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N^6-(Δ^2-Isopentenyl)adenosine. A Component of the Transfer Ribonucleic Acid of Yeast and of Mammalian Tissue, Methods of Isolation, and Characterization*

Morris J. Robins,† Ross H. Hall,‡ and Roosevelt Thedford

ABSTRACT: A nucleoside has been isolated from yeast transfer ribonucleic acid (tRNA) which has been identified as 6-*N*-(3-methylbut-2-enylamino)-9-β-D-ribofuranosylpurine (I). tRNA (60 g) was hydrolyzed enzymatically to its constituent nucleosides and the hydrolysate was subjected to partition chromatography on a column. The separated nucleoside was isolated and crystallized (15 mg). The nucleoside was assigned structure I on the basis of elemental analysis, mass spectroscopy, and nuclear magnetic resonance (nmr) spectroscopy. Compound I was synthesized by condensing γ,γ-dimethylallylamine with 6-chloro-9-β-D-ribofuranosylpurine. The natural compound was identical with the synthetic nucleoside. In mild acid solution I loses the ribose residue and undergoes hydration of the allylic double bond to form 6-*N*-(3-hydroxy-3-methylbutylamino)purine which on further acid treatment yields 3H-7,7-dimethyl-7,8,9-trihydropyrimido-[2,1-*i*]purine. Mild permanganate oxidation of I yields 6-*N*-(2,3-dihydroxy-3-methylbutylamino)-9-β-D-ribofuranosylpurine. In concentrated ammonium hydroxide I is converted to adenosine. Compound I exhibits potent cytokinin activity; that is, it stimulates cell division and cell differentiation in plant systems.

Transfer ribonucleic acid contains many components which are structural modifications of the four major nucleosides. Methylation represents the most common form of modification known and the occurrence of 19 different methylated nucleosides in tRNA has now been reported. The function of the methylated components of RNA remains unknown but from physicochemical considerations the methyl groups could be instrumental in maintaining the secondary and tertiary structure of RNA molecules. Other types of modified nucleosides exist in which a larger and chemically more active group than a methyl is attached to the parent nucleoside, as for example, 2′-(3′)-*O*-ribosyladenosine (Hall, 1965), N^6-(aminoacyl)adenosine (Hall and Chheda, 1965), and N^4-acetylcytidine (Zachau *et al.*, 1966a). These nucleosides represent nucleic acid components that are capable of undergoing a variety of unique biochemical reactions.

We recently isolated from yeast tRNA another nucleoside containing a functional group attached

