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Purine Ring Rearrangements Leading to the Development of Cytokinin Activity. Mechanism of the Rearrangement of 3-Benzyladenine to N^6-Benzyladenine[1]

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Abstract: Under autoclaving conditions, certain 3-substituted adenines undergo rearrangement, in low conversion, to the corresponding N^6-substituted adenines which are cytokinins or plant growth substances. Using the 3-benzyladenine to N^6-benzyladenine conversion as illustration, we determined that the rearrangement was not due to a trivial sequence of N-3-α-C solvolysis followed by N^6-α-C alkylation under 2 atm of steam at 120° at pH 5.8. Rather, the rearrangement follows a contortional route involving ring opening and ring closing, during which the side chain does not leave its original nitrogen. In the process both rings open, the pyrimidine ring more readily than the imidazole ring. The solution to the problem of the route of rearrangement was based on the synthesis of specifically ^{15}N-labeled 3-benzyladenines and comparison of the products of their autoclaving, by mass spectrometry and NMR ^{15}N-^{1}H coupling, with N^6-benzyladenines specifically labeled with ^{15}N. Thereby, we determined that the products of autoclaving 3-benzyladenine-$^{15}N^6$ are N^6-benzyladenine, with label distributed about equally between N-3 and N-9, and 9-benzyladenine, with label distributed about equally between N-1 and N^6. Additional facts obtained in the course of this investigation establish the fragmentation pathways of substituted adenines in the mass spectrometer, provide diagnostic ^{15}N-^{1}H nuclear magnetic spin couplings for atom location, relate to the mutability of the adenine nucleus in a steam atmosphere, and suggest a chemical route for natural conversion to cytokinin-active substances.

Cytokinin is the generic name used to designate plant growth substances that play a major role in cell division and cell differentiation.[2-11] The measurement of cytokinin activity may be based on the growth of tobacco callus tissue, soybean callus tissue, or carrot tissue, the germination of seeds, or the retardation of leaf senescence. Cytokinin activity referred to in this paper will have been determined by the tobacco bioassay.[12,13] Whereas the existence of a specific cell division factor was postulated as early as 1892,[14] the modern era of research on the cytokinins began in 1955 with the isolation of kinetin, 6-furfurylaminopurine [N^6-(2-furfuryl)adenine], from old preparations of yeast DNA or from autoclaved, freshly prepared DNA.[15] The structure of the artefact was confirmed by synthesis, and this was quickly followed by the synthesis of other active analogs, especially N^6-substituted adenine derivatives and including 6-benzylaminopurine [N^6-benzyladenine or bzl^{6}Ade (**2**)].[16,17]

It has been established[18] that the cytokinin activity which develops when pure 1-benzyladenine (**1**) is autoclaved (pH 5.8, 120°, 2 atm of steam) either alone or in the plant growth medium is due to its conversion to 6-benzylaminopurine (**2**). The conversion pathway involves a 1–2 ring opening and 2-N^6 reclosure, as shown schematically by formulas **1a** → **2a** which identify the nitrogens, and it is representative of the general class of Dimroth rearrangements.[19-24] The autoclaving of 1-(Δ^2-isopentenyl)adenine similarly leads to the development of cytokinin activity due to its rearrangement to N^6-(Δ^2-isopentenyl)adenine (i^{6}Ade),[18] which may also be accomplished simply by refluxing in aqueous solution.

The task of establishing the reaction path or paths for the

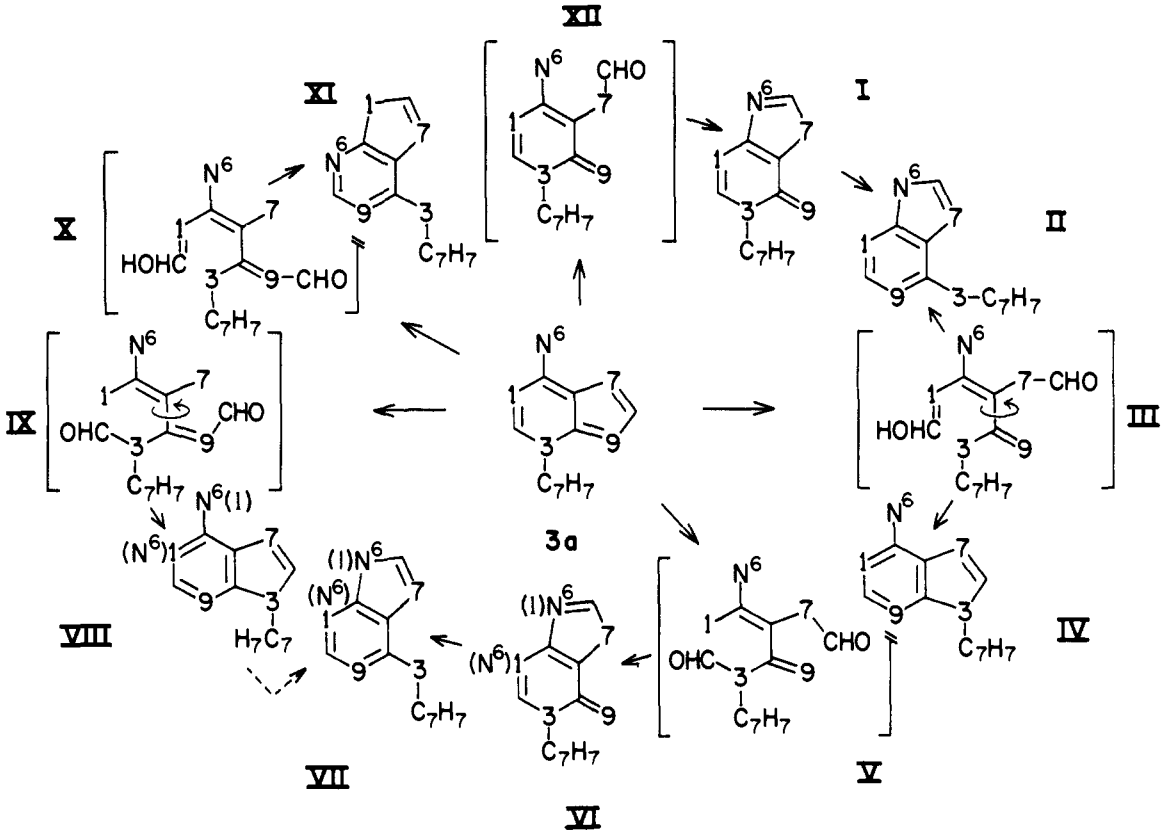
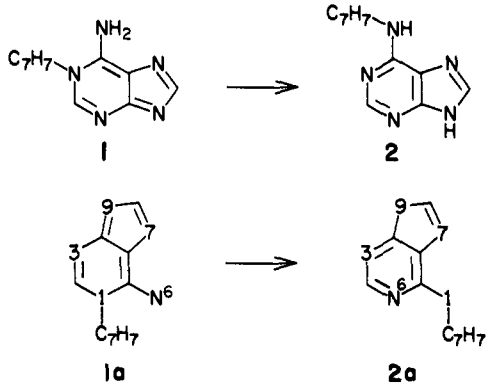
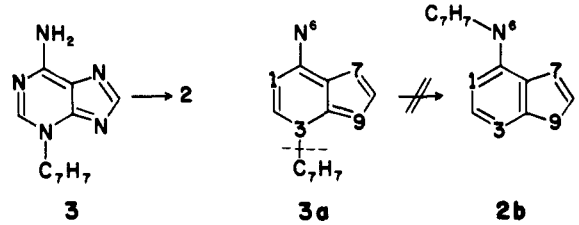


Figure 1. "The clock". Possible routes of the 3-benzyladenine to N^6-benzyladenine autoclaving rearrangement involving ring opening–reclosure sequences.



