The results are presented in Table 2. We found that about 10 percent of the magnesium ions are associated with sulfate ions. This result is in good agreement with those of Garrels and Thompson, Thompson, and Fisher (*1*) which were obtained by a variety of methods. The values of the steady-state *p*H obtained in the present work are lower than those obtained earlier by Pytkowicz *et al.* (*1*).

The apparent (quasi-stoichiometric) solubility product of brucite in solution B, $K'_{SP} = (Mg^{2+})_T(a_{OH})^2$, was found to be $(2.4 \pm 0.2) \times 10^{-11}$. The thermodynamic solubility product may be expressed as $K_{SP} = (f_{Mg})_F(Mg^{2+})_F(a_{OH})^2$. According to Garrels and Thompson (*1*), $(f_{Mg})_F = 0.36$. Therefore, from our values of $(Mg^{2+})_F$ and a_{OH}, $K_{SP} = (7.8 \pm 0.5) \times 10^{-12}$. This result is only approximate because a_{OH} obtained from *p*H measurements is not exactly the thermodynamic activity of hydroxyl ions. However, the arbitrariness in the operational definition of *p*H does not detract from its practical value in self-consistent potentiometric studies of seawater (*4*). Our result is in good agreement with that of Hostetler (*5*), who found that $K_{SP} = (7.1 \pm 0.5) \times 10^{-12}$.

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28 June 1968

Cytokinin from Soluble RNA of Escherichia coli: 6-(3-Methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine

Abstract. *We have isolated a compound responsible for the cytokinin activity of soluble RNA from* Escherichia coli. *The structure, indicated as 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-*D*-ribofuranosylpurine, $C_{16}H_{23}N_5O_4S$, on the basis of low- and high-resolution mass spectrometry, was established by unequivocal synthesis. The mass spectra, chromatographic behavior, and ultraviolet spectra of the compounds from natural and synthetic sources were identical.*

Acid hydrolyzates of *Escherichia coli* tRNA (*1*) were found to exhibit cytokinin activity in the tobacco callus bioassay (*2*), but since 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (2iPA), the cytokinin-active minor riboside of several tRNA's (*3*), was not detectable in the enzymic hydrolyzates of *E. coli* tRNA (*4*), it was of special interest to isolate and identify the compound or compounds responsible for this activity. The approach, then, was to locate the component by following its route through the isolation procedure by means of the tobacco bioassay (*5, 6*) of aliquots.

Thirty grams of partially degraded *E. coli* B sRNA (*7*), containing 9.7 $O.D._{260}$ units per milligram, were hydrolyzed to nucleosides with lyophilized snake venom (*Crotalus adamanteus*, Sigma) and alkaline phosphatase (*8*). The hydrolysis procedure was similar to that of Hall (*9*). The RNA was dialyzed for 24 hours at 4°C before hydrolysis.

The nucleoside mixture was fractionated by partition chromatography on Celite-545 columns (*9*); the upper phase of a mixture of ethyl acetate, methoxyethanol, and water (4:1:2) was used for elution. The elution profile was similar to that reported by Hall (*10*) for yeast sRNA. Cytidine and guanosine were eluted together by washing the column with distilled water.

A small $O.D._{260}$ peak (fraction 1) emerging with the solvent front was highly active in the tobacco bioassay for cytokinins (*2*). The activity in fraction 1 was increased by acid hydrolysis (0.1*N* HCl, 100°C, 45 minutes) before bioassay. Under these conditions, activity could also be detected in the methyladenosine peak and, very weakly, in the adenosine peak. The uridine peak and water eluate were inactive, as were all fractions eluted from a control column to which a sample of the enzyme solution, incubated in the absence of RNA, was applied.

Thin-layer chromatography of samples of fraction 1 in Cellulose MN 300 F_{254} plates, with distilled water as a solvent, revealed four ultraviolet-absorbing spots. Cytokinin activity was associated with two poorly resolved spots moving at R_F 0.06 and 0.12 (compounds 1′ and 2′). Two fast-moving spots (compounds 3′ and 4′, R_F 0.64 and 0.82, respectively) were inactive in the tobacco bioassay. Compounds 1′ and 2′ could be resolved by thin-layer chromatography in 10 percent ethanol (R_F 0.10 and 0.31, respectively). Only compound 2′ exhibited cytokinin activity.

