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A COMPARATIVE INVESTIGATION OF KINETIN (6-FURFURYLAMINOPURINE) AND SOME SIMILARLY SUBSTITUTED PURINES AND PYRIMIDINES WITH *LEMNA MINOR* (L.)

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Abstract—The effect of kinetin on the growth of *Lemna minor* (L.), cultivated under sterile conditions, was compared with that of 2-furfurylamino-6-hydroxypurine (furfurylguanine), 2-benzylamino-6-hydroxypurine (benzylguanine), 2-hydroxy-4-benzylaminopyrimidine (benzylcytosine) and 2-mercapto-4-furfurylamino-pyrimidine (furfurylthiocytosine). Only kinetin proved to be active as a growth stimulant. Neither were the other compounds active in maintaining the level of chlorophyll in detached leaves. Kinetin-type activity thus seems to be restricted to adenine derivatives.

INTRODUCTION

THE course of events in the investigations on compounds promoting cell division in plants termed kinins (on the assumption of a physiological function), has been the reverse of the normal one with physiologically acting factors. In most cases the investigation starts with the isolation of a pure substance whose structure and function are established. Subsequently an investigation is made as to whether the physiological activity can be imitated by synthetic analogues (cf. e.g. the auxins). When kinetin was isolated[1] it soon became clear that, although it was endowed with a striking stimulatory activity in a number of tests, it was an artefact. This led to the postulation of the existence of a naturally occurring compound(s) having a physiological function, and initiated several investigations aiming at the isolation of substances from plants with kinetin-type of activity.

Though certain facts may admittedly be used as arguments for the thesis that some of the active compounds are of natural origin (cf. the discussion in a recent paper from the laboratories of Skoog and Strong[2]), in our opinion they do not constitute a decisive proof. We feel that it is still undecided whether we really do have physiologically functioning compounds (rightly indicated as (phyto)kinins) or are merely concerned with an interesting chapter on phytopharmacology. It might be, for example, that with the highly active substituted adenines we are not imitating a natural function, but interfering with one (of adenine?).

In this respect it seemed interesting to see whether or not kinetin-like activity might be found with compounds containing purine skeletons different from adenine, or with pyrimidines, both substituted with, for example, furfuryl- and benzyl-groups. For this purpose the compounds listed in Table 1 were synthesized and tested for growth-promoting activity in *Lemna minor*. We preferred using an intact plant in order that our comparisons referred to a complete functioning system. To obtain information about a different aspect of kinetin

[1] C. O. MILLER, F. SKOOG, F. S. OKUMURA, M. H. VON SALTZA and F. M. STRONG, *J. Am. Chem. Soc.*, **78** 1375 (1956).

[2] J. H. ROGOZINSKA, J. P. HELGESON, F. SKOOG, S. H. LIPTON and F. M. STRONG, *Plant Physiol.* **40**, 469 (1965).

action, the compounds were assayed for their effect on chlorophyll retention in detached leaves according to Mothes *et al.*[3]

RESULTS

The results of a growth experiment are summarized in Table 1. Evidently neither the substituted guanines nor the substituted pyrimidines exert any growth stimulating action.

TABLE 1. EFFECT OF SUBSTITUTED PURINES AND PYRIMIDINES (1 μM) ON THE INCREASE IN WEIGHT OF *Lemna minor* AFTER 18 DAYS OF GROWTH AT 1000 LUX

Compound	Δ Fresh weight in mg	Δ Dry weight in mg
Control	260	28
Kinetin	625	49
N-Furfurylguanine	256	27
N-Benzylguanine	268	27
N-Benzylcytosine	258	27
N-Furfuryl-thiocytosine	280	29

That this is not caused by lack of uptake into the plant was proved in the case of benzylguanine by using the ^{14}C-labelled compound. It was interesting to note that under our experimental conditions 90 per cent had been taken up from the medium after 4 hr (measured as disappearance from the medium) in the case of ^{14}C-adenine and of ^{14}C-guanine, whereas only 10 and 35 per cent of ^{14}C-kinetin and ^{14}C-benzylguanine respectively were adsorbed.

