The Metabolism and Biological Activity of a 9-Substituted Cytokinin$^{1,2}$

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Received for publication May 27, 1970

ABSTRACT

In order to test the metabolic stability of 9-substituted cytokinins, 6-benzylamino-9-methyl purine has been synthesized and labeled with $^{14}$C in the 9-methyl carbon or doubly labeled with $^{14}$C in the 9-methyl carbon and $^3$H in the methylene moiety of the side chain. Although the 6-benzylamino-9-methylpurine is chemically stable, cytokinin-requiring tissues begin removing the 9-substituent in as little as 10 minutes. Among the various metabolic products is free benzylaminopurine. Thus, the biological activity of 9-substituted cytokinins could be accounted for by their conversion to the free base.

The widespread presence of cytokinins in the transfer RNA of animals, plants, and microorganisms is well established (see review by Key [14]). Furthermore, in those cases where the primary structure is known, the cytokinin is found immediately adjacent to the presumed anticodon (I, 5, 17). The continuing debate as to whether or not the physiological activity of exogenously supplied cytokinins is mediated by their presence in tRNA seems to have been recently resolved by the work of Chen and Hall (4). Here it was shown that the potent cytokinin N$^{9}$-(α$^3$-isopentenyl) adenosine occurs in the tRNA of cultivated tobacco tissue even though the tissue requires an exogenous cytokinin in order to grow. The fact that the cytokinin supplied in the medium is not the metabolic precursor of the cytokinin in the tRNA argues that the role of the former is distinct in at least some important way from that of the latter.

This work, however, cannot be used as evidence against the notion that exogenous cytokinins act through their observed incorporation into polynucleotides (7, 9, 10). One would need to demonstrate that cytokinins which are prevented from entering into nucleotide formation nevertheless retain all of their biological activity. It occurred to us that the use of blocking agents at the 9 position of the purine moiety would result in compounds which might yield information concerning the relation between nucleotide formation and biological activity of the cytokinins. Accordingly, in July of 1967, we reported (11) the results of tests with the substance 6-benzylamino-9-(3-hydroxypropyl) purine. Although this material retains cytokinin activity, it is 10 to 100 times less effective than the corresponding free base; it is possible that the activity of the compound is due to its conversion to the free base in the plant tissue by removal of the substituent at the 9 position.

Earlier, Weaver et al. (22) reported that substituting benzyladenine at the 9 position with a tetrahydropyran ring yielded a compound with good biological activity, while Guern et al. (12) pointed out that several 9-substituted benzyladenines are active though seemingly not hydrolyzed in vivo. More recently Kende and Tavares (13) described studies with 6-benzylamino-9-methyl purine-benzyl-$^3$H. Although radioactivity was incorporated into the RNA of soybean callus tissue when the free base was employed, methylation at the 9 position apparently prevented incorporation while in no way reducing biological activity of the cytokinin. Letham (16) and Young and Letham (23) reported that the 9-cyclohexyl analog of isopentenyladenine possesses considerable cytokinin activity; Letham considers that, “It is extremely unlikely that this compound with a group not subject to hydrolytic cleavage on the 9 position could be converted to a ribotide and incorporated into RNA as a nucleotide.” (16).

Likewise, Shaw et al. (21) reported that 9-methylzeatin gave biological activity of the same order as zeatin and concluded that, “The results imply that the mechanism of cytokinin activity in substituted adenines does not require prior formation of nucleotide derivatives.”

Clearly, however, such conclusions are premature unless it is demonstrated directly that the substituent at the 9 position is not removed by the plant tissue and thus constitutes a truly effective block to ribonucleotide synthesis. In this paper the synthesis and biological activity of 9-methyl benzyladenine is described. In addition, evidence is presented which indicates that soybean and tobacco tissues remove substituents at the 9 position within a few minutes of incubation.

MATERIALS AND METHODS

Biological Tests. The origin of the soybean and tobacco tissues used here and their growth on various cytokinin and auxin levels have previously been described (8). The basal medium and culture methods have been reported (6). In some short term metabolic studies with labeled compounds, liquid media were used and the flasks were shaken. Compounds tested for cytokinin activity were sterilized at room temperature by filtration.