Compound 2′ was isolated from fraction 1 (see above) by chromatography on Whatman No. 1 paper. The composition of eluates from the chromatograms was monitored by bioassay and thin-layer chromatography. Chromatography of fraction 1, with distilled water as a solvent, separated the fast-moving compounds 3′ and 4′ from the cytokinin activity which was streaked from the origin to about R_F 0.4. Compound 2′ and a large amount of fluorescent material were present in the eluate from R_F 0.06 to 0.40. The chromatographic properties of the fluorescent substance were very similar to those of compound 2′. As a result, it was convenient to isolate the latter compound from the eluate of the region below R_F 0.06. The solids from this eluate were extracted twice with 1.5 ml volumes of chloroform-petroleum ether (2:1). The supernatant from this extraction contained considerable colored material together with most of compound 1′ and a small amount of compound 2′. The residue, consisting primarily of compound 2′, was chromatographed in 50 percent ethanol. One ultraviolet-absorbing band (R_F 0.85) was observed. The eluate from this band was rechromatographed in 20 percent ethanol and gave a single ultraviolet-absorbing band at R_F 0.58, which was again rechromatographed in 20 percent ethanol. Elution of the ultraviolet-absorbing band (R_F 0.58) yielded about 1 mg of white solids containing compound 2′ and a small amount of fluorescent material. This sample was used for determination of the structure of compound 2′.

The low-resolution mass spectrum of the active compound showed a fragmentation pattern parallel to that of

2iPA (*11*) and (in the *m/e* region corresponding to the loss of the pentose moiety) of 2iP, 6-(3-methyl-2-butenylamino)purine (*12–14*) but consistently 46 mass units greater. This feature suggested the presence of CH_3S- substituent which survived in many of the fragments resulting from electron bombardment. The high-resolution mass spectrum confirmed the presence of sulfur and showed a molecular-ion peak at 381.149, corresponding to the formula $C_{16}H_{23}N_5O_4S$ (calculated 381.147). We therefore deduced that the compound was 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**I**). The fragment ions at low resolution of *m/e* 249, 234, 206, 194, and 181 for the $(B+1)^+$ purine portion (*15*), parallel to those for 6-(3-methyl-2-butenylamino)purine with the change in *m/e* being +46 (Fig. 1), were supported by the high-resolution figures, and metastables were observed for the conversions 249→234 and 249→206. This series of peaks and their relative intensities supported the 3-methyl-2-butenylamino (Δ^2-isopentenylamino) side-chain structure and ruled against the isomeric 3-methyl-3-butenylamino (Δ^3-isopentenylamino) side chain (*13*). The *m/e* values of 381, 366, 338, and 313 represented similar side-chain fragmentation at the riboside level, while *m/e* values of 292 and 278 represented retention of the hydrocarbon side chain and typical cleavage of the ribose moiety, yielding $(M-89)^+$ and $(B+30)^+$, respectively (*15*) (Fig. 1). Minor peaks indicated loss of CH_2 from the methylthio group: *m/e* 235 (0.8 percent total abundance), 180 (1.0 percent), 151 (1.2 percent); and others, loss of CH_2S, giving fragments like those from 2iP (*12, 13*): 203 (0.2 percent), 188 (0.2 percent), 160 (0.3 percent), 148 (0.2 percent), 135 (2.2 percent), all confirmed in formulation by the high-resolution *m/e* values.