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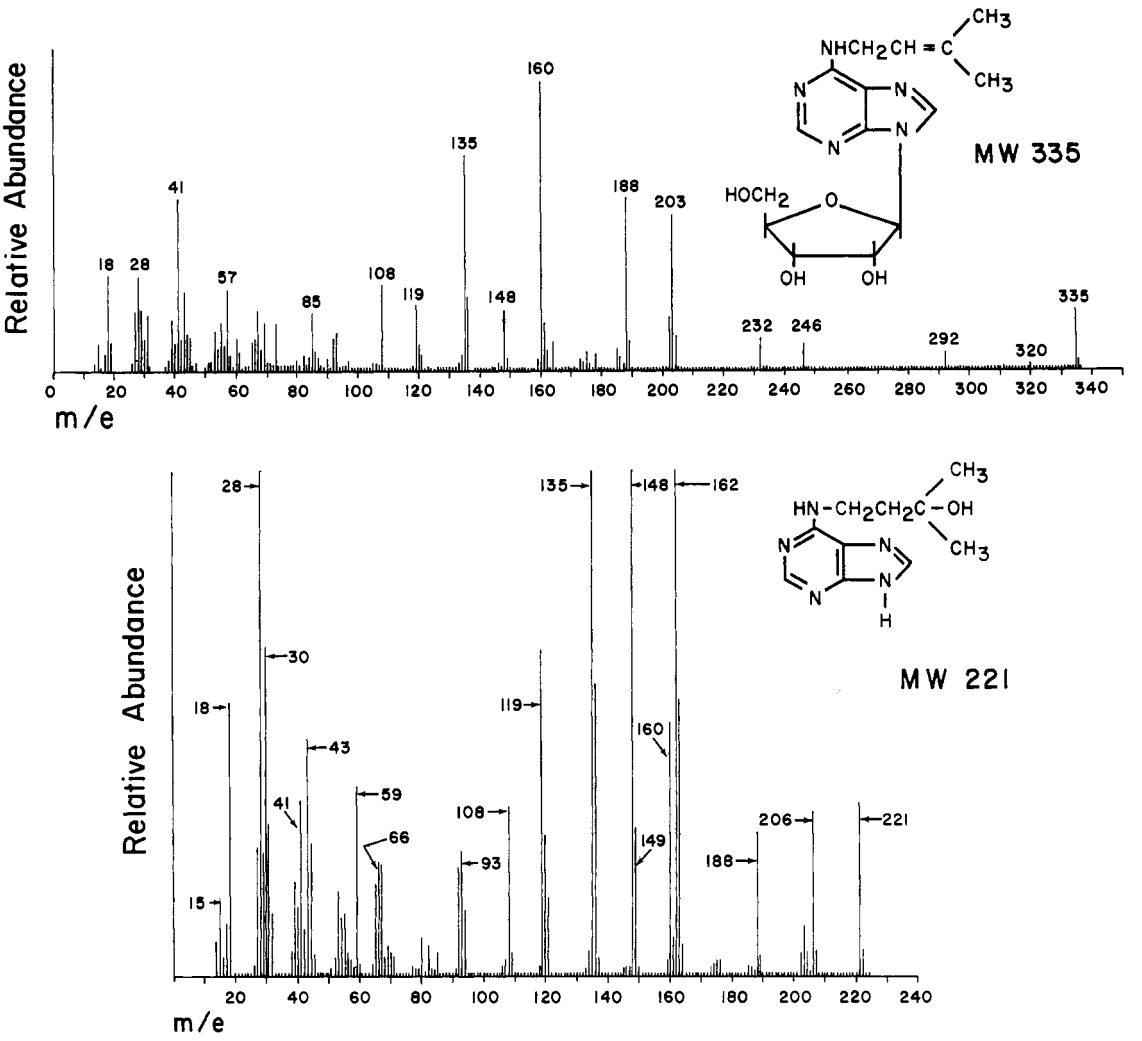


FIGURE 1: Mass spectra (a) (top) IPA, direct inlet, 170°, 70 ev. Spectrum obtained on isolated sample. Major peak assignments *m/e*: 320 (−15), loss of CH_3; 292 (−43), loss of $C(CH_3)_2$ and H; 246 (−89) and 232 (−103), loss of sugar fragments (see Biemann and McCloskey, 1962); 203, free base; 188, free base less CH_3; 160 (−175), loss of ribosyl and $C(CH_3)_2$; and 135, adenine. (b) (bottom) N^6-(3-hydroxy-3-methylbutyl)adenine, indirect inlet 250°, 70 ev. Major peak assignments (*m/e*): 206 (−15), loss of CH_3; 203 (−18), loss of H_2O; 188 (−33), loss of CH_3 and H_2O; 162 (−59), loss of COH $(CH_3)_2$; 148 (−73), loss of $CH_2COH(CH_3)_2$; and 135, adenine.

to a major nucleoside, namely, 6-*N*-(3-methylbut-2-enylamino)-9-β-D-ribofuranosylpurine (I)[1] (Hall *et al.*, 1966). Zachau and Biemann and their co-workers (Biemann *et al.*, 1966) independently identified this component in yeast tRNA and furthermore, Zachau *et al.* (1966a,b) and Feldman *et al.*, 1966) located IPA[2] in a specific sequence of yeast seryl-tRNA I and II. This paper reports methods for the isolation of IPA from the tRNA of yeast and mammalian tissue and gives details of its characterization, chemical synthesis, and properties.