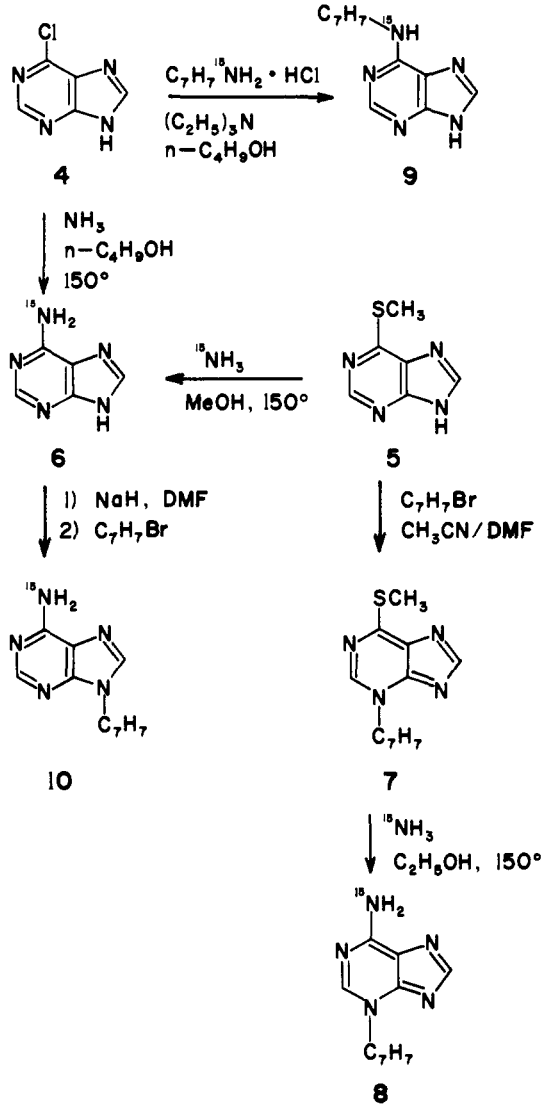
conversion of 3-substituted adenines [e.g., 3-benzyl (**3**), 3-(Δ^2-isopentenyl), 3-furfuryl] to the corresponding N^6-substituted adenines[25-27] has proved to be more arduous after we learned that the rearrangement was apparently not due to a trivial sequence of N-3–α-C solvolysis followed by N^6–α-C alkylation (**3a** ⇌ **2b**). Although we addressed our-



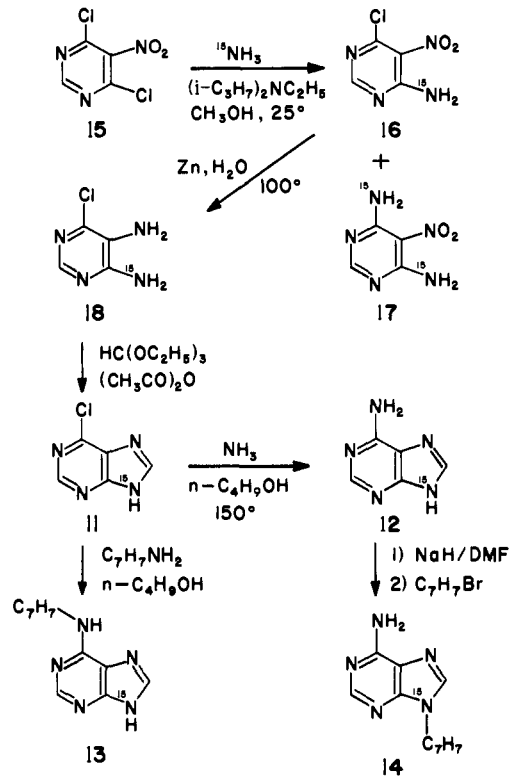
selves to the problem some years ago in this Laboratory, it was not until improvements and refinements in chromatographic separation, high- and low-resolution mass spectrometry with data processing equipment, and nuclear magnetic resonance, combined with lower pricing of ammonia-^{15}N, that we were able to solve the problem to our satisfaction. The method of solution involved synthesis of specifically ^{15}N-labeled 3-benzyladenines and comparison of the products of their autoclaving with N^6-benzyladenines also specifically labeled with ^{15}N. By this means it was possible to trace the movement of a strategically placed heavy nitrogen atom from starting material to product. Additional facts obtained in the course of this investigation establish the fragmentation pathways of substituted adenines in the mass spectrometer, provide diagnostic ^{15}N-^{1}H nuclear magnetic spin couplings for atom location, and relate to the mutability of the adenine nucleus in a steam atmosphere (a stage in prebiotic conditions).

Possible Mechanisms of 3-Benzyladenine to N^6-Benzyladenine Conversion. In addition to a solvolysis–realkylation route and an intramolecular [1,5] sigmatropic rearrangement, both of which would leave the nitrogens of the N^6 product in the same relation (**2b**) as the nitrogens of the starting N-3 compound (**3a**), we considered conversion mechanisms that involved more profound structural reorganization such as a series of ring openings and reclosures analogous to the Dimroth rearrangement. A number of ring opening–reclosure sequences may lead from a 3-substituted adenine to an N^6-substituted product as shown in Figure 1 ("the clock"). The sequences illustrated involve intermediates, but not necessarily all intermediates, in which the imidazole ring is opened (Figure 1, XII) or both rings are opened according to the four possible permutations (Figure 1, III, V, IX, and X). Ring opening of the pyrimidine ring alone would lead only to scrambling of N-1 and N^6 in the 3-benzyladenine (see later) and would not result in conversion to N^6-benzyladenine. Any sequence involving the intermediacy of a 1-benzyladenine may be considered (Figure

Scheme I



Scheme II



1, I and VI) since, as stated earlier, 1-benzyladenine (**1**) is converted to N^6-benzyladenine (**2**) under the autoclaving conditions. All of the pathways illustrated for conversion of 3-benzyladenine (**3a**) to N^6-benzyladenine (Figure 1, II, VII, and XI) have in common the following features: (1) a nitrogen atom at the 3 position in the starting material becomes N^6 in the product; (2) N^6 in the starting material becomes N-3 or N-9 in the product; (3) N-7 does not change position. Since the greatest information was to be obtained by following the migration particularly of N^6, 3-benzyladenine-$^{15}N^6$ was autoclaved and key rearrangement products were isolated. The location of the ^{15}N label in these products was determined by comparison of their NMR and mass spectra with those of specifically labeled model compounds.

Results and Discussion

Preparation of Labeled Compounds. The syntheses of starting material and model compounds labeled at N^6 are shown in Scheme I. ^{15}N-Amination of 3-benzyl-6-methylthiopurine (**7**), prepared according to the method of Neiman and Bergmann,[28] gave 3-benzyladenine-$^{15}N^6$ (**8**). Treatment of 6-chloropurine with benzylamine-^{15}N hydrochloride in 1-butanol in the presence of triethylamine yielded N^6-benzyladenine-$^{15}N^6$ (**9**), and treatment of the sodium salt of adenine-$^{15}N^6$ (**6**) with benzyl bromide in dimethylformamide[29] gave 9-benzyladenine-$^{15}N^6$ (**10**). The NMR spectra of these compounds in dimethyl sulfoxide corresponded to the spectra of the unlabeled standards.[30]

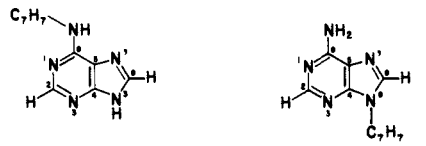
Model compounds bearing a ^{15}N label at N-9 were prepared in essentially the same manner from 6-chloropurine-$^{15}N^9$ (**11**) as outlined in Scheme II, furnishing adenine-$^{15}N^9$ (**12**), N^6-benzyladenine-$^{15}N^9$ (**13**), and 9-benzyladenine-$^{15}N^9$ (**14**). These compounds had melting points which corresponded to those of their $^{15}N^6$-labeled isomers, and their NMR spectra were similar except for the presence of a doublet (J = 8–10 Hz) in each case for the proton at C-8 (in Chart I the recorded chemical shifts and coupling constants serve as a check on each synthesis). Treatment of 4,6-dichloro-5-nitropyrimidine (**15**)[31] with ^{15}N-enriched ammonia in methanol at 25° for 1 hr gave 4-amino-6-chloro-5-nitropyrimidine-$^{15}N^4$ (**16**) and 4,6-diamino-5-nitropyrimidine-$^{15}N^{4,6}$ (**17**). The former was converted to 6-chloro-4,5-diaminopyrimidine-$^{15}N^4$ (**18**), mp 247–248°, by reduction with zinc in boiling water[32] and thence to 6-chloropurine-$^{15}N^9$ (**11**).[33]

N^6-Benzyladenine-$^{15}N^1$ (**19**) was prepared as shown in Scheme III, applying the Dimroth rearrangement to our advantage. 9-Pivaloyloxymethyladenine-$^{15}N^6$ (**20**) was prepared from adenine-$^{15}N^6$ (**6**) by the method of Rasmussen and Leonard.[34] In accordance with the known site of alkylation of 9-substituted adenines,[35] treatment of **20** with benzyl bromide in acetonitrile gave 1-benzyl-9-pivaloyloxymethyladenine-$^{15}N^6$ hydrobromide (**21**). Treatment with 0.2 N sodium hydroxide at reflux for 1 hr effected the Dimroth rearrangement and removed the directing group. The NMR spectrum of compound **19** corresponded to that of its unlabeled counterpart except that a doublet (J = 16 Hz) was observed for the proton at C-2 (Chart I).

