KINETIN-LIKE GROWTH-PROMOTING ACTIVITY OF 1-SUBSTITUTED ADENINES [1-BENZYL-6-AMINOPURINE AND 1-(\(\gamma,\gamma\)-DIMETHYLALLYL)-6-AMINOPURINE]*

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Biological activity of the type exhibited by kinetin (I) in promoting growth (cell division, organ formation, etc.) in plant tissues has been found to be limited almost entirely to 6-substituted purine derivatives. Activity has been reported for a few other substances including a 3-substituted adenine derivative, the alkaloid triacanthine, isolated from honey locust leaves and identified by procedures including synthesis as 3-(\(\gamma,\gamma\)-dimethylallyl)-adenine. This alkaloid may be widely distributed in plants as it also has been isolated under the names chidlovine and togholamine from two other species. In tests carried out in our laboratory, however, triacanthine was active only when it had been heated in the test procedures and, even so, only in relatively high concentrations (1–100 \(\mu\)M/l compared with ca. 0.001–0.05 \(\mu\)M/l of kinetin). It was established that the activity was due not to triacanthine but to a product formed by autoclaving it with the nutrient medium as in routine bioassay procedures or merely by heating it in weakly acid aqueous solutions. The active product has an Rf higher than that of triacanthine (Rf 0.73) and in the same region as that of 6-(\(\gamma,\gamma\)-dimethylallylamino)-purine (II) (Rf 0.95) in a \(n\)-butanol:water:glacial acetic acid 75:20:5 v/v/v solvent system. This 6-isomer of triacanthine has ten times higher specific activity than kinetin in the range from barely detectable to maximum response in the tobacco callus bioassay; i.e., 0.0001–0.01 \(\mu\)M/l of 6-(\(\gamma,\gamma\)-dimethylallylamino)-purine compared with ca. 0.001–0.10 \(\mu\)M/l of kinetin. Thus it was estimated that a conversion of 0.01 per cent or less of the tested dosages of triacanthine to the 6-isomer would account for the observed biological activities.

Certain kinetin analogues and other adenine derivatives substituted in position two, seven, or nine have been negative in tests for growth-promoting activity.

Recently, Leonard and Fujii have synthesized 1-(\(\gamma,\gamma\)-dimethylallyl)-adenine (III) and 1-benzyladenine (IV). Evidence is presented here that these compounds are biologically active. Quantitative activities of these, the corresponding 6N-isomers, and of kinetin have been compared in tests of growth promotion in callus cultures and chlorophyll retention in leaf tissue.

Materials and Methods.—The test chemicals 1-benzyladenine (IV), 6-benzylaminopurine (V), 1-(\(\gamma,\gamma\)-dimethylallyl)-adenine (III) and 6-(\(\gamma,\gamma\)-dimethylallylamino)-purine (II) were prepared by Leonard et al., University of Illinois. Synthesis and biological activity of the 6N-substituted adenines are well established.†-§ Synthesis of the 1-substituted adenines is described in this issue. Kinetin was prepared locally. Growth-promoting activity was measured in terms of yields and observed morphogenetic effects in bioassays with tobacco tissue cultures.

The procedures and media employed were essentially as reported by Murashige and Skoog. The organic supplements were 2.0 mg/l 3-indoleacetic acid (IAA), 0.5 mg/l niacin, 0.5 mg/l pyridoxine, 0.1 mg/l thiamin-HCl, 100 mg/l myo-
inositol, 2.0 mg/1 glycine, 30 g/1 sucrose, and 10 g/1 Difco agar. However, the stock callus tissue (Nicotiana tabacum var. Wis. #38) had been previously maintained on modified White's medium\textsuperscript{10} with 0.2 mg/1 of kinetin and 2.0 mg/1 IAA, and had then been transferred for two passages on the high-yielding revised medium with low (0.04 mg/1) kinetin and 2.0 mg/1 IAA. This stock callus gives a definite growth response to kinetin concentrations down to 0.001 \( \mu \text{M/1} \) and gives a linear increase in yield in the range from ca. 0.005 to 0.08 \( \mu \text{M/1} \). Four replicate cultures each with 3 pieces of callus (each ca. 30 mg fresh weight) planted on 50 ml of agar medium were used for each treatment. Note that to minimize chemical breakdown, solutions of the test substances were sterilized by filtration and added to the medium after it had been autoclaved and allowed to cool nearly to the gelation point.