Experimental Section

General. Celite 545 and Microcel-E are Johns-Manville Co. brands of diatomaceous earth. Snake venom (*Crotalus adamanteus*) was obtained from Ross Allen's Reptile Institute, Silver Springs, Fla. Bacterial alkaline phosphatase (BAP-C grade) was obtained from Worthington Biochemicals. We prepared yeast tRNA using the large-scale method of Holley (1963). Fertile hen eggs were obtained from a local hatchery. Partition chromatography on columns, which was used extensively in this work, was carried out according to the general method published previously (Hall, 1962). Melting points are corrected. The nuclear magnetic resonance (nmr) spectra of natural IPA were run on a Varian Model HA-100 spectrometer through the courtesy of Mr. Ross Pitcher of Varian Associates.

[1] This compound is referred to in the text as N^6-(Δ^2-isopentenyl)adenosine to denote its relationship to adenosine.

[2] Abbreviations used: IPA, N^6-(Δ^2-isopentenyl)adenosine; DMSO-d_6, deuterated dimethyl sulfoxide

The other spectra were run on the Varian Model A-60. Ultraviolet spectra were obtained on a Cary Model 14 spectrophotometer, infrared spectra on a Beckman Model 9 instrument, mass spectra on a Hitachi Perkin-Elmer RMU-6D mass spectrometer (Morgan and Schaffer Corp., Montreal), and the optical rotations on a Bendix-Ericson polarimeter. Microanalyses were performed by Galbraith Laboratories, Inc.

Paper Chromatography. Whatman No. 1- or 3MM filter paper was used and chromatography was carried out in the descending manner. The R_F values of the compounds associated with this work are listed in Table I.

TABLE I: Paper Chromatography and R_F Values.

Compound	Solvent System				
	A	B	C	D	E
IPA (natural)	0.80	0.87	0.80	0.87	0.80
IPA (synthetic)	0.80	0.87	0.80	0.87	0.80
N^6-(Δ^2-Isopentenyl)adenine	0.86	0.90	0.83	0.88	0.86
II	0.73	0.81	0.63	0.79	0.57
III	0.51	0.66	0.36	0.62	0.07
N^1-(Δ^2-Isopentenyl)adenosine	0.69	0.67	0.64	0.78	0.24
N^1-(Δ^2-Isopentenyl)adenine	0.77	0.80	0.53	0.77	
N^6,N^6-Dimethyladenosine	0.63	0.75	0.52	0.73	0.54
N^6,N^6-Dimethyladenine	0.71	0.78	0.43	0.73	0.67
Zeatin[a]	0.75	0.87	0.61	0.77	0.61
Ribosylzeatin[a]	0.63	0.79	0.56	0.77	0.41

[a] Shaw *et al.* (1966).

The solvent systems were (measurement by volume): (A) 1-butanol–water–concentrated ammonium hydroxide (86:14:5), (B) 1-butanol–glacial acetic acid–water (5:3:2), (C) 2-propanol–concentrated hydrochloric acid–water (680:170:144), (D) 2-propanol–water–concentrated ammonium hydroxide (7:2:1), (E) ethyl acetate–1-propanol–water (4:1:2), (F) ethyl acetate–water (5:1), (G) ethyl acetate–1-propanol–1% aqueous ammonium hydroxide (4:1:2), and (H) ethyl acetate–2-ethoxyethanol–2% aqueous formic acid (4:1:2).

Isolation of IPA from Yeast tRNA. tRNA (60 g) was dissolved in 4 l. of 0.005 M $MgCl_2$ solution, contained in a 5-l., three-necked, round-bottom flask, equipped with a large paddle stirrer. Whole *C. adamanteus* venom (4.5 g) and bacterial alkaline phosphatase (100 mg) were added and the pH of the solution was adjusted to 8.6 with 0.5 N NaOH. A few milliliters of toluene was added. The mixture was stirred gently