The mass spectra of the labeled adenines and benzyladenines differed from those of authentic samples of unlabeled compounds available in our Laboratory as standards or from published data only in ways directly related to the introduction of a heavy nitrogen atom and dependent upon its position (see below).

Chart I. $^{15}N{=}C{-}^{1}H$ Coupling Constants in Substituted Adenines

	2-H	8-H	2-H	8-H
Unlabeled	δ 8.15 (s)	δ 8.11 (s)	δ 8.53 (s)	δ 7.76 (s)
$^{15}N^{6}$	8.15 (s)	8.11 (s)	8.54 (s)	7.77 (s)
$^{15}N^{9}$	8.15 (s)	8.13 (d, 10Hz)		

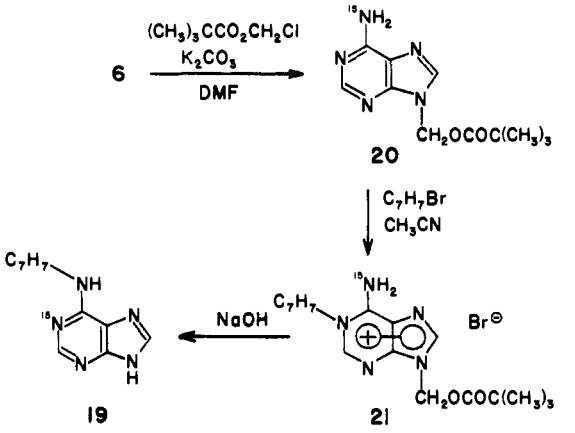


	2-H	8-H	2-H	8-H
Unlabeled	δ 8.18 (s)	δ 8.10 (s)	δ 8.17 (s)	δ 8.25 (s)
$^{15}N^{6}$	8.18 (s)	8.11 (s)	8.18 (s)	8.25 (s)
$^{15}N^{9}$	8.18 (s)	8.11 (d, 9Hz)	8.17 (s)	8.24 (d, 8Hz)
$^{15}N^{1}$	8.16 (d, 16Hz)	8.09 (s)		

Autoclaving Experiments. The autoclaving of organic compounds, i.e., heating at 120° under 2 atm of steam at pH 5.8, is rather an unusual method of effecting chemical conversion. Indeed, we would not have been led to investigate these conditions if it had not been for the cytokinin activity that developed as a result of the autoclaving of certain 3-substituted adenines, either alone or in the plant growth medium.[25-27,36] Among the 3-substituted adenines studied, 3-(Δ^2-isopentenyl)adenine or triacanthine,[37-42] from the new leaves of the honey locust, *Gleditsia triacanthos* L., was inactive in the sensitive tobacco bioassay but developed cytokinin activity upon autoclaving. The compound present in the autoclaved mixture in small amount and responsible for this activity was shown to be N^6-(Δ^2-isopentenyl)adenine, first by chromatography and then by uv, NMR, and mass spectra and melting point and mixture melting point.[26,36,43] 3-Furfuryladenine, inactive in the tobacco bioassay, developed cytokinin activity on autoclaving, and the product responsible was identified as N^6-furfuryladenine (6-furfurylaminopurine or kinetin).[26,27,36,43] We concentrated our efforts on 3-benzyladenine (**3**, inactive) which, when autoclaved, developed cytokinin activity[36] for which the N^6-benzyladenine (**2**) produced was responsible.

3-Benzyladenine-$^{15}N^6$ (**8**), adenine-$^{15}N^6$ (**6**), adenine-$^{15}N^9$ (**12**), and unlabeled 9-benzyladenine were autoclaved separately by heating solutions of each, ca. 0.01 *M*, in distilled water or, in the case of adenine-$^{15}N^9$, in phosphate buffer in a pressure cooker at 120°. The heating time was extended to 48 hr in order to ensure the isolation of sufficient product in the case of the substituted adenines and to provide controls in the case of the adenines. After the autoclaving of 3-benzyladenine, starting material was first separated from other benzyladenines by application to a short Aminex A.5 column (4.5 × 0.25 in.), elution with 25% aqueous DMF, 0.3 *M* in formate, pH 4. The N^6-benzyladenine and 9-benzyladenine were then separated from each other and from the other products of autoclaving on a longer Aminex column (15 × 0.5 in.), elution with the same buffer system. Desalting was effected on a P.2 gel column. The yields of N^6-benzyladenine and 9-benzyladenine, calculated as percentage of theoretical for complete conversion of **3** to one isomer, were 0.1 and 1%, respectively. The conversion of **3** to **2** would neither have been observed nor studied if the product had not had biological activity detectable at concentrations as low as 1×10^{-9} *M*![25] Test mixtures containing all of the *N*-benzyladenines and adenine itself were resolvable on the longer Aminex column. In spite of this, neither adenine itself nor other *N*-benzyladenines were observed as products from the autoclaving. Numerous other products were observed in very low conversion in the cytokinin-inactive fractions but were not isolated.

Scheme III



Adenine-$^{15}N^6$ (**6**) was homogeneous on the short Aminex column after autoclaving in distilled water and the solvent was simply removed by evaporation. Adenine-$^{15}N^9$ (**12**) was separated from the phosphate buffer by chromatography on silica gel, elution with DMF, and was further purified by sublimation. The results of the mass spectrometric and NMR studies on all the compounds resulting from the autoclaving experiments are reported below.

The autoclaving of unlabeled 9-benzyladenine for 48 hr produced no uv-detectable amount of material at the elution volume characteristic of N^6-benzyladenine on the longer Aminex A.5 column. For the concentration used, the limit of detection was 0.004% conversion of 9-benzyladenine to N^6-benzyladenine. Slight biological activity did develop on autoclaving 9-benzyladenine for 120 hr, but the level of conversion was so low as to indicate that the major route from **3** to **2** did not pass through 9-benzyladenine (i.e., in Figure 1, VIII → VII would be negligible).[44] It is further significant that 7-benzyladenine did not develop any cytokinin activity on autoclaving, completing the series 1 > 3 > 9 > 7 ≈ 0 in terms of ease of rearrangement to the N^6 isomer.

Mass Spectrometry Results. The results of high-resolution isotope ratio studies of N^6-benzyladenine isolated from the autoclaving rearrangement of 3-benzyladenine-$^{15}N^6$ and of model compounds with specific ^{15}N labels are summarized in Table I. The data, obtained by comparison of peak heights of unlabeled ions with those of ions bearing a ^{15}N atom in place of a ^{14}N, represent relative amounts of incorporation of one ^{15}N atom for key fragmentation products. The ratios were determined at resolutions up to 70,000. Insofar as it was possible to determine, all interfering elemental compositions, especially those due to ^{13}C or protonation, were resolved. Of particular interest were the molecular ion, *m/e* 225 (226), the $C_7H_8N^+$ ion, *m/e* 106 (107), and the two ions resulting from loss of the side chain as C_7H_7N plus one or two molecules of HCN: *m/e* 93 (94), 66 (67).