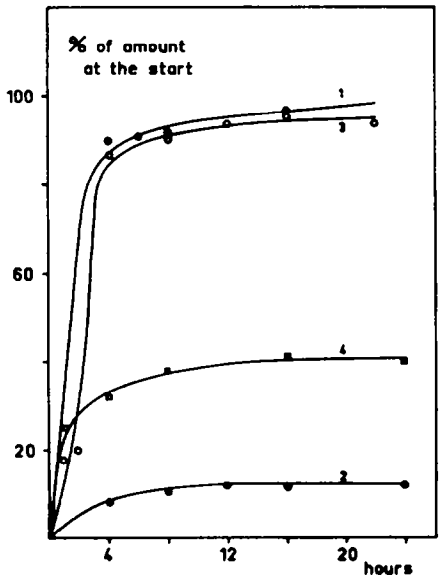

FIG. 1. UPTAKE OF ^{14}C-LABELLED COMPOUNDS (1 μM) FROM THE GROWTH MEDIUM, MEASURED AS DISAPPEARANCE FROM THE MEDIUM

1. ^{14}C-adenine; 2. ^{14}C-kinetin; 3. ^{14}C-guanine; 4. ^{14}C-*N*-benzylguanine.

[3] K. MOTHES, *Naturwissenschaften* **47**, 337 (1960). K. MOTHES, L. ENGELBRECHT and H. R. SCHÜTTE, *Physiol. Plantarum* **14**, 72 (1961).

The initial rate of uptake is higher for adenine than for kinetin; the proportions are reversed for guanine and benzylguanine (cf. Fig. 1). Benzylguanine (50 μM) does not interfere with the uptake of kinetin (1 μM), whereas adenine (100 μM) and benzyladenine (50 μM) do. When the latter compound is tested at a lower concentration (1 μM), and also when kinetin (1 μM) is combined with 6-S-benzylmercaptopurine (both at 1 and 50 μM), which lacks kinetin activity, the uptake of kinetin is enhanced. These differences between adenine and guanine derivatives and the effect of benzylmercaptopurine are paralleled by those found in growth experiments, the results of which are summarized in Table 2 and 3. It is clear that adenosine is able to counteract the growth stimulating action of kinetin, guanosine being devoid of this capacity.

TABLE 2. INCREASE IN WEIGHT OF *Lemna minor* AFTER 19 DAYS OF GROWTH AT 1000 LUX. INTERACTION BETWEEN KINETIN (1 μM) AND ADENOSINE OR GUANOSINE (both 100 μM)

Compound	Δ Fresh weight in mg	Δ Dry weight in mg
Control	281	27
Kinetin	896	64
Adenosine	351	30
Guanosine	321	36
Kinetin + adenosine	293	22
Kinetin + guanosine	877	65

TABLE 3. INCREASE OF WEIGHT OF *Lemna minor* AFTER 2 WEEKS OF GROWTH IN THE PRESENCE OF KINETIN (1 μM), *S*-BENZYLMERCAPTOPURINE (1 μM) OR OF BOTH AT 1000 LUX

Compound	Δ Fresh weight in mg	Δ Dry weight in mg
Control	220	20
Kinetin	490	29
S-Benzylmercaptopurine	255	22
Kinetin + *S*-benzylmercaptopurine	545	34

When applied to detached leaves of *Nicotiana tabacum, Samsun NN.* or *Phaseolus vulgaris, Cultivar Berna*, according to the technique of Mothes *et al.*,[3] from the compounds furfuryl- and benzylguanine (at 83·3 and 50 μM respectively), adenine and benzyladenine, -guanine and -cytosine (at 100 μM), only benzyladenine was capable of maintaining a high level of chlorophyll, at the point of application.