Chlorophyll retention, an index of vigor (keeping quality) of tissue, was measured in leaf disks of Raphanus sativus (var. White icicle) treated with serial concentrations of the test substances and extracted largely as described by Osborne.\textsuperscript{11} Disks (12 mm in diameter) were punched from areas in between the large veins of the youngest, fully expanded leaf of 25–30-day-old radish plants grown in the greenhouse. The disks were floated on water for 1 hr and then distributed four per dish into 5-cm Petri dishes. The disks were mounted on a \#50 Whatman filter paper which had been placed on the bottom of the dish and had been thoroughly wetted with a 0.50-ml sample of test solution. Duplicate sets of dishes were prepared for a series of dilutions of the test substance. After 3 days of incubation in a covered plastic tray with high humidity in the dark at 22°C, each set of 4 disks was extracted with a total volume of 10 ml of 85% ethanol, and the optical
density of the extract at 665 m\(\mu\) was measured in a Bausch & Lomb Spectronic 20 colorimeter.

Results.—(a) Yields: The effects of increasing concentrations of 1-(\(\gamma,\gamma\)-dimethylallyl)-adenine, 1-benzyladenine, and kinetin on the growth of tobacco callus cultures are illustrated in Figure 1, and mean fresh and dry weight yields per flask obtained in one experiment are shown in Table 1. It may be seen that even

<table>
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<tr>
<th>Concentration ((\mu)M/l)</th>
<th>Kinetin Fresh wt.</th>
<th>1-((\gamma,\gamma)-Dimethylallyl)-adenine Fresh wt.</th>
<th>1-Benzyladenine Fresh wt.</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>0.004</td>
<td>0.60</td>
<td>0.40</td>
<td>0.66</td>
</tr>
<tr>
<td>0.02</td>
<td>4.30</td>
<td>1.57</td>
<td>0.84</td>
</tr>
<tr>
<td>0.1</td>
<td>19.00</td>
<td>11.30</td>
<td>6.65</td>
</tr>
<tr>
<td>0.5</td>
<td>6.00</td>
<td>18.60</td>
<td>13.10</td>
</tr>
<tr>
<td>2.5</td>
<td>1.00</td>
<td>15.10</td>
<td>2.86</td>
</tr>
<tr>
<td>12.5</td>
<td>0.16</td>
<td>8.10</td>
<td>2.87</td>
</tr>
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0.004 \(\mu\)M/l, the lowest tested concentration, of all three substances definitely increased the size of the treated tissue as compared with the controls, and this increase was accompanied by a 5–10-fold increase in fresh and dry weights.

In the case of kinetin optimal volumes as well as optimal fresh and dry weights of tissue were consistently reached at the 0.10 \(\mu\)M/l level. This level of 1-(\(\gamma,\gamma\)-dimethylallyl)-adenine also permitted good growth, but its optimum level was closer to 0.50 \(\mu\)M/l. In this experiment (Table 1), the maximum fresh weight yield for 1-benzyladenine was only 13 gm/flask as compared with 19 for each of the other two substances. This lower value might mean that the maximum effectiveness of the 1-benzyladenine fell in the range between 0.10 and 0.50 \(\mu\)M/l, or perhaps the effectiveness of the substance happened to be less than normal in this
particular experiment. In two other experiments, 1-benzyladenine gave as high
optimal yields as the other two substances.

The two or three highest concentrations of the substances gave less than optimal
yields but did not decrease the vigor of the tissue. In fact, the cultures with the
highest levels would survive the longest if allowed to continue to grow. It is
clear that also in the suppression of growth by high levels, mole for mole kinetin
was more effective than the 1-substituted adenines.

Comparisons show that the dry weight yields closely paralleled the fresh weights
throughout the entire range. In summary, it may be said that in the region of
sensitive response, kinetin was roughly 2–5 times more active than either of the
1-substituted adenines.

(b) Organ formation: Morphogenetic effects of the 1-substituted adenines
have not been studied specially, but in cultures intended for yield determinations
organ formation comparable to that obtained with appropriate levels of kinetin
was already evident after 4–5 weeks also in some cultures which received either
6N- or 1-substituted adenines. In Figure 2 examples are shown of bud formation
obtained with high levels of (6-γ,γ-dimethylallylamino)-purine and root formation
obtained with 2.5 and 12.5 μM/1 of 1-(γ,γ-dimethylallyl)-adenine. In this
experiment, similar rooting effects were obtained with ca. 5 times lower levels of
kinetin. A few buds and appearances of the callus characteristic of early budding
stages have been observed in cultures treated with high levels of either one of the
1-substituted adenines. Both with respect to growth and organ formation, therefor
the 1-substituted adenines are somewhat weaker than kinetin but otherwise
very similar to it in their actions:

(c) Comparison of 1- and 6N-isomers: The relative effectiveness of the 1- and
6N-isomers in promoting growth of tobacco tissue may be judged from cultures
supplied with low concentrations of the benzyladenines (Fig. 3A), and of the
(γ,γ-dimethylallyl)-adenines (Fig. 3B). In each case the 6N-isomer was the more
active. Note that 0.1 μM/1 of 6-benzylaminopurine was high enough to induce
the compact growth form characteristic of much higher kinetin levels. Unfortu
nately the 0.1 μM/1 treatment with 6-(γ,γ-dimethylallylamino)-purine in this
experiment was lost due to infection. The fresh and dry weight data are summa
rized in Table 2. It is clear that the 6-(γ,γ-dimethylallylamino)-purine was con
sistently ahead of its 1-isomer and also ahead of the 1-benzyladenine, which
latter in this case was the more active of the 1-isomers. In this experiment,
6-benzylaminopurine was less than ordinarily effective. Nevertheless, disregar

<table>
<thead>
<tr>
<th>Conc. of chemical</th>
<th>Weight (gm/flask)</th>
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<tr>
<td>adenosine</td>
<td>1-(γ,γ-Dimethylallyl)-</td>
</tr>
<tr>
<td>0.00</td>
<td>0.26</td>
</tr>
<tr>
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<tr>
<td>0.10</td>
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<tr>
<td>0.50</td>
<td>13.89</td>
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<tr>
<td>2.5</td>
<td>19.60</td>
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<tr>
<td>12.5</td>
<td>12.00</td>
</tr>
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</table>
Fig. 2.—Examples of organ formation in tobacco callus cultures treated as specified: 1, basal nutrient control; 2 and 3, bud formation in cultures treated with 12.5 μM/l of 6-(γ,γ-dimethylallylamo)-purine; 4 and 5, root formation in cultures treated, respectively, with 2.5 and 12.5 μM/l of 1-(γ,γ-dimethylallyl)-adenine; 6, root formation in control culture with 0.5 μM/l kinetin. Age of cultures—5 weeks.

ing its failure to reach a high optimum yield, and considering all other aspects, it is still judged to be more active than its 1-isomer even in this test.

(d) Chlorophyll retention: In tests of chlorophyll retention in excised leaf disks, the activities of the five substances were as indicated in Figure 4, in which are plotted O.D. values of extracted chlorophyll against log_{10} the concentration of the test substances. In this graph, all curves are from one experiment except the one for 6-benzylaminopurine which is from a separate experiment and has been corrected by an appropriate factor based on the slight but real difference in activity of kinetin in the two experiments. Although individual points on the curves are subject to considerable chance variation, mole for mole 6-benzylaminopurine is more active than kinetin, and it in turn is definitely more effective than 1-benzyladenine and either one of the 1- and 6N-(γ,γ-dimethylallyl)-derivatives in maintaining the chlorophyll content and general vitality of the leaf disks. This holds true even though 6-(γ,γ-dimethylallylamino)-purine is ten times more active than kinetin in promoting growth of the tobacco callus. It should be noted that the leaf disk test requires ca. 1000 times higher concentration than the callus growth test.

Discussion and Conclusion.—Qualitatively, both 1-benzyladenine and 1-(γ,γ-dimethylallyl)-adenine possess the same type of growth-promoting activity as

kinetin, and quantitatively they may be nearly as effective. On the other hand, numerous tests in this laboratory with 6-benzylaminopurine since it was first synthesized and tested here in 1956 have established that it is more active than kinetin in the tobacco bioassay, and data accumulated over the past two years fix the activity of 6N-(γ,γ-dimethylallyl)-adenine at close to 10 times that of kinetin. The present results, though less conclusive, are in agreement with this, and they clearly show that each of the 1-substituted adenines mole for mole is less active than its 6N-isomer. More tests in the region of high sensitivity would be required to determine exact differences in specific activity, but from the available data it is estimated that each 6N-isomer is at least 3 times and not over 20 times as active as its 1-isomer. At least as large differences in activity between the 1-substituted adenines and their 6N-isomers are indicated in the chlorophyll retention test. These data, however, also bring out the important point that the relative activities of active substances may be quite different, even in reverse order, in different tests. For example, the 6-(γ,γ-dimethylallylamino)-purine which has been consistently more active than kinetin in the tobacco bioassay also has been consistently less active than kinetin in chlorophyll retention tests with Raphanus leaves and also in comparable tests with other tissues and species.
The data as a whole permit the conclusion that the 6N-substituted adenines are biologically active and the interpretation that the 1-isomers may be converted to the 6-isomeric- or other active 6-substituted forms. Evidence for 1-R to 6-NHR conversion in vitro and possible chemical mechanisms or pathways by which it may be achieved are discussed by Leonard and Fujii. It may perhaps be even more readily achieved enzymatically in vivo. In any case, it is clear from the present results that if such a conversion is required to confer activity on the 1-substituted adenines, the reaction must be at least about 10 per cent efficient, even for minute concentrations and at room temperature. The mechanism, therefore, must be quite specific, and it would seem to require a directed transfer of the substituent, i.e., an intramolecular rearrangement. Random breakdown and recombination, respectively, at two reactive positions, which might be enough in the formation of kinetin from DNA or for the “activation” of triacanthine under suitable conditions of high temperature, etc., would hardly suffice.