The first row of numbers, in parentheses, in Table I shows the actual levels of incorporation of one ^{15}N for the molecular ions of three different ^{15}N-labeled N^6-benzyladenines and for two separate autoclaving runs starting with 3-benzyladenine-$^{15}N^6$ (**8**). The levels differ slightly because of the varying isotopic purity of $^{15}NH_3$ used in the preparation of models and of starting material for the rearrangement. In order to provide a more meaningful compari-

Table I. Isotope Ratio Results with Labeled N^6-Benzyladenines

Fragment ion	Percent of ions bearing ^{15}N label[a]				
				bzl^{6}Ade-$^{15}N^?$[b]	
	bzl^{6}Ade-$^{15}N^9$	bzl^{6}Ade-$^{15}N^6$	bzl^{6}Ade-$^{15}N^1$	(1)	(2)
$M^{\cdot+}$	(95.5)	(95.5)	(97.6)	(93.1)	(93.7)
226/(225 + 226)	100	100	100	100	100
$C_7H_8N^+$ 107/(106 + 107)	0[c]	89	14	0[c]	0[c]
$[M - C_7H_7N - HCN]^{\cdot+}$ 94/(93 + 94)	99	3	94	53	54
$[M - C_7H_7N - 2HCN]^{\cdot+}$ 67/(66 + 67)	85	7	79	49	49

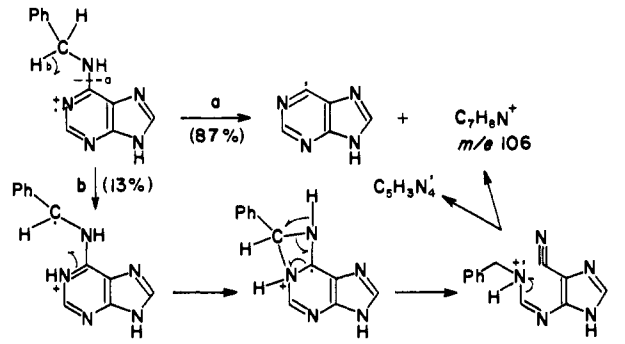
[a] Numbers are normalized to 1.00 ^{15}N atom per molecule. Numbers in parentheses indicate the exact level of ^{15}N enrichment. [b] Isolated from the autoclaving rearrangement of 3-benzyladenine-$^{15}N^6$ (**8**). [c] Within detectable limits, i.e., <0.5%.

son, all the other numbers in Table I have been normalized, i.e., 100% = 1.00 ^{15}N atom per ion.

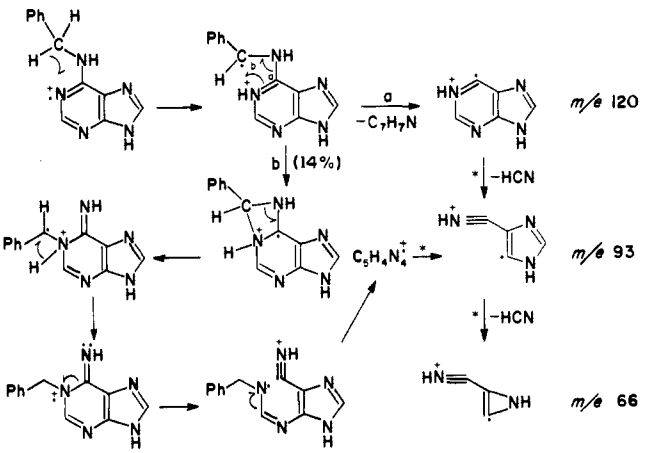
It can be seen in Table I that the $C_7H_8N^+$ ion fragment resulting from the electron bombardment of bzl^{6}Ade-$^{15}N^9$ (**13**) contains no ^{15}N while that from bzl^{6}Ade-$^{15}N^6$ (**9**) contains 89% ^{15}N on a relative basis. Accordingly, the most important result lies in the finding that the $C_7H_8N^+$ fragment from the autoclaved product of 3-benzyladenine-$^{15}N^6$ (**8**) contains no ^{15}N. *In the rearrangement product the label that was originally at N^6 in the starting material must be buried within the purine nucleus.* This fact eliminates any rearrangement mechanism, for example, one terminating with N^6-benzylation (or N-1 benzylation followed by a Dimroth rearrangement) from hydrolysis components adenine and benzyl alcohol,[45] that does not involve disruption of the adenine ring. That the label originally at N^6 in **8** has not migrated exclusively to either N-1 or N-9 may be seen by comparison of the isotopic ratios for the fragments corresponding to the loss of C_7H_7N plus one or two molecules of HCN from the autoclaved product of **8**, i.e., 53% for *m/e* 94 and 49% for *m/e* 67. The mass spectral data alone do not rule out location of the label exclusively at N-3 or N-7 or scrambling among N-3, N-7, and N-9. However, very little, if any, of the label in the rearrangement product is located at N-1 since in that case the $C_7H_8N^+$ ion would not be completely lacking in ^{15}N as observed (cf. bzl^{6}Ade-$^{15}N^6$, bzl^{6}Ade-$^{15}N^1$, and the two runs on the autoclaved product). The combination of the mass spectral results with those of the NMR spectra to be described below allowed definite location of the label.

In terms of the mass spectral fragmentation pattern of N^6-benzyladenine, the fact that the $C_7H_8N^+$ ion from bzl^{6}Ade-$^{15}N^6$ does not contain 100% of the ^{15}N label indicates a rearrangement in the mass spectrometer in which N^6 is formally interchanged with one of the other nitrogen atoms. The finding of a complementary amount of ^{15}N in the $C_7H_8N^+$ fragment produced from bzl^{6}Ade-$^{15}N^1$ (Table I) is confirmatory. This behavior can be rationalized (Scheme IV) by assuming partitioning of the molecular ion between two reaction paths: (a) the major route (the figure 87% is approximate) involving direct loss of a purine radical; (b) the minor route (ca. 13%) involving a hydrogen shift leading to a four-center intermediate, followed by ring opening and loss of $^{15}N^1$ in the $C_7H_8N^+$ fragment. A related four-center intermediate was proposed by von Minden et al.[46] to account for the loss of methylenimine from certain dimethylamino heteroaromatic compounds using deuterium labeling. However, unlike the process they described for derivatives of 6-dimethylaminopurine, in the case of 6-benzylaminopurine it is clearly N-1 that is involved and lost as well as N^6 (Table I, Scheme IV). Formation of the cation radical, $C_5H_4N_4^{\cdot+}$, *m/e* 120, from **2** is also accompanied by rearrangement since the loss of C_7H_7N from 6-benzylaminopurine-$^{15}N^6$ yields $C_5H_4N_4^{\cdot+}$ that contains 14% of the original label, while 6-benzylaminopurine-$^{15}N^1$ yields $C_5H_4N_4^{\cdot+}$ that contains 86% of the original label (not shown in Table I). Hydrogen transfer and similar partitioning mechanisms will account for this distribution of label, as shown in Scheme V.

Scheme IV

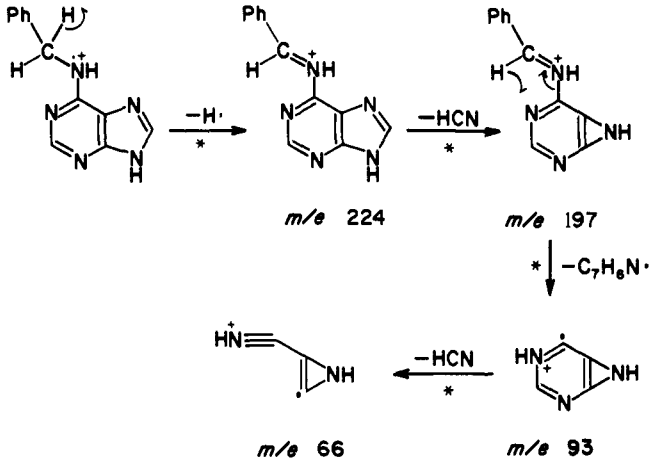


Scheme V



Metastable ion peaks were observed for the further fragmentations *m/e* 120(121) → 93(94) → 66(67). As can be seen from Table I, the successive losses of HCN do not involve N-9 or N-1 along the major fragmentation path. This feature is incorporated in the possible Scheme V. The fact that the fragment at *m/e* 93(94) in the spectrum of N^6-benzyladenine-$^{15}N^1$ bears a greater proportion of the original ^{15}N label (93%) than does the *m/e* 120(121) fragment (86%) is indicative of a second fragmentation path to *m/e* 93. A sequence involving loss of H, HCN, and C_7H_6N is one possibility (a-type route), as shown in Scheme VI. Metastable ion peaks were observed for the transitions *m/e* 197 → 93 → 66. The initial ion reactions *m/e* 225 → 224 → 197 had been postulated for 6-benzylaminopurine by Shan-

Scheme VI



non and Letham,[47] who observed metastable ion peaks for these transitions.