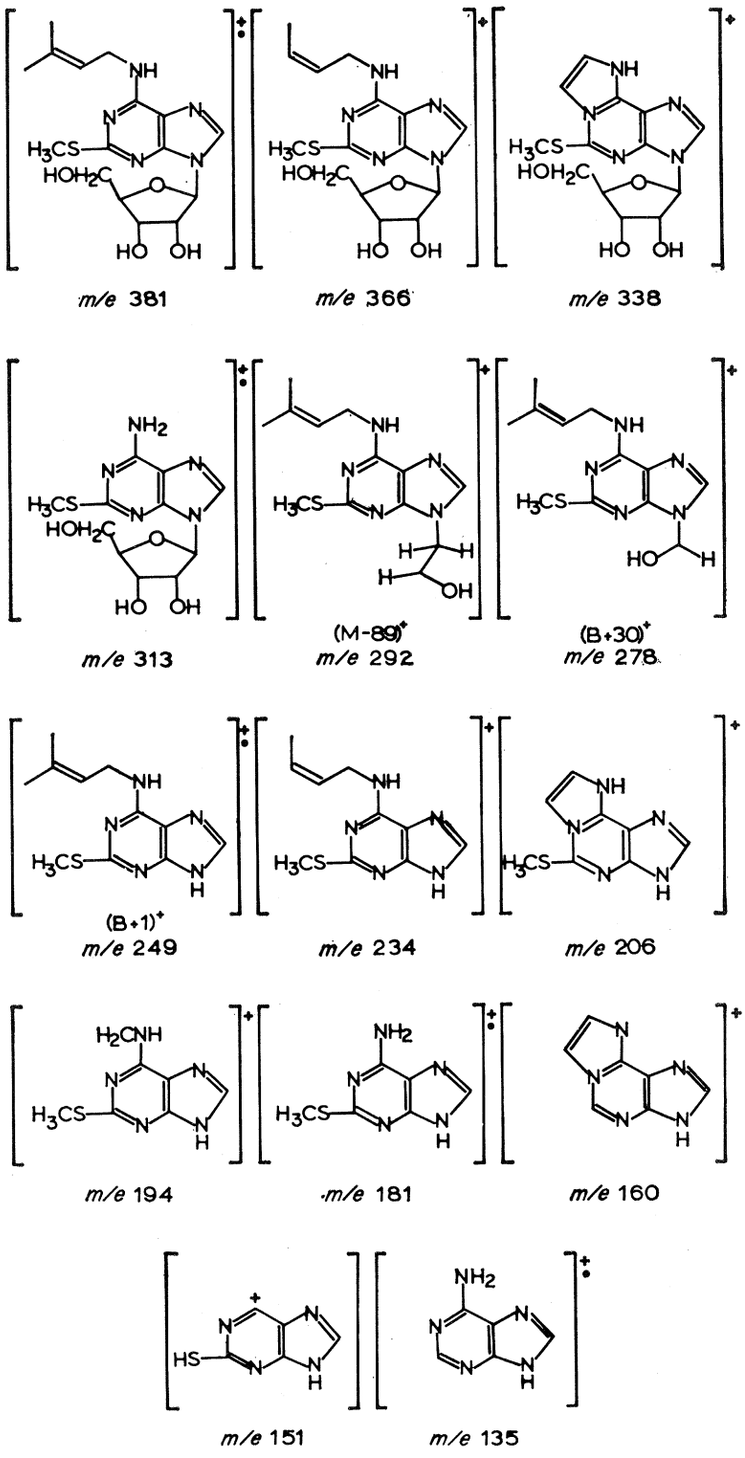
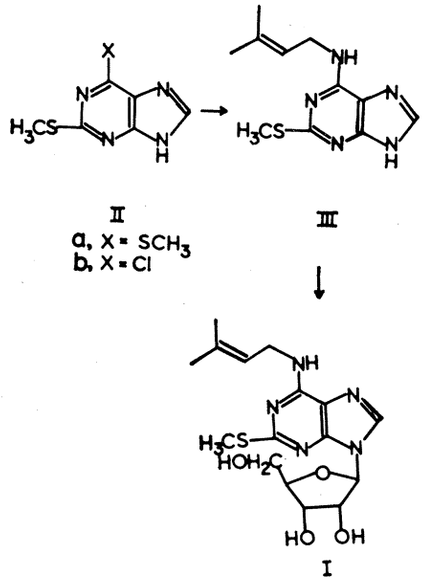


Fig. 1. Fragments of cytokinin from *Escherichia coli* determined by mass spectrometry at 70 ev.

The selection of the 2-position of the adenine moiety for methylthio substitution rather than the 8-position was made by comparison of the qualitative ultraviolet spectra of the active cytokinin in neutral, acidic, and basic solution with the corresponding spectra for 2-methylthio- and 8-methylthio-N^6-substituted adenines (*16, 17*).