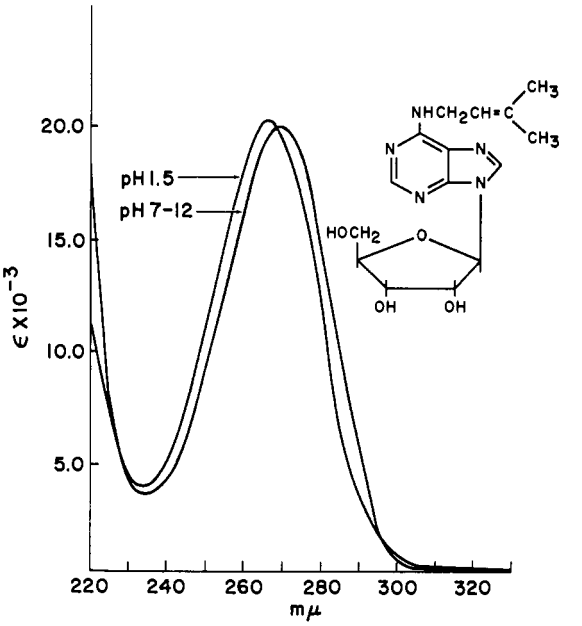


FIGURE 2: Ultraviolet absorption spectra of IPA, I.

at 37° and the pH was maintained at 8.6 by periodic addition of 0.5 N NaOH. After the first 7 hr more venom (2 g) and $MgCl_2$ (5 mmoles) were added. Incubation was continued for a total of 24 hr, at which time the uptake of sodium hydroxide had ceased. (Some 300 ml was consumed.) The hydrolysate was kept at 4° for 3 hr and then centrifuged at 13,000*g* for 30 min. The clear solution was lyophilized.

IPA was isolated from the hydrolysate by means of partition chromatography on a column. The lyophilized hydrolysate was stirred for 1 hr at 25° in 200 ml of water saturated with ethyl acetate and the solution was clarified by centrifugation at 13,000*g* for 10 min. The clear brownish solution (150 ml) was thoroughly mixed with 300 g of Celite 545 and the mixture was packed on top of a previously prepared column (500 g of Celite 545 was mixed with 232 ml of water saturated with ethyl acetate and packed into a precision-bore glass tube; 5.08 (diameter) × 95 cm). The column was developed with ethyl acetate saturated with water (solvent F) at a flow rate of 800 ml/hr. The IPA was eluted in the first 700 ml of solvent. No significant amount of ultraviolet-absorbing material was detected in the next 3 l. of eluate and after this point the major ribonucleosides began to appear in the eluate.

Chromatographic analysis of the first fraction in system E showed IPA to be the major ultraviolet-absorbing compound (R_F 0.80). Two minor ultraviolet-absorbing bands were observed; R_F 0.37 and 0.95 (*p*-hydroxybenzaldehyde).[3] This fraction contained a total of 37 mg of IPA (spectrophotometric analysis).

[3] *p*-Hydroxybenzaldehyde is a contaminant, originating from either the snake venom or bacterial alkaline phosphatase preparation.

TABLE II: Nmr Spectral Data.

Compound	Solvent	CH_3 CH_3>C	Chemical Shifts, ppm (δ)									
			α-C	β-C	N^6-H	C-2	C-8	C-1′	C-2′	C-3′	C-4′	C-5′
	D_2O–acetone-d_6[a,b,c]	1.75	4.22	5.4	—	8.28	8.28	5.97	4.78	4.4		3.80
I	DMSO-d_6[c,d,e]	2.0	—		8.15	8.47	8.62	6.18	4.92	—	—	3.92
II	DMSO-d_6[e,f,g]	1.19	3.72	1.80	7.58	8.36	8.23	—	—	—	—	—
III	DMSO-d_6–D_2O[f,g,h]	1.82	3.75	2.25	—	8.62	8.2	—	—	—	—	—
IV	DMSO-d_6[e,f,g]	1.75	4.25	5.45	7.65	8.35	8.25	—	—	—	—	—
VI	DMSO-d_6[e,f,g]	1.13	3.45	—	7.40	8.2	8.4	5.82	4.58	4.20	4.03	3.63
	D_2O[e,f,g]	1.31	3.66	—	—	8.05	8.19	5.98	—	—	3.89	3.89

[a] Tetramethylsilane internal standard. [b] Temperature at 35°. [c] 100 Mcycles. [d] Tetramethylsilane external standard. [e] Ambient. [f] DSS internal standard. [g] 60 Mcycles. [h] Temperature at 80°.