Nuclear Magnetic Resonance Results. All the mass spectral results taken together indicate that the ^{15}N label originally at N^6 in 3-benzyladenine-$^{15}N^6$ becomes a ring nitrogen in the N^6-benzyladenine formed by autoclaving rearrangement, and the results are compatible with location of the label at N-3 and N-9. We sought confirmation in an examination of the ^1H NMR spectra at 220 MHz assisted by the CAT, particularly with reference to ^{15}N-^1H spin-spin coupling. The chemical shift assignments for the aromatic protons were based on the earlier work of Fox in this Laboratory.[30]

For all the compounds which are labeled at N^6, both H-2 and H-8 are singlets (Chart I). For all those labeled at N-9, H-2 is a singlet and H-8 is a doublet (J = 8–10 Hz). For N^6-benzyladenine labeled at N-1, H-2 is a doublet (J = 16 Hz). The spin-spin coupling constant of a proton with its adjacent ^{15}N is larger for the pyrimidine proton than for the imidazole proton, a finding that is consistent with but not dependent upon the J = 12 Hz coupling previously observed for $^{15}N^3{=}C^4{-}H$ in 2,6-dichloropyrimidine doubly labeled with ^{15}N.[48] For N^6-benzyladenine isolated from the rearrangement of 3-benzyladenine-$^{15}N^6$ (**8**), both H-2 and H-8 appeared as pseudo-triplets with area ratios of approximately 1:2:1, consisting each of the doublet and a singlet. The coupling constants of the doublets were observed to be J = 16 Hz, therefore the H-2, and J = 10 Hz, therefore the 8-H. Accordingly, about half of the ^{15}N label is in the pyrimidine ring because it splits H-2, but we know it is not at N-1 because in that case the mass spectrum would have shown some label in the $C_7H_8N^+$ fragment.

The combination of mass spectral and ^1H NMR data therefore fixes the label at N-3 in about half the product molecules. The splitting of the proton signal for H-8 indicates that the label may also be at N-7 or N-9. The nitrogen at 7 is the only one that does not move in all of the rearrangement possibilities (Figure 1) and therefore would not be labeled in the N^6-benzyladenine product. Even aside from mechanistic possibilities, it is unlikely that the label would be distributed between N-3 and N-7 because in that event, since we know that N-3 and N-7 are readily lost in the mass spectrometer (Table I), the fragments due to loss of side chain and two molecules of HCN would necessarily contain less ^{15}N label than actually observed. Thus, the presence of label at N-9 is responsible for the 10-Hz coupling of the doublet portion of the H-8 signal.

Returning to "the clock" (Figure 1), we see that the autoclaving reaction path **3a** → V → VI → VII would account directly for distribution of the original N^6 label between the 3 and 9 positions in the product. Reaction path IX → VIII → VII has been ruled out earlier (see section on Autoclaving Experiments). The sequence **3a** → X → XI cannot operate alone since it would lodge the original N^6 label at the 3 position. Similarly, the sequences **3a** → III → II and **3a** → XII → I → II cannot operate alone since they would lodge the original N^6 label at the 9 position. In order for these paths leading separately to 3- and 9-labeled N^6-benzyladenine to account for the observed equal distribution of label between N-3 and N-9 in the product, they would have to occur with equal probability. Alternatively, if the pyrimidine ring undergoes opening and reclosure between N-1 and C-2 prior to rearrangement, scrambling of the label between N-1 and N^6 would occur.

We therefore checked this possibility and found that the pyrimidine ring does open faster than the imidazole ring under the autoclaving conditions. The ^1H NMR spectrum of the 3-benzyladenine recovered from the autoclaving of 3-benzyladenine-$^{15}N^6$ (**8**) showed a singlet for H-8 and a singlet and doublet (signal with areas 1:2:1) for H-2. It was also ascertained that the 9-benzyladenine product of this autoclaving similarly showed a singlet for H-8 and a singlet and doublet, roughly equal in total area, for H-2. Prior scrambling of label between N-1 and N^6 permits the retention of intermediate III in Figure 1 as a possibility for both N^6-benzyladenine and 9-benzyladenine with N-1 and N^6 scrambled (II, IV with N-1, N^6 scrambled). Prior scrambling in 3-benzyladenine also permits the retention of intermediates X and XII in Figure 1 as possibilities to account for the distribution of label in N^6-benzyladenine (XI, II with N-1, N^6 scrambled) but they fail to account for the 9-benzyladenine product. It is difficult to delineate between the admissable and requisite pathways shown in Figure 1, but we have provided evidence that both rings open and that the pyrimidine ring opens faster than the imidazole ring to bring about the intramolecular rearrangement.

Autoclaving of Adenine. Finally, we examined the mutability of the adenine nucleus itself under autoclaving conditions, using adenine-$^{15}N^9$ (**12**) and adenine-$^{15}N^6$ (**6**). If the imidazole ring of adenine were to open between the 8 and 9 positions under autoclaving conditions, the symmetrical intermediate, 4,6-diamino-5-formylaminopyrimidine, could reclose to the original N^6 or N-9 with equal probability. In this event, adenine-$^{15}N^9$ (**12**) would be partially converted to adenine-$^{15}N^6$ (**6**) on autoclaving. However, as we see in Table II, this does not take place. The autoclaving of adenine-$^{15}N^9$ for 48 hr produced no change in the mass spectrum. By contrast, similar autoclaving of adenine-$^{15}N^6$ (**6**) produced a change in the mass spectrum indicative of some pyrimidine ring opening between N-1 and C-2 and reclosure to either N-1 or N^6 of the original. After 48 hr, there was a maximum of 24% scrambling, that is, a maximum of 12% of ^{15}N label appearing at N-1 in the product, according to the mass spectral results (Table II). In the ^1H NMR spectrum a doublet began to appear corresponding to H-2 (J = 16 Hz) after this period of autoclaving. Thus, for unsubstituted adenine the pyrimidine ring opens faster than the imidazole ring with steam under pressure but not as readily as the pyrimidine ring in either 1- or 3-benzyladenine. It is interesting to speculate that the adenine formed prebiotically from anhydrous hydrogen cyanide and ammonia by electric discharge or heat[49-51] showed mutability within the pyrimidine ring, in preference to the imidazole ring, when exposed to steam or water, but was structurally unchanged.

The mass spectral data in Table II were obtained at a solid probe temperature of about 135°, but the ratios of the major fragment ions did not change appreciably with a vari-

Table II. Isotope Ratio Results with Labeled Adenines

Fragment ion	Percent of ions bearing ^{15}N label[a]			
	Adenine-$^{15}N^9$		Adenine-$^{15}N^6$	
	Before autoclaving	After autoclaving[b]	Before autoclaving	After autoclaving[b]
$M\cdot^+$	(95.5)	(95.7)	(98.0)	(97.9)
136/(135 + 136)	100	100	100	100
$[M - HCN]\cdot^+$ 109/(108 + 109)	99	99	97	85
$[M - 2HCN]\cdot^+$ 82/(81 + 82)	39	38	78	68
$[M - HCN - CH_2N_2]\cdot^+$ 67/(66 + 67)	35	35	53	46
$[M - 3HCN]\cdot^+$ 55/(54 + 55)	16	16	58	53

[a] Numbers are normalized to 1.00 ^{15}N atom per molecule. Numbers in parentheses indicate the exact level of ^{15}N enrichment. [b] 120°, 2 atm of steam, pH 5.8, 48 hr.

ation in the solid probe temperature from 110 to 180°. In terms of establishing the mechanisms of ionic reactions in the mass spectrometer, specific ^{15}N labeling of polynitrogen heterocycles offers obvious advantages over other types of labeling, as illustrated in Table II. We can agree with Occolowitz,[52] for example, that C-8 is completely retained in the $[M - HCN]\cdot^+$ fragment ion, since N-9 is completely retained, and that C-8 is about 35% retained in the $[M - 2HCN]\cdot^+$ ion, since N-9 is 38–39% retained. However, our data show that N^6 and therefore C-6 are almost fully retained in the first loss of HCN, not 45% retained,[52] and that some N-1 is lost in this process. Moreover, N^6 is retained to a greater extent than N-9 in the fragmentations leading to loss of 2HCN and 3HCN, although both nitrogens undergo loss in the radical cation chain, and in the fragments leading to loss of HCN + CH_2N_2 [N=CNH$_2$ (?)], although both undergo loss in this chain as well.