Synthesis and Characterization of 6-Benzylamino-9-methyl Purine. The 6-benzylaminopurine used in this and following synthetic procedures was prepared as previously described (9). The methylated derivative was synthesized by alkylation of the

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1 Supported by National Science Foundation Grants GB-6010 and GB-8319 and National Institutes of Health Grant GM-09902.
2 Part of this material is from a thesis presented by Chander Sood in partial fulfillment of the requirements for the Ph.D. degree at the University of Kansas.
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potassium salt of BA. This procedure is similar in many respects to that of Carraway et al. (3) who report an increase in the selectivity of the alkylation which yields an increased ratio of the 9- to the 3-isomer as compared with earlier methods. In a typical synthesis \(5.8 \times 10^{-4}\) moles (130.2 mg) of BA were dissolved in 15 ml of anhydrous tetrahydrofuran to which were added \(7.0 \times 10^{-4}\) moles (27.3 mg) of metallic potassium. This mixture was stirred at room temperature under nitrogen for 12 hr after which \(6.4 \times 10^{-4}\) moles (912 mg) of methyl iodide was added, and the whole mixture was stirred for an additional 12 hr. The reaction flask was fitted to a condenser through which water at 0 C was circulated to prevent the escape of gaseous methyl iodide. The reaction mixture was then taken to dryness at 30 C under reduced pressure in a rotary evaporator. The dried material was dissolved in a small amount of ethyl acetate and streaked on 1-mm layers of aluminum oxide G (Merck) on glass plates activated at 60 C for 24 hr. The thin layer chromatogram was developed in a mixture of chloroform and benzene (9:1). In this system ultraviolet-absorbing material presumed to be 6-benzylamino-9-methyl purine migrated to an \(R_F\) of 0.25 while the unreacted potassium salt of BA remained at the base line. In addition, at least two other ultraviolet-absorbing compounds could often be detected at \(R_F\) 0.55 and at the solvent front; these were presumed to be other methylated derivatives although they were not further characterized. The ultraviolet quenching band at \(R_F\) 0.25 was eluted from the alumina with ethanol and taken to dryness in vacuo. In the run described here 38 mg of product were recovered, which on the basis of the expected compound MBA, constitutes a 27% yield.

For further characterization and tests of biological activity,
the product was dissolved in ethanol and precipitated as the hydrochloride. The precipitate was dissolved in water and reconverted to the free base by titration with sodium bicarbonate. MBA was then extracted from the mixture with diethyl ether and recovered in crystalline form as colorless needles by evaporating the solvent. Final purification was achieved by sublimation of crystalline material. The sublimed material melted at 138 to 138.5 C. Elemental analysis yielded the following results: C: 65.27; H: 5.85; N: 29.09; calculated for C_{19}H_{19}N_{5}: C: 65.26; H: 5.48; N: 29.27.

A mass spectrum of the compound (Fig. 1) shows a molecular ion at m/e 239 which is consistent with a methyl-substituted benzyladenine. In addition, the fragmentation pattern of the compound is not unlike that of BA (Fig. 2) with strong peaks in both at m/e 91 and m/e 106 identifiable respectively as the tropilium ion (C_{6}H_{5}^{+}) and a species (C_{6}H_{5}CH_{2}N^{+}H) formed by the loss of the purine ring system (2).

Paramagnetic resonance spectra (Table 1, Fig. 3) indicate that the purine 2 and 8 positions are unsubstituted and support a 9-substituted structure assignment. That the substance is a 6-, 9-disubstituted adenine is likewise indicated by the ultraviolet absorption spectrum (Fig. 4). Features of the spectrum which permit this conclusion are the shoulder on the high wavelength side of the maximum under alkaline conditions, and maxima in the region of 270 with little shift of variation in extinction coefficient with change in pH (15).

Further evidence that the compound is substituted at the 9 position is provided by the infrared absorption spectrum (Fig. 3). The IR spectrum of the hydride (Fig. 3, solid line) indicates the presence of the methyl group characteristically in the range 2950 to 2850 cm. The IR spectrum of the free base (Fig. 3, dashed line) shows the vibrations of the benzyl group between 1600 and 1500 cm, which is consistent with the purine C8 vibration at 1600 cm.