Irrespective of the mode or need for conversion of the 1-substituted adenines to exhibit activity, it appears from the above results that the 1- and 6N-substituted purines must be set apart as distinct from all other purine derivatives with respect to their growth-promoting activity. Serious consideration must be given to the alternative that also the 1-substituted adenines are in fact highly active as such. Investigation of this possibility, which may have important bearing on structure-activity relationships of this group of substances, is continuing.

* Supported in part by the University Research Committee of the Graduate School with funds from the Wisconsin Alumni Research Foundation.
† Predoctoral fellowship from the Ministry of Education of the Syrian Arab Republic and the Institute of International Education is gratefully acknowledged.

1 Strong, F. M., in Topics of Microbial Chemistry (New York: John Wiley and Sons, 1958), chap. 3.
6 Rogozinska, J. H., J. Helgeson, and F. Skoog, Physiol. Plant., in press.
7 2-Furfuryladenine synthesized by F. S. Okumura, University of Kyoto, and tested in our laboratory (unpublished).
9 Laboratories of the Wisconsin Alumni Research Foundation.
SEQUENCES OF RNA AND PROTEIN SYNTHESIS DURING EARLY ESTROGEN ACTION

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That RNA synthesis is a major aspect of early estrogen action in the uterus of a previously ovariectomized rat is now well documented.\textsuperscript{1,4} Following administration of a single dose of estradiol-17b to such experimental animals, synthesis within the uterine cells of a variety of biological molecules is accelerated, and evidence exists that this increase is one of qualitative\textsuperscript{4} as well as of quantitative\textsuperscript{1} differences in comparison to the control system. By four hours, rises in levels of RNA, nucleotides, protein, and phospholipids can be discerned,\textsuperscript{2,6–8} and it is well known that this \textit{in vivo} uterine-response system is a valuable one for unraveling of the biochemical basis of early estrogen action in particular, and of hormone action in general.

Parallels between the biochemical basis of hormone actions in vertebrate and invertebrate organisms are now apparent,\textsuperscript{8,10} and it is a major goal of contemporary endocrinology—which seeks to explain on a molecular basis the ability of hormones to influence both the \textit{synthesis} and \textit{activity} of other biological molecules—to unify cellular theories for hormonal mechanisms of representative organisms throughout the animal kingdom. The current direction of thinking in many laboratories is that ontogenetic as well as phylogenetic tissue specificities\textsuperscript{11,12} to particular hormones result from genetic programming of intracellular responses to these. Thus, DNA–RNA interactions are involved in hormone actions, and the suggestions of Jacob and Monod\textsuperscript{13} and Monod \textit{et al.}\textsuperscript{14} concerning the relation of hormone action to protein synthesis assume a new relevance, posing models which should be capable of testing in the near future.

Below are reported isotopic and inhibitor experiments which indicate the following about estrogen-induced synthesis of RNA and proteins throughout the four-hour \textit{in vivo} response of uterine cells to single injections of 10 \(\mu\text{g}\) of estradiol-17b at zero hour in ovariectomyzed rats: (1) that between 30 min and 1 hr a small rise in protein synthesis occurs, lasting until 2\(1/2\) hr, at which time a marked acceleration of protein synthesis occurs; (2) that RNA synthesis during these test periods is accelerated by the time of 1 hr, rising markedly to a peak at 3–4 hr; (3) that actinomycin D, by preventing DNA-dependent RNA synthesis, prevents the typical estrogen-induced, 4-hr rise in RNA and protein synthesis with the RNA, \textit{but not the protein} synthesis being restricted to levels below those of controls; and (4) that the time-sequence studies (1, 2) and inhibitor studies (3) illustrate (a) the involvement