more than 5% of the values as determined.[13] Moreover, the osmotic value of the solutes extracted from the cells (as calculated from routine determinations of K^+, Na^+, Cl^-, organic acid, sugars and non-protein nitrogen compounds) have consistently agreed to within 10% of those directly determined on the extracts[8,9].

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