The eluate containing IPA was evaporated to dryness *in vacuo* in a rotating flash evaporator and the residue was dried by reevaporation with absolute ethanol several times. The residue was crystallized from 1.3 ml of absolute ethanol (1–2 mg of Norit A was added), yield 28 mg. The residue was crystallized three times from acetonitrile–ethanol (3:1), yield 15 mg, mp 139°, $[\alpha]_{546}^{25}$ −100° (*c* 0.07, ethanol). *Anal.* Calcd for $C_{15}H_{21}N_5O_4$ (335.4): C, 53.72; H, 6.31; N, 20.89. Found: C, 53.68; H, 6.25; N, 20.36. The mass spectrum is shown in Figure 1a, and ultraviolet absorption spectra are shown in Figure 2. Paper chromatographic data are given in Table I. The nmr spectrum was obtained in both acetone-d_6 and DMSO-d_6 (Table II). The chemical shifts of the protons are assigned on the basis of those assigned to the protons of adenosine and other nucleosides (Jardetzky and Jardetzky, 1960; Bhacca *et al.*, 1962). The C-1 and C-2 coupling constants are similar to those of adenosine. The N-6 proton shows a triplet which is additional proof the side chain is attached at N-6. The infrared spectra of this sample and that of synthetic IPA are identical. Major absorption bands occur at 3340, 3150, 2910, 1630, 1470, 1420, 1380, 1340, 1295, 1220, 1100, 1055, 985, 945, 870, 825, 790, 765, 670, 635, and 625 cm^{-1}.

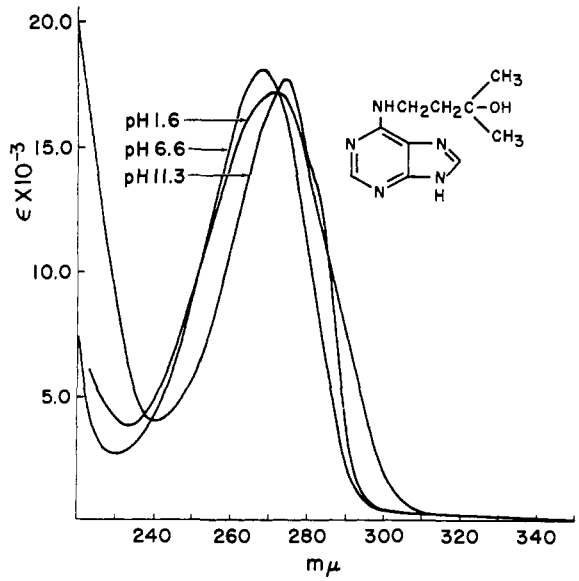


FIGURE 3: Ultraviolet absorption spectra of II.

Preparation of the Free Base from Natural IPA. A solution of 0.6 mg (1.8 μmoles) of the natural IPA in 0.2 ml of water was mixed with 2 ml of H_2O containing 12 μmoles of $NaIO_4$. The solution was heated for 1 hr at 95°, after which it was made 1 N with respect to sodium hydroxide. The solution was heated for 45 min at 95°, cooled, and 50 μl of ethylene glycol was added. After 2 hr the sample was streaked on a 7-in. sheet of Whatman No. 3MM paper which was developed for 24 hr in solvent A. Three ultraviolet-absorbing bands were obtained; the slowest consisted of IPA (about 25%) and the second consisted of a material with ultraviolet absorption spectra corresponding to those of an N^6-(alkyl-substituted)adenosine. The fastest moving material (60%) was characterized as the free base of IPA by cochromatography with the synthetic sample of 6-*N*-(3-methylbut-2-enylamino)-purine (IV), in solvent systems A–E. The isolated material has ultraviolet absorption spectra identical with those of the authentic sample IV.

3-Methylbut-2-enylamine-1. β,β-Dimethylacrylonitrile (K & K Chemical Co.) (4.05 g, 50 mmoles) was dissolved in 40 ml of ether and the solution was added dropwise with stirring and cooling to a slurry of lithium aluminum hydride (1.9 g, 50 mmoles) in 100 ml of ether. The mixture was stored overnight and water (3.6 ml, 0.2 mole) in 50 ml of tetrahydrofuran was added dropwise with cooling. The greenish suspension was filtered through Celite and the clear solution was dried over anhydrous sodium sulfate. The product distilled at 109–110° at atmospheric pressure, yield 1.2 g. Semenow *et al.* (1958) report a boiling point of 110.5° for this compound.