Conclusion

The control experiments on the autoclaving of adenine indicate that any N-1, N^6 scrambling could not alone have accounted for the mass spectral and ^1H NMR results obtained in the rearrangement of 3-benzyladenine to N^6-benzyladenine via a solvolysis–realkylation mechanism. The specific products of autoclaving 3-benzyladenine-$^{15}N^6$, namely N^6-benzyladenine, with label distributed about equally between N-3 and N-9, and 9-benzyladenine, with label distributed about equally between N-1 and N^6, require an intramolecular mechanism or mechanisms (Figure 1). We would like to stress that we have not run a pH profile on the reaction, which may take a different course under modified conditions, nor have we tried to optimize the conversion to rearranged products. We have simply adhered to the exact conditions which produced cytokinin activity from an inactive precursor so that we could explain this observation. In the rearrangement, both rings must open during the process, and the pyrimidine ring opens more readily than the imidazole ring. The establishment of this contortional route involving ring opening and closing, during which the side chain does not leave its original nitrogen, provides the detailed basis for the development of cytokinin activity in the autoclaving of 3-benzyladenine. In a natural setting, it suggests that the triacanthine or 3-(Δ^2-isopentenyl)adenine present in the new leaves of the honey locust, *Gleditsia triacanthos* L.,[37-42] and other species may serve as a storage source of cytokinin activity through (chemical) rearrangement to the N^6-substituted isomer. Further, it provides one possible intramolecular route from 3-furfuryladenine to kinetin, N^6-(2-furfuryl)adenine,[26,27] the former being potentially available from old yeast DNA or autoclaved fresh yeast DNA by a reasonable relay route through an anhydronucleoside followed by hydrolysis and dehydration.[26,53] It is intriguing to reflect that in the debris of dying plant cells there may be specific compounds which influence the growth and differentiation of new cells.

Experimental Section

Melting points were taken on a Büchi melting point apparatus and are uncorrected. Ultraviolet spectra were recorded on a Cary Model 15 spectrophotometer. NMR spectra in $(CD_3)_2SO$ were determined on Varian Associates Model A-60 and HR-220 spectrometers, the latter with the special attention of Mr. Michael L. Miller. Chemical shifts are given in parts per million relative to Me_4Si as an internal standard. Low-resolution mass spectra were obtained on a Varian-MAT CH-5 spectrometer by Mr. Joseph A. Wrona and high resolution on a Varian-MAT 731 spectrometer by Mr. J. Carter Cook and Mr. Paul Matejcek. Both instruments were coupled with a 620i computer and STATOS recorder. The ion abundance measurements reported represent the average of at least five consecutively recorded partial spectra. The isotope ratios were determined at a resolution of 70,000 for the molecular ion, and the compositions of the fragment ions were determined by peak matching at sufficiently high resolution to differentiate among the isotopic components at each *m/e* value.

The $^{15}NH_3$ was obtained from either Bio-Rad or Analytical Supplies Development Corp.

4-Amino-6-chloro-5-nitropyrimidine-$^{15}N^4$ (16) and 4,6-Diamino-5-nitropyrimidine-$^{15}N^{4,6}$ (17). These compounds were made by ammonia displacement of halogen by a modification of the method of Boon, Jones, and Ramage.[31] A flask containing 1.0 l. (44.7 mmol at STP) of N-15 enriched ammonia (96.5 atom % ^{15}N) was cooled to −78°, and a cold (−78°) solution of 6.35 g (49.2 mmol, 10% excess) of *N,N*-diisopropylethylamine in 60 ml of methanol was added. The resulting solution was added dropwise over 1 hr to a solution of 8.67 g (44.7 mmol) of 4,6-dichloro-5-nitropyrimidine (Aldrich 97%) in 80 ml of anhydrous ether in a round-bottomed flask cooled in ice water and fitted with a Dewar condenser (Dry Ice–acetone). The reaction mixture was allowed to warm to room temperature and was stirred for 1 hr. The resulting precipitate was filtered, washed with ether, and extracted with hot ethyl acetate (2 × 25 ml). The remaining solid was collected and dried in vacuo over P_2O_5 at 56° for 16 hr to give 0.74 g (21%) of **4,6-diamino-5-nitropyrimidine-$^{15}N^{4,6}$ (17)**, mp >300°. The combined filtrates were evaporated and the oily residue was extracted with light (bp 30–60°) petroleum ether (3 × 100 ml). The residue was taken up in benzene (800 ml), washed with saturated salt solution (6 × 50 ml) and then with water (3 × 50 ml), dried over Na_2SO_4, concentrated to 75 ml, and cooled, and the crystals were collected, yielding 3.90 g (50%) of **4-amino-6-chloro-5-nitropyrimidine-$^{15}N^4$ (16)**, mp 152–154° (lit.[31] 155–156°).

6-Chloro-4,5-diaminopyrimidine-$^{15}N^4$ (18). Previous directions for the synthesis of unlabeled compound[32] were applied to the ^{15}N compound. To a well-stirred mixture of 25 g of zinc dust and 120 ml of boiling water was added in portions, with care, 3.66 g (20.8 mmol) of 4-amino-6-chloro-5-nitropyrimidine-$^{15}N^4$ (**16**). The mixture was boiled for 10–15 min and then filtered immediately. The hot filtrate was brought to pH 10 with ammonium hydroxide, and the solution was cooled to room temperature and then in an ice bath for 1 hr. The orange crystals were collected, washed with cold water, and dried under vacuum over P_2O_5 for 16 hr at room temperature, yielding 1.14 g of 6-chloro-4,5-diaminopyrimidine-$^{15}N^4$ (**18**), mp 247–248° (lit.[32] 252° dec).

6-Chloropurine-$^{15}N^9$ (11). A suspension of 1.13 g (7.8 mmol) of 6-chloro-4,5-diaminopyrimidine-$^{15}N^4$ (**18**) in 10 ml of a 1:1 mixture of ethyl orthoformate and acetic anhydride[33] was heated slowly to reflux and was maintained at reflux for 1 hr after all the solid had disappeared. Solvent was removed in vacuo, and the residue was taken up in 10% sodium hydroxide solution and heated for 10 min at 40°. The solution was treated with Darco, warmed for another 1–2 min, filtered, acidified (pH 5) with concentrated HCl, and kept in the refrigerator overnight. The precipitated 6-chloropurine-$^{15}N^9$ was filtered, washed with cold water, and dried, yielding 0.85 g of orange powder. This material was chromatographed on 100 g of silica gel with 9:1 chloroform–ethanol, yielding 0.722 g

of chromatographically pure 6-chloropurine-$^{15}N^9$, characterized by its mass spectrum: *m/e* (rel intensity) 155 (100), 157 (31.21) (M^+), 120 (M − Cl), 93 [M − (Cl + HCN)], 66 [M − (Cl + 2HCN)].

Adenine-$^{15}N^9$ (12). A solution of 156 mg (1 mmol) of 6-chloropurine-$^{15}N^9$ (**11**) in 15 ml of 10% ammoniacal 1-butanol was heated in a sealed tube at 150° for 17 hr.[54] Solvent was removed in vacuo, and the residue was purified by chromatography on 100 g of silica gel (9:1 chloroform–ethanol) yielding 138 mg of adenine-$^{15}N^9$ (**12**). Part of this material was purified further by two successive sublimations (195°, <0.005 Torr) with an overall recovery of 57%, mp >300°. Identity and position of ^{15}N label were established by source, mass spectrum, and NMR (see Results and Discussion).

Adenine-$^{15}N^6$ (6). Method A. A flask containing 1.00 l. (44.7 mmol at STP) of N-15 enriched ammonia (99.2 atom % ^{15}N) was cooled to −78°, and 40 ml of cold 1-butanol was added. The resulting solution was heated with 2.03 g (13.15 mmol) of 6-chloropurine (**4**) in a sealed tube at 150° for 24 hr. Solvent was removed in vacuo, and the dark gray-green residue was taken up in 350 ml of methanol and boiled with Darco for 2 hr. Removal of solvent in vacuo after filtration gave 1.15 g of chromatographically pure adenine, mp >320°. Identity and position of label were established by source, mass spectrum, and NMR.

Method B. A mixture of 4.98 g (30 mmol) of 6-methylthiopurine (**5**) (Sigma) and 30 ml of methanolic N-15 ammonia (1.53 g, 90 mmol, 95 atom % ^{15}N) was heated in a sealed tube at 155° for 24 hr. After cooling, the solvent was removed in vacuo and the remaining solid was extracted with boiling ethanol (3 × 50 ml) to remove unreacted 6-methylthiopurine. The less soluble adenine-$^{15}N^6$ (**6**) was purified by recrystallization from water. The product had an identical mass spectrum with that prepared by method A (Table II).

Autoclaving of Adenine-$^{15}N^9$ (12). A solution of 14 mg (0.1 mmol) of adenine-$^{15}N^9$ in 10 ml of a 0.1 *M* phosphate buffer (pH 5.8) was heated in a pressure cooker for 48 hr at 120°. Solvent was removed in vacuo. The residue was extracted with 2 ml of dimethylformamide at 60° and chromatographed on 25 g of silica gel, elution with dimethylformamide. The adenine-containing fractions were combined. Solvent was removed in vacuo, and the residue was sublimed at 195° and <0.005 Torr, yielding 11 mg of adenine, mp >300°. The mass spectrum is mentioned in Results and Discussion.