DISCUSSION

All the data from our experiments lead to the conclusion that the effects of kinetin show an exclusive "adenine story", which cannot be "translated" in terms of other purines or of pyrimidines. In our opinion this means that it is unlikely that the action of kinetin is connected with some role in metabolism in which all of these constituents are concerned (e.g. nucleic acid). Though the outcome of our experiments do not permit us to make a definite choice between a physiological function (of naturally occurring kinetin analogues) or a phytopharmacological action (of substituted adenines), it seems to us that they encourage one to pursue the possibility that kinetin interferes with a balance of adenine functions

which has to do with cell-division, the shift of which (triggering division?) normally may come about by different means, that is not necessarily by the action of one special natural kinin.

EXPERIMENTAL

Plant material. Lemna minor (L) was used for the experiments. The plants were cultivated under sterile conditions in Clark's[4] medium, from which peat extract and caseine hydrolysate were omitted to rule out the possibility of including some unknown growth factor. The effect of kinetin was studied on normally growing, photosynthesizing plants. For this purpose the optimal conditions for light intensity had to be determined. The experiments were carried out at 1000 lux (daylight fluorescent tubes, 40 W, Philips) and 20° ± 1°.[5]

Uptake experiments. To the growth medium (10 mg), on which the plants (200 mg wet weight) were floating, the labelled compounds were added in a given concentration. After certain intervals of time, a small amount was taken from the medium, charcoal (cf.[6]) was added for absorption, and filtered on a Popják filter, washed with water, dried and the charcoal spread on planchets and counted with a Nuclear Chicago gas flow counter with micromil window (correction to infinite thinness was made on the basis of the charcoal absorption curve for ^{14}C). The counts were expressed as the percentage of the number found at the start.

Detached leaves. By means of a pipet 0·1 ml of the solution of the compound to be tested was put as a droplet on the leaf (*Nicotiana tabacum, Phaseolus vulgaris*). After 20 min the remainder of the droplet was taken up in filter paper and the leaves were subsequently incubated in the dark in a most atmosphere at room temperature. After 3 days the retention of chlorophyll was recorded.

Synthesis of the Compounds

Kinetin[7] A mixture of 1 g 6-chloropurine (Dr Theodor Schuchardt, GmbH, München, Germany), 10 ml methyl cellosolve and 2 ml freshly distilled 2-furfurylamine Fluka AG, Bucho, Switzerland was refluxed in an oil-bath for 2 hr. After standing overnight at 4° the crystals were filtered off and washed with methylcellosolve, water, absolute alcohol and ether. The yellow crystalline powder weighing 1·2 g was recrystallized from 95% alcohol; yield 0·8 g.

^{14}C-kinetin. In a microcarius tube with enclosed filtration needle a mixture of 2·3 mg 6-chloropurine-8-^{14}C (50 μc spec. act. 3·6 mc/m-mole, Calbiochem. U.S.A.) and 0·03 ml methylcellosolve–furfurylamine mixture (4:1) was heated for 0·75 hr at 110°. During the reaction, crystallization of kinetin starts; when the reaction is finished the microcarius tube is kept in the oil-bath in order to effect slow cooling and growth of big crystals of kinetin. The carius tube was opened at both ends and the kinetin washed with water, alcohol and ether. The kinetin was purified by vacuum sublimation at 7·5–8 mm, 220° and the yield (2·5 mg 78 per cent) was determined spectrophotometrically at 267 mμ.

Radiochemical Purity of ^{14}C-kinetin

The ^{14}C-kinetin was tested for radiochemical purity by means of high voltage electrophoresis. This was performed on Whatman No. 1 paper in an ammonium formate buffer

[4] N. A. CLARK, *Iowa State Coll. J. Sci.* **7**, 13 (1932).
[5] J. VAN EYK, Investigations on the mode of action of kinetin with *Lemna minor* (L). Thesis, University of Leyden, (1963).
[6] C. J. THRELFALL, *Biochem J.*, **65** 694 (1957).
[7] M. W. BULLOCK, J. J. HAND and E. L. R. STOKSTAD, *J. Am. Chem. Soc.* **78**, 3693 (1956).

at pH 3·5, 2000 V, during 2·5 hr. After electrophoresis the radioactivity was counted on paper and shown to be 96·7 per cent pure kinetin.