N^6-(Δ^2-Isopentenyl)adenine [*6-N-(3-Methylbut-2-enyl-*

TABLE III: Ultraviolet Absorption Spectra.

Compound	pH 1.0		pH 7.0		pH 12.0	
	λ_{max} (mμ)	$\epsilon \times 10^{-3}$	λ_{max} (mμ)	$\epsilon \times 10^{-3}$	λ_{max} (mμ)	$\epsilon \times 10^{-3}$
IPA I	265	20.4	269	20.0	269	20.0
N^6-(Δ^2-Isopentenyl)adenine (IV)	273	18.6	269	19.4	275	18.1
II	272	16.8	268	17.7	274	17.3
III	264	14.7	270	13.1	276	14.7
VI	264		266		266	
VIII (IPA I)	265		265		267) (310 s)	

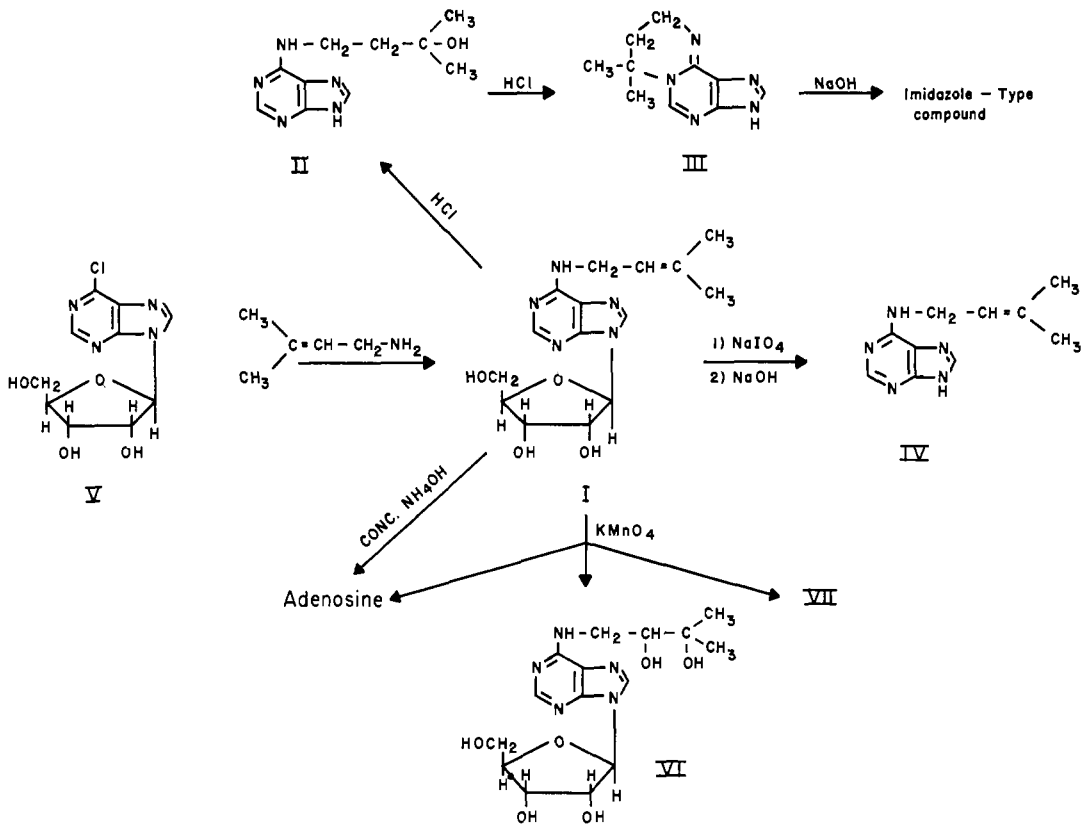
amino)purine] (*IV*). A solution of 6-chloropurine (0.16 g, 1.04 mmole) and 3-methylbut-2-enylamine-1 (0.17 g, 2.0 mmoles) in 10 ml of 2-methoxyethanol was refluxed for 3.5 hr. Sodium hydroxide (1.04 mmole) was added and the solution was evaporated *in vacuo* to dryness. The residue was crystallized twice from water containing 10% ethanol (charcoal). The yield was 85 mg of colorless platelets, mp 212–214°. *Anal.* Calcd for $C_{10}H_{13}N_5$: C, 59.09; H, 6.45; N, 34.46. Found: C, 59.02; H, 6.34; N, 34.65. (Leonard and Fujii (1964) report a melting point of 213–215° for this compound.) The ultraviolet absorption spectra are similar in pattern to those of II (Figure 3), the λ_{max} and extinction coefficients are presented in Table III. The peaks in the nmr spectrum are tabulated in Table II. The peaks due to the C-2 and C-8 protons of this compound and those of II were assigned on the basis of the assignments for adenine made by Bullock and Jardetzky (1964) and confirmed by Fox (1965).