![Fig. 3. Proton magnetic resonance spectrum of 6-benzylamino-9-methyl purine in deuterated chloroform determined on a Varian A 60A PMR spectrophotometer.](image)

![Fig. 4. Ultraviolet absorption spectrum of 6-benzylamino-9-methyl purine at a concentration of 2.1 \times 10^{-4} M in water determined on a Bausch and Lomb 505 recording spectrophotometer. \( \lambda_{\text{max}} \), pH 2.0 (HCl) 271.5, \( \lambda_{\text{max}} \), pH 6.0 (H_{2}O) 270, \( \lambda_{\text{max}} \), pH 11.0 (NH_{4}OH) 271.5.](image)
5). The infrared spectrum of BA exhibits typical weak absorption
between 2800 to 2400 cm\(^{-1}\) due to the acidic hydrogen on the 9
nitrogen (2) whereas the corresponding spectrum of the com-
pound in question lacks this feature. Finally, the compound was
not precipitated with AgNO\(_3\) under acidic conditions (0.05 N
acetic acid) although BA precipitated immediately with this
treatment.

MBA was also synthesized by a second method which pro-
vided still more evidence for its structure. In a typical experiment
81.5 mg of 6-chloro-9-methyl purine (Cyclo Chemical Co.)
were refluxed in 20 ml of water with 0.1 ml of benzylamine for 6
hr. The reaction mixture was taken to dryness in a rotary evap-
orator and washed with a small amount of water at 0 C to remove
residual benzylamine. The product was converted to the HCl salt
and purified as described above. Crystalline material was re-
covered in a 68% yield and proved identical with the material
synthesized by method 1.

Synthesis of 6-Benzylamino-9-methyl Purine-methyl\(^{14}\)C. The
preparation of the \(^{14}\)C-labeled compound was performed as in
the first method described above except that the synthesis was
carried out in a sealed reaction bomb. In this procedure 7.2 \(\times\)
10\(^{-5}\) moles of the potassium salt of BA were reacted with 0.5
mc of \(^{14}\)CH\(_3\)I (Amersham Searle, batch 95) sp. act. 41.2 mc/
mole, \(\text{i.e.,} 12.1 \mu\)moles. After purification, 0.62 mg (2.6 \(\times\) 10\(^{-6}\)
moles) of the desired product were recovered. The specific radio-
activity of the product as estimated with the aid of a Packard
model 3375 liquid scintillation spectrometer was 43.0 mc/mM.

Synthesis of 6-Benzylamino-9-methyl Purine-methyl\(^{14}\)C-methyl-
olene-\(^{3}\)H. The tritium, carbon 14 doubly labeled compound was
synthesized by reducing \(N^8\)-benzoylaminopurine (18) with lithium
aluminum tritide (New England Nuclear Corp., specific radio-
activity 125 mc/mM). The reactants were refluxed in \(N\)-methyl
morpholine overnight, taken to a small volume \(\text{in vacuo, and}
subjected to chromatography on thin layers of silica gel in chloro-
form-methanol-1 N NH\(_2\)OH (5:2:1), a procedure which
conveniently separates benzyladenine from unreacted benzoyladenine.
The tritiated BA then became the starting compound for methyl-
atation at the 9 position with \(^{14}\)CH\(_3\)I as described above.

From this procedure 0.5 mg of doubly labeled MBA was
recovered with specific radioactivities of \((^{14}\)C) 10 mc/mM and
(\(^{3}\)H) 37 mc/mM.

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Table II. Effect of BA and MBA on the Growth of Soybean Callus
Tissue/ 2

<table>
<thead>
<tr>
<th>Conc of Cytokinin</th>
<th>BA</th>
<th>MBA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh wt/flask</td>
<td>Dry wt/flask</td>
</tr>
<tr>
<td>(M)</td>
<td>(g)</td>
<td>(g)</td>
</tr>
<tr>
<td>0</td>
<td>0.16</td>
<td>0.01</td>
</tr>
<tr>
<td>(1 \times 10^{-9})</td>
<td>0.17</td>
<td>0.01</td>
</tr>
<tr>
<td>(1 \times 10^{-8})</td>
<td>0.66</td>
<td>0.04</td>
</tr>
<tr>
<td>(1 \times 10^{-7})</td>
<td>1.63</td>
<td>0.10</td>
</tr>
<tr>
<td>(1 \times 10^{-6})</td>
<td>1.42</td>
<td>0.08</td>
</tr>
<tr>
<td>(1 \times 10^{-5})</td>
<td>1.19</td>
<td>0.07</td>
</tr>
<tr>
<td>(1 \times 10^{-4})</td>
<td>0.20</td>
<td>0.01</td>
</tr>
</tbody>
</table>