6-*Benzylaminopurine*[8, 9] was prepared as described under kinetin. Purification by recrystallization from methanol, 96% alcohol, methylcellosolve or vacuum sublimation, 8 mm 210°. (Found: C, 63·7; H, 5·4; N, 30·8. Calc. for: C, 64·00; H, 4·88; N, 31·11%)

6-*Benzylaminopurine*-^{14}C.[10, 11] Usually reactions of the type described are carried out in the presence of a large excess of amine, part of the amine serving for the neutralization of the HCl formed in the reaction. In order to find amine-sparing reaction conditions, we used equimolecular amounts of chloropurine and benzylamine in the presence of MgO or $KHCO_3$. These experiments proved to be unsuccessful. Better yields were obtained by the use of tris-ethanolamine or tris-isopropanolamine as proton-accepting bases, though they did not raise the yield much above the 50 per cent, based on benzylamine. 1·6 mg benzylamine-1-^{14}C (0·1 mc spec. act. 6·7 mc/m-mole, Calbiochem. U.S.A.) in 0·55 ml water was dried over P_2O_5 in a CO_2 atmosphere in a desiccator. The product was mixed with 0·05 ml of a solution of 11·6 mg 6-chloropurine (recrystallized from *n*-butanol) in 0·25 ml *n*-butanol; about 7 mg trisethanolamine was added. The mixture was refluxed in an oil-bath during 45 min and evaporated to dryness *in vacuo*. The residue was washed with water and methanol, dried and vacuum sublimed, 8 mm, 210°. In "cold" experiments with the same quantities and under the same conditions yields of benzylaminopurine of 45-60 per cent based on benzylamine were obtained. The yields were determined spectrophotometrically. When considering the reaction efficiency it is to be noticed that there is a loss of benzylamine as a result of drying and transferring the benzylamine carbonate to the reaction-vessel.

6-*S-benzylmercaptopurine*.[7, 12, 13] 1 g 6-mercaptopurine (Nederlandse Combinatie voor Chemische Industrie, Amsterdam) was dissolved in one equivalent of dilute NaOH. 1·2 g benzylchloride (Fluka AG) was slowly added under vigorous stirring. After 30 min the oily phase disappeared and a crystalline precipitate of 6-benzylmercaptopurine was formed. After 1·5 hr the precipitate was filtered by suction, washed with water and light petrol, dried and recrystallized from toluene. Yield 1 g of 6-benzylmercaptopurine, m.p. 191·5–192° (lit.: 178–180°,[12] 193–194°,[7] 193°[13]). The u.v. spectra in water and 95% alcohol are identical with those given in literature.

2-*Furfurylamino-6-hydroxypurine* (N-*furfurylguanine*).[14, 15] 0·9 g 2-methylthioxanthine (prepared by methylation of 2-thioxanthine (Fluka AG) after G. B. Elion) was heated for 24 hr at 140° with 1·5 g of freshly distilled furfurylamine (Fluka AG) in a carius tube. The filter-cake was washed with 20 ml of acetone and dissolved in hot 4 N HCl. After treatment with charcoal (norit) the solution was adjusted to pH 7 with concentrated ammonia. The precipitate was filtered off and washed with water. This procedure was repeated once more. After drying over P_2O_5 the yield was 0·5 g, m.p. >325°. (Found: C, 51·7; H, 4·0; N 29·9. Calc.: C, 51·95; H, 3·89; N, 30·30%.)