Accordingly, 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**I**) was considered the proper candidate for synthesis. In the first of two methods which were followed, 2,6-bis-(methylthio)purine (**IIa**) (*16*) was heated under reflux with an excess of γ,γ-dimethylallylamine for 30 hours, the mixture was diluted with water, and the solid product was triturated with hot ethanol to give 6-(3-methyl-2-butenylamino)-2-methylthiopurine (**III**) [or 6-(γ,γ-dimethylallylamino)-2-methylthiopurine], m.p. 259° to 260°C. This compound had the correct elemental analysis for $C_{11}H_{15}N_5S$, and the structure was confirmed by the ultraviolet and NMR spectra. The other route involved the treatment of 2-methylthiohypoxan-

thine (*18*) with phosphorus oxychloride under reflux (*19*). The crude 6-chloro-2-methylthiopurine (**IIb**) obtained after cooling, filtration, and drying was heated at reflux in *t*-butanol with γ,γ-dimethylallylamine hydrochloride and triethylamine. The product **III** was isolated by removal of the solvent in a vacuum and was purified by chromatography of the residue over silica gel and recrystallization from ethanol. The benzoylated riboside of **III** was prepared by formation of the chloromercuri derivative and treatment of this with 1-bromo-2,3,5-tribenzoylribofuranose ac-



cording to the general method of Davoll and Lowy (*20*). The riboside 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**I**) was isolated after treatment of the tribenzoyl derivative with methanolic ammonia in a sealed tube at 120°C for 9 hours and recrystallized from aqueous ethanol, m.p. 194° to 195°C. The ultraviolet spectrum of synthetic **I** was qualitatively the same as that of the naturally occurring material and showed the following quantitative absorption maxima (mμ): in absolute ethanol 283 (ε 18,000), 244 (ε 25,300); (H^+) 286 (ε 16,100), 246 (ε 18,600); (OH^-) 283 (ε 18,000), 243 (ε 24,900). The low-resolution mass spectra of natural and synthetic compounds were identical, and thin-layer chromatography over cellulose in three systems showed identical R_F values. The molecular formula for the synthetic 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**I**) was confirmed as $C_{16}H_{23}N_5O_4S$ by high-resolution mass spectrometry (*21*), and the structure was further characterized by its NMR spectrum (*13*).

The structure of an active cytokinin in *E. coli* sRNA has thus been fully identified as **I** (possible abbreviation: ms2iPA). Its presence and exact location as a minor base in a specific tRNA of *E. coli* is of special interest with respect to the work of Harada *et al.* (*22*) on sequencing of $tRNA^{tyr}$. Moreover, the A* in the position adjacent to the anticodon in the oligonucleotide sequence ACU*CUAA*A*ψCUG* of $tRNA^{tyr}$ from su^+_{III} has been considered by Goodman *et al.* (*23*) to be derived from adenylic acid and, because it can be labeled with sulfate-^{35}S and methionine-^{14}C-methyl, is presumed to contain both sulfur and a methyl group. This A*, which is also present in the su^-_{III} and species **I** and **II** (*24, 25*) of *E. coli* $tRNA^{tyr}$, is probably 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine. The biosynthetic precursor of the hydrocarbon side chain is presumably mevalonic acid, as in the case of its incorporation into the same side chain of 2iPA in *Lactobacillus acidophilus* (*4, 26*).

The finding of another N^6-Δ^2-isopentenyl-substituted adenosine next to the anticodon of a tRNA (*27*) provides further ground for speculation as to the role of the "anticodon-adjacent base" in general. Chemical and biological changes involving the side chain of compounds **III** and **I** have not yet been quantitatively compared with those of 2iP and 2iPA (*4, 6, 13, 28*).

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References and Notes

1. Abbreviations used in this paper include: 2iPA, 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine, or 6-(γ,γ-dimethylallylamino)-9-β-D-ribofuranosylpurine, or 6-(Δ^2-isopentenylamino)-9-β-D-ribofuranosylpurine; 2iP, 6-(3-methyl-2-butenylamino)purine or 6-(γ,γ-dimethylallylamino)purine; tRNA, transfer ribonucleic acid; sRNA, soluble ribonucleic acid; *m/e*, mass-to-charge ratio; A, adenine; C, cytosine; U, uracil; G, guanine; ε, molar extinction; NMR, nuclear magnetic resonance; O.D.$_{260}$, optical density at 260 mμ; su^+_{III}, mutant strain of *E. coli*.
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