1 Tissues were cultured for 22 days under standard conditions
(see text).

2 Each figure is an average of 4 flasks with 2 pieces of tissue in
each.
RESULTS AND DISCUSSION

Biological Activity. The ability of MBA to support growth in cytokinin-requiring tissues approached but never equaled that exhibited by BA itself. The latter exhibited an optimum at 1 × 10^{-4} M for both soybean and tobacco cultures, while the 9-substituted derivative was most active at 1 × 10^{-4} M in tobacco tissue and perhaps at slightly higher concentration in soybean (Table II, Fig. 6). This relative difference has been a consistent feature in repeated tests.

In addition, the activity of MBA has been contrasted with that of BA in the assay for cytokinins depending upon the initiation of deoxyisoflavone synthesis in soybean cultures described by Miller (19). This test, which was performed by Carlos Miller in Bloomington, Indiana, indicated that the presence of a methyl group at the 9 position greatly interfered with the ability of BA to promote synthesis of deoxyisoflavones in soybean tissues incubated 45 hr in liquid medium (Fig. 7). Some activity was noticed, however, at concentrations of MBA 10 to 100 times higher than the unsubstituted compound.

Metabolic Stability of the 9-Substituent. In order to test for metabolic cleavage of the 9-methyl moiety, soybean and tobacco tissues were incubated for varying periods on liquid or solid media containing MBA labeled with ^14C in the 9-methyl carbon. The tissues employed in these studies were in the log phase of growth but starved for 72 hr prior to use on a basal medium, complete except for the cytokinin. At the end of the incubation period, the gases present in the flask were passed through a column containing finely divided filter paper soaked in a concentrated solution of barium hydroxide in order to trap CO_2.

Table III. ^14CO_2 Evolution from Tobacco and Soybean Tissues Incubated on a Culture Medium Containing MBA-methyl-^14C

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Incubation Period</th>
<th>CPM—background</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid medium</td>
<td>Tobacco</td>
<td>10 min</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>10 min</td>
</tr>
<tr>
<td></td>
<td>Tobacco</td>
<td>60 min</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>60 min</td>
</tr>
<tr>
<td></td>
<td>Tobacco</td>
<td>360 min</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>360 min</td>
</tr>
<tr>
<td></td>
<td>Tobacco</td>
<td>48 hr</td>
</tr>
<tr>
<td></td>
<td>Soybean</td>
<td>48 hr</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>48 hr</td>
</tr>
<tr>
<td>Solid medium</td>
<td>Tobacco</td>
<td>48 hr</td>
</tr>
<tr>
<td></td>
<td>Soybean</td>
<td>48 hr</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>48 hr</td>
</tr>
</tbody>
</table>

1 See text for details of trapping and radioactivity estimation of released CO_2.
2 Experimental manipulations of the control flask were identical with the test flask except that the tissue was omitted.

3 Tissue (approximately 3 gm fresh weight/flask) was incubated with 10 ml of a standard culture medium containing 1 mg/l, i.e., 4.15 × 10^{-4} moles/10 ml, 6-benzylamino-9-methylpurine-methyl-^14C sp. ac. 43 mc/mmole, i.e., 3.5 × 10^4 cpm/10 ml. Tissues were starved on a medium complete except for a cytokinin for 72 hr prior to use.

Tobacco cultures, while never equaled, did exhibit some activity at 9-methyl moieties, higher than the unsubstituted compound.

Fig. 7. Influence of BA and MBA on the synthesis of deoxyisoflavones in soybean tissue; 0.6 g of finely chopped tissue was incubated for 45 hr at 26 C in 20 ml of medium and then extracted by the addition of 80 ml of 95% ethanol. The increase in absorbance at 260 nm of the extract is due largely to the synthesis of deoxyisoflavones stimulated by active cytokinins (19). Each point is the average of three cultures. See Miller (19) for details of procedure.