Autoclaving of Adenine-$^{15}N^6$ (6). A solution of 136 mg (1 mmol) of adenine-$^{15}N^6$ in 100 ml of distilled water was heated in a pressure cooker at 120° for 48 hr. Solvent was removed in vacuo, yielding 136 mg of adenine, mp >300°.

Compounds Labeled at N^6. Benzylamine-^{15}N Hydrochloride. Phthalimide-^{15}N, prepared from phthalic acid,[55] was converted to benzylphthalimide-^{15}N[56] and thence to benzylamine-^{15}N hydrochloride.[57] A solution of 0.835 g (3.5 mmol) of *N*-benzylphthalimide-^{15}N in 0.170 g (3.62 mmol) of 85% aqueous hydrazine hydrate plus 25 ml of methanol was heated at reflux for 1.5 hr. Solvent was removed in vacuo and water was added. The mixture was cooled in an ice bath and the pH was adjusted to 1.0 with hydrochloric acid. The mixture was allowed to stand for several minutes, and the solid was filtered. The aqueous phase was evaporated in vacuo and recrystallized from ethanol–ether, yielding 0.136 g of colorless crystalline material, mp 258–260° (lit.[58] mp 256°).

6-Benzylaminopurine-$^{15}N^6$ (N^6-Benzyladenine-$^{15}N^6$, 9). A solution of 124 mg (0.8 mmol) of 6-chloropurine (**4**) and 116 mg (0.8 mmol) of benzylamine-^{15}N hydrochloride in 10 ml of a 20:1 mixture of 1-butanol and triethylamine was heated at reflux for 3 hr. Solvent was removed in vacuo. The residue was shaken with 10 ml of water, filtered, and washed with water and cold ether, yielding 149 mg of white powder, mp 228.5–230°. Several recrystallizations from ethyl acetate brought the melting point to 232–233°. Identity and position of label were established by source, melting point, NMR, and mass spectrum.

9-Benzyladenine-$^{15}N^6$. A suspension of 250 mg (1.9 mmol) of adenine-$^{15}N^6$ (**6**) and 88 mg (2.1 mmol) of a 57% mineral oil suspension of sodium hydride in 3 ml of dimethylformamide[29] was stirred for 15 min under dry nitrogen, and then 0.25 ml (0.36 g, 2.1 mmol) of benzyl bromide was added. Stirring at room temperature was continued for 16 hr. After cooling the reaction mixture in an ice bath, the precipitate was collected and washed with cold ethanol, yielding 198 mg of white powder. This was further purified by chromatography on 80 g of silica gel, elution with ethanol, to give 171 mg, mp 230–232°. Recrystallization from ethanol gave 124 mg, mp 231–232°. Identity and position of label were established by source, melting point, NMR, and mass spectrum.

3-Benzyladenine-$^{15}N^6$ (8). A flask containing 1.00 l. (44.7 mmol at STP) of N-15 enriched ammonia (96.5 atom % ^{15}N) was cooled to −78°, and 15 ml of cold absolute ethanol was added. The resulting solution was heated with 3.84 g (15 mmol) of 3-benzyl-6-methylthiopurine (**7**)[28] in a sealed tube at 152° for 24 hr. Solvent was removed in vacuo, yielding 3.39 g of pale yellow crystals, homogeneous on TLC (silica gel, 9:1 chloroform–ethanol). Two recrystallizations from ethanol gave 2.50 g of colorless plates, mp 280–281°, homogeneous on the Aminex columns used for isolation of 3-benzyladenine rearrangement products (see below). Identity, purity, and position of label were established by source, mass spectrum, NMR, and analysis.

Anal. Calcd for $C_{12}H_{11}N_4{}^{15}N$ (96.5 atom % ^{15}N): C, 63.78; H, 4.91; N, 31.31. Found: C, 63.87; H, 4.84; N, 31.45.

A similar sequence had been tested and used in this Laboratory earlier to synthesize 3-(3-methyl-2-butenyl)-6-methylthiopurine from 6-methylthiopurine, followed by conversion to 3-(3-methyl-2-butenyl)adenine (triacanthine).

Compounds Labeled at N-9. 6-Benzylaminopurine-$^{15}N^9$ (N^6-Benzyladenine-$^{15}N^9$, 13). To a solution of 202 mg (1.3 mmol) of 6-chloropurine-$^{15}N^9$ (**11**) in 10 ml of 1-butanol was added 278 mg (2.6 mmol) of benzylamine, and the solution was heated at reflux for 3.25 hr.[59] After cooling to room temperature, 25 ml of water was added to dissolve precipitated benzylamine hydrochloride, and the mixture was extracted with ethyl acetate (500 ml). The organic phase was washed with water (2 × 50 ml), dried over $MgSO_4$, and evaporated in vacuo. Recrystallization from ethyl acetate gave 187 mg of colorless crystalline material, mp 230.5–232.5°. Two additional crystallizations from ethyl acetate brought the melting point to 232–233°.

9-Benzyladenine-$^{15}N^9$ (14). A suspension of 310 mg (2.3 mmol) of adenine-$^{15}N^9$ (**12**) and 105 mg (2.5 mmol) of a 57% mineral oil suspension of sodium hydride in 5 ml of dimethylformamide was stirred for 15 min under dry nitrogen, and then 0.31 ml (0.45 g, 2.6 mmol) of benzyl bromide was added.[29] Stirring at room temperature was continued for 16 hr. After cooling the reaction mixture in an ice bath, the precipitate was collected and washed with cold ethanol, yielding 212 mg of white powder. This was further purified by chromatography on 85 g of silica gel, elution with ethanol, yielding 190 mg. Two recrystallizations from ethanol gave 118 mg of ultrapure compound, mp 230.5–231.5°.

Compound Labeled at N-1. 6-Benzylaminopurine-$^{15}N^1$ (N^6-Benzyladenine-$^{15}N^1$, 19). 9-Pivaloyloxymethyladenine-$^{15}N^6$ (20) was prepared according to the directions of Rasmussen and Leonard[34] from adenine-$^{15}N^6$ (6) and chloromethyl pivalate in the presence of potassium carbonate. Chromatography of the crude product on silica gel with 9:1 chloroform–ethanol yielded (50%) compound of low melting point, 179–184° (lit.[34] 197–198°), homogeneous on TLC and having the same R_f as authentic material. Recrystallized material was then subjected to identical conditions with those given below for unlabeled material, with identification at the intermediate, **1-benzyl-9-pivaloyloxymethyladenine-$^{15}N^6$** hydrobromide (**21**), and final stage, **6-benzylaminopurine-$^{15}N^1$ (19)**, by direct comparison. The overall yield was 22% for labeled and 49% for unlabeled material.

Pilot Preparation of 1-Benzyl-9-pivaloyloxymethyladenine Hydrobromide. The directions of Rasmussen and Leonard[34] for benzyloxymethylation were applied to benzylation. To a suspension of 498 mg (2 mmol) of 9-pivaloyloxymethyladenine in 30 ml of acetonitrile was added 684 mg (4 mmol) of benzyl bromide (distilled from Na_2CO_3) and the mixture was heated at reflux (the starting material dissolved in a few minutes) for 17 hr under nitrogen. Crystals formed on cooling to room temperature. The mixture was allowed to stand 48 hr and was then filtered. The crystals were washed with acetonitrile and ether, yielding 527 mg (63%) of 1-benzyl-9-pivaloyloxymethyladenine hydrobromide, mp 208–211°. An analytical sample recrystallized from ethanol had mp 212–214°: NMR [$(CD_3)_2SO$] δ 9.00 (s, 1, H-2), 8.65 (s, 1, H-8), 7.35 (s, 5, C_6H_5), 6.22 (s, 2, NCH_2O), 5.70 (s, 2, $PhCH_2$), and 1.13 ppm [s, 9, $C(CH_3)_3$]; mass spectrum *m/e* (rel intensity) 340 (17.1, M − Br), 339 (78.6), 338 (60.9), 225 (45.9, M − HBr −

$C_4H_9CO_2CH_2$), 224 (100.0), 106 (14.0), 91 (81.7).