2-*Benzylamino-6-hydroxypurine* (N-*Benzylguanine*). For the synthesis of this new compound the procedure of the preparation of *N*-furfurylguanine was followed. 0·9 g 2-methyl-

[8] M. W. Bullock, J. J. Hand and E. L. R. Stokstad, *J. Org. Chem.* **22**, 568 (1957).
[9] Ch. G. Skinner and W. Shive, *J. Am. Chem. Soc.* **77**, 6692 (1955).
[10] S. Hünig and M. Kiessel, *Chem. Ber.* **91**, 380 (1958).
[11] S. Hünig and M. Kiessel, *J. Prakt. Chem.* **5**, 224 (1958).
[12] Ch. G. Skinner, R. G. Ham, D. C. Fitzgerald, Jr. and R. E. Eakin, *J. Am. Chem. Soc.* **78**, 5097 (1956).
[13] Th. P. Johnston, L. B. Holum and J. A. Montgomery, *J. Am. Chem. Soc.* **80**, 6265 (1958).
[14] G. B. Elion, W. H. Lange and G. H. Hitchings, *J. Am. Chem. Soc.* **78**, 217 (1956).
[15] L. Almirante, *Ann. Chim. (Rome)* **49**, 333 (1959).

thioxanthine gave 0·45 g *N*-benzylguanine, m.p. >325°. (Found: C, 59·2; H, 4·7; N, 29·6. Calc.: C, 59·75; H, 4·56; N, 29·05%.)

2-(*Benzylamino*-1-^{14}C-6-*hydroxypurine* (^{14}C-N-*benzylguanine*). The solution of 1·6 mg benzulamine-1-^{14}C (0·1 mc) in 0·55 ml water as acidified with 4 N HCl and dried over P_2O_5 in a desiccator. In a microcarius tube was weighed 2·25 mg 2-methylthioxanthine. This was mixed with about 0·8 mg benzylamine-1-^{14}C-HCl and 1·5 μl benzylamine. The mixture was heated for 25 hr at 140° in the closed carius tube. After cooling and opening of the carius tube the solid was washed with acetone, water and acetone and dried. The resulting ^{14}C-*N*-benzylguanine is a white crystalline compound with a specific activity of 0·5 mc/mM. Cold experiments with 4·5 mg of 2-methylthioxanthine gave yields of 3–4 mg of *N*-benzylguanine.

2-*Hydroxy*-4-*benzylaminopyrimidine* (N-*benzylcytosine*).[16] The method of Fidler *et al.*[16] was applied to 1 g 2·4-dimercaptopyrimidine (Fluka AG) yielding 0·4 g *N*-benzylcytosine. (Found: C, 65·3; H, 5·3; N, 21·3. Calc.: C, 65·67; H, 5·47; N, 20·89%) After several recrystallizations from alcohol the compound is paper-chromatographically pure. On melting its crystal form changes at 228–232° and melts without decomposition at 239–242° (lit. after one recrystallization from alcohol; m.p. 213–217° (decomp.)[16]).

2-*Mercapto*-4-*furfurylaminopyrimidine* (N-*furfurylthiocytosine*). In a 25 ml Erlenmeyer flask 1 g 2·4-dimercaptopyrimidine (Fluka AG) is mixed with 5 ml freshly distilled furfurylamine. The flask is stored unstoppered *in vacuo* during 3 days in a desiccator over KOH pellets. A yellowish precipitate is formed. This is filtered and washed with a small quantity of 96% alcohol. 1·5 g precipitate is recrystallized from 300 ml 96% alcohol, using norit. After one more recrystallization the yield is 0·7 g white crystals of *N*-furfurylthiocytosine, m.p. 235–238°. (Found: C, 52·3; H, 4·4; N, 20·3. Calc.: C, 52·17; H, 4·35; N, 20·20%). Prolonged heating during the recrystallization is to be avoided, as it results in browning of the substance. We did not succeed in substituting the SH group by an OH group by the method described by Fidler and Wood for the preparation of *N*-benzyl-cytosine from the corresponding SH-compound by heating with chloroacetic acid and concentrated HCl solution at 100°. In our hands this method only yielded black tars when applied to *N*-furfurylthiocytosine.

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[16] W. E. FIDLER and H. C. S. WOOD, *J. Chem. Soc.* 3980 (1957).