Fig. 6. Effect of BA and MBA on the growth of soybean callus tissue. The tissues were grown for 3 weeks on a basal medium (see text) to which was added the appropriate concentration of the cytokinin sterilized at room temperature by filtration.
sibility of microbial contamination during the manipulations required by the experiment, a control flask complete except for the tissue was subjected to the same operations for each experiment.

The results (Table III) indicate that both soybean and tobacco tissue on either liquid or solid media metabolize MBA in such a way that at least some portion of the methyl moiety is released as CO₂. Furthermore, the amount of ¹⁴CO₂ released is a function of time and the process begins to occur within at least 10 min.

Only a tiny fraction of the counts supplied to the tissue as MBA-methyl-¹⁴C are represented by ¹⁴CO₂. Extracts of the tissue chromatographed on a variety of systems have made it clear, however, that MBA persists for only a very short time and is quickly metabolized by the tissue. The most instructive experiments were done with tobacco tissue incubated with MBA doubly labeled with ¹⁴C and ³H. The tissue was extracted with sufficient 95% ethanol at room temperature to achieve a final concentration of 70% by grinding with a mortar and pestle. Insoluble material was separated by centrifugation and re-extracted twice. The combined supernatants were reduced in vacuo to a small volume and spotted on precoated sheets of alumina for thin layer chromatography (Eastman-Chromatogram sheet 6062). The chromatogram was developed in chloroform-ethanol (97:3, v/v), a solvent system chosen for its ability to separate widely BA (Rᶠ 0.15) and MBA (Rᶠ 0.75). The developed chromatogram was then air-dried, cut into small strips, and eluted at 50°C with 50% ethanol for 1 hr. The eluate was dried at 30°C in counting vials to which was then added 15 ml of a standard solution for liquid scintillation spectrometry. The vials were assayed for both ¹⁴C and ³H.

The results (Fig. 8) indicate that even within 1 hr, less than 10% of the ¹⁴C is associated with MBA; the bulk of the radioactivity is distributed across several fractions. By 4 days there is little, if any, intact MBA in tobacco tissue. The compound is apparently much more stable in soybean tissue however, so that, although other metabolites appear early, as much as 40% of the MBA taken up by the tissue on a liquid medium may persist up to 7 days. In a control run in which manipulations were identical with the experiment except that no tissue was placed in the flask, over 99% of the radioactivity recovered from the medium after 4 days was coincident with MBA in three chromatographic systems.

A question crucial to this study is whether or not free BA appears during the metabolism of MBA. One ought to be able to detect this compound easily since it would be tritium labeled but devoid of ¹⁴C. However, Figure 8 shows that ¹⁴C is present in substantial amounts even at the Rᶠ of BA. Presumably, though, the ¹⁴C from the methyl group of MBA enters into the methyl pool and subsequently into a number of metabolites, some of which migrate into the same general area of the chromatogram as BA. In order to test for free BA, portions of the chromatogram having the proper Rᶠ were eluted in ethanol and the eluate co-crystallized with authentic, unlabeled BA from mixtures of ethanol and water. The product was sublimed and the resulting crystals were scraped from the walls of the sublimation tube directly into counting vials. Similar studies were performed on the incubation medium after tissue had been growing for either 6 hr or 4 days. In all cases the product was radioactive and contained largely tritium although a small amount of ¹⁴C was observed as well, probably due to a tenaciously bound contaminant.

Thus, in one instance a sample which contained 3600 dpm tritium was also registered 178 dpm of ¹⁴C. As much as 10% of the tritium counts in the liquid medium after 6 hours incubation with tobacco tissue was identified with BA.

The biological activity of MBA, which in our hands is at least 10 times less active than BA, is therefore probably best explained by its conversion in the tissue to the free base. The methyl group at the 9 position does not constitute an effective block as has been assumed by previous workers but is in fact rather readily metabolized by plant tissues. This finding makes the presumption of stability of other 9-substituted cytokinins in the absence of information regarding their metabolic stability, one cannot, therefore, use the biological activity of 9-substituted cytokinins as evidence against the possibility that cytokinin activity depends upon nucleotide formation.

LITERATURE CITED

METABOLISM OF A CYTOKININ