Anal. Calcd for $C_{18}H_{22}BrN_5O_2$: C, 51.44; H, 5.28; N, 16.66; Br, 19.01. Found: C, 51.15; H, 5.33; N, 16.66; Br, 19.17.

Pilot Preparation of 6-Benzylaminopurine. A suspension of 105 mg (0.25 mmol) of 1-benzyl-9-pivaloyloxymethyladenine hydrobromide in 10 ml of 0.2 *N* sodium hydroxide was heated at reflux (starting material dissolved in a few minutes) for 1 hr.[18] After cooling to room temperature the pH was adjusted to 7 with 2 *N* hydrochloric acid. A precipitate formed immediately. The mixture was kept in the cold for 2 hr and was then filtered, yielding 43 mg (77%) of chromatographically pure **6-benzylaminopurine,** mp 229–231°.

Autoclaving of 3-Benzyladenine. The same autoclaving procedure was used for unlabeled 3-benzyladenine (**3**) and for all the N-15 labeled 3-benzyladenines. For example, a solution of 250 mg (1.1 mmol) of **3-benzyladenine-$^{15}N^6$** (**8**) in 100 ml of distilled water was heated in a pressure cooker for 48 hr at 120°. Solvent was removed in vacuo, and the residue was taken up in 10 ml of a buffer solution [25% in dimethylformamide and 0.3 *M* in formate (ammonium formate–formic acid), pH 4.0 calcd, 4.2 measured]. This solution was applied in 2-ml fractions to a 4.5 × 0.25 in. Aminex A.5 column and eluted with the same buffer solution at 60 ml/hr (column temperature 51°). Crude product was separated into two fractions having retention times less than and greater than 1.25 hr. The low-retention-time fractions were combined and carefully neutralized with concentrated ammonium hydroxide. Solvent was removed in vacuo, and the residue was taken up in 3 ml of 50% aqueous ethanol (total volume of solution, 12 ml). This solution was applied in 2-ml fractions to a 63 × 0.5 in. P.2 gel column and eluted with 50% aqueous ethanol at 40 ml/hr at room temperature. The fractions from zero time to 3.4 hr excluded ammonium formate and residual dimethylformamide. These fractions were combined and solvent was removed in vacuo. The residue was taken up in 4 ml of the buffer solution described above, applied to a 15 × 0.5 in. Aminex A.5 column, and eluted with the same buffer at 60 ml/hr and a column temperature of 51°. Fractions containing N^6-benzyladenine (5.8 hr) and 9-benzyladenine (4.45 hr) were pooled separately, and the two solutions were each desalted on the P.2 gel column described above and were identified by comparison of their uv and mass spectra with those of authentic samples. Yields, calculated as percentage of theoretical values for complete conversion to one isomer, were measured by comparison of peak areas on the 15 × 0.5 in. Aminex column (N^6-benzyladenine, 0.075%; 9-benzyladenine, 1.46%) and by quantitative uv of isolated material (N^6-benzyladenine, 0.11%; 9-benzyladenine, 0.76%). Starting material was recovered by combining the high-retention-time fractions (1.25–6.00 hr) from the short Aminex column, neutralizing with ammonium hydroxide, and desalting on the P.2 gel column. This proved more difficult than for N^6-benzyladenine or 9-benzyladenine because 3-benzyladenine is retained longer on P.2 gel and overlaps the salt peak. This situation is aggravated by overloading. Several successive passes on P.2 followed by recrystallization from ethanol led to 35% recovery of material with mp 278–279°, although close to 90% of the 3-isomer was calculated to be present in the crude mixture.

Autoclaving on the scale described above provided sufficient products for determination of the mass spectra. The procedure was repeated on a larger scale (1.50 g) in each case in order to obtain enough N-15 labeled N^6-benzyladenine for determination of the proton magnetic resonance. The ^1H–^{15}N couplings, or the absence of such, were indicative of the location of the labeled nitrogen.

The autoclaving of unlabeled 9-benzyladenine for 48 hr was followed by the usual work-up and chromatographic procedure. No N^6-benzyladenine was detectable on elution from the 15 × 0.5 in. Aminex A.5 column, setting a limit of <0.004% for the conversion of **9** to N^6. Another sample was autoclaved for 120 hr at 120°, after which the total product was collected and sent to Professor Skoog at the University of Wisconsin for tobacco bioassay. It showed only slight cytokinin activity (at 20 and 60 μM). As a control, the 9-benzyladenine sample prior to autoclaving showed no activity in the concentration range applied (up to 40 μM).

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Effect of Molecular Weight on Hydrogen–Deuterium Exchange in a Nonhelical Polyamide

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Abstract: Hydrogen-deuterium exchange kinetics have been measured for poly(isopropylacrylamides) over a molecular weight range from 100 to 200,000. A sharp drop in rate appears as soon as one goes from monomer to trimer. Thereafter a very slow progressive decrease is displayed. The local environment of the exchanging amide residue is largely (although not completely) established by the mutual interaction of as few as three residues in these polymers of random conformation.

The rate of exchange of the N-H hydrogen of an amide reflects its local environment, in the ground state and in the transition state. To understand exchange kinetics in proteins, one must have some knowledge of the factors governing these rates in small amides and in model polymers. Previous investigations[1-3] have demonstrated that the N-H in a nonhelical polyamide undergoes exchange at a much slower rate than in a corresponding monomeric residue. The rate constants drop by a factor of about 10^2 when the amide is transferred from a small molecule of molecular weight near 100 to a nonhelical polymer of molecular weight near 200,000. An intriguing question thus arises: at what stage in molecular weight does the transition in behavior appear? The present study had been designed to answer this question and to determine whether the change in behavior occurs gradually or appears abruptly at some critical range in macromolecular weight.

Experimental Section

Materials and Methods. Reagent grade benzoyl peroxide was obtained from commercial sources. *n*-Butyl mercaptan (1-butanethiol) was purchased from Aldrich Chemical Co. [n^{20}D 1.4437 (lit.[4] n^{25}D 1.4401)]. Deuterium chloride, 38% in D_2O (warranted to contain 99.5 mol % D), and deuterium oxide (warranted to contain 99.9 mol % D) were purchased from Bio Rad Laboratories. Raney nickel-aluminum catalyst, 42-58 pulverized ingot (Gilman Paint and Varnish Co.), was a gift from Dr. A. S. Hussey.

N-Isopropylacrylamide was obtained from American Cyanamid Co. or Eastman Chemical Products, Inc., and was recrystallized twice from a toluene–hexane mixed solvent (mp 66-67°). Dioxane was purified by the method of Fieser[5] and freshly distilled before use. Anhydrous sodium acetate (J. T. Baker Co.) was dried at 120° in vacuo for 4-12 hr before use. Benzene and hexane were purified by fractional distillation; only the fractions boiling at 80 and 69°, respectively, were used. Other solvents were purchased in reagent grade from commercial sources and used without further purification.

Bio Beads S-X2, X3, and X8, Bio-Gel A-0.5m, and AG11A8 Ion Retardation Resin were purchased from Bio-Rad Laboratories. Sephadex G-10 and G-25 were purchased from Pharmacia Fine Chemicals, Inc. Thin-layer plates (0.25 mm, Silica Gel H, Brinkmann Instruments, Inc.) were prepared according to Stahl.[6] High purity Q-Gel was obtained from Quantum Industries. Silica gel grade 950, 60-200 mesh, was purchased from Davison Chemical Division, W. R. Grace & Co., washed with boiling methanol, and activated at 120° in vacuo.

Ultrafiltration membranes were obtained from Amicon Corp. and used in their Model 401 filtration cell.

Infrared spectra in the fundamental region were scanned with a Beckman IR 10 spectrophotometer. Overtone infrared spectra were obtained with a Cary 14R spectrophotometer equipped with nitrogen-purged optical and cell compartments and a thermostated cell holder. All chromatographic columns were eluted at flow rates of 1-3 ml/min. Aqueous column eluents were monitored with a Waters Associates Model R4 differential refractometer. With organic column eluents, selected fractions were spotted on thin layers of analytical silica gel, developed in 12% (v/v) ethanol-hexane, and stained with iodine vapor. A Beckman Spinco Model E analytical ultracentrifuge was used for sedimentation equilibrium experiments. Elemental analyses were determined by Micro Tech Laboratories, Inc. Solution pH was measured with a Radiometer Model PHM4c meter in conjunction with a Corning semimicro combination electrode. The pH readings were changed to pD values with the relationship of Glasoe and Long:[7] pD = pH + 0.40.