IPA. METHOD A. A solution of 6-chloro-9-β-D-ribofuranosylpurine (0.3 g, 1.05 mmole) and 3-methylbut-2-enylamine (0.2 g, 2.36 mmoles) in 15 ml of ethanol was refluxed for 3 hr. The solution was kept in the refrigerator overnight and 0.3 g of product was filtered off. This material was crystallized from absolute ethanol (charcoal) to yield 0.2 g of colorless needles. An analytical sample was recrystallized three times from acetonitrile–ethanol (3:1), mp 145–147°, $[\alpha]_{546}^{25}$ −97° (*c* 0.07, ethanol). *Anal.* Calcd for $C_{15}H_{21}N_5O_4$: C, 53.72; H, 6.31; N, 20.89. Found: C, 53.50; H, 6.36; N, 21.01. (Leonard *et al.* (1966) report a melting point of 142–143° and $[\alpha]_D^{28}$ −103° (*c* 0.14, ethanol).) Our synthetic sample prepared by method A was used for comparison with the sample isolated from the tRNA.

METHOD B. IPA was also synthesized in this laboratory by Dr. M. H. Fleysher using a modification of the method of Leonard *et al.* (1966). A solution of adenosine (32 g, 0.12 mmole) and γ,γ-dimethylallyl bromide (31 g, 0.21 mole) (γ,γ-dimethylacrylic acid was reduced to the alcohol (Knights and Waight, 1955) which was converted to the bromide according to Kuhn and Schinz (1952)) in 400 ml of freshly distilled *N,N*-dimethylacetamide stirred at 33° for 24 hr. The solvent was evaporated to dryness *in vacuo*. Analysis of this residue by paper chromatography in solvent E showed that 60% of the adenosine had been converted to IPA. The syrupy reaction mass was dissolved in 1.0 l. of water and the pH was adjusted to 10.0 with ammonium hydroxide and the solution was refluxed for 3.0 hr. The pH of the solution was maintained at 10.0 by periodic additions of ammonium hydroxide. Completeness of the reaction was assayed by chromatography in solvent E. The solution was cooled to room temperature and was extracted with six 250-ml portions of ethyl acetate. Chromatographic analysis (solvent E) showed complete extraction of IPA into the ethyl acetate while adenosine remained in the aqueous phase. The ethyl acetate solution was dried over sodium sulfate, evaporated to dryness *in vacuo*, and the residue was crystallized from 100 ml of acetonitrile–ethanol (3:1), yield 17.11 g, mp 145–147°. An additional 1.08 g was recovered from the mother liquor. The sample was recrystallized from 170 ml of acetonitrile–ethanol (3:1) to give 16.8 g (42.5% yield), mp 148°.

Oxidation of IPA with Potassium Permanganate. A solution of IPA (1 mmole) in 500 ml of water at 25° was treated with a 0.1% aqueous solution of potassium permanganate (250 ml). After 10 min the solution was diluted with 500 ml of ethanol and left for 16 hr at 25°. The reaction products were separated by chromatography on Whatman No. 3MM paper in solvent D. The R_F values and percentage of the product in each band, based on optical density units, were as follows: band 1, 0.34, 9%; band 2, 0.54, 30%; band 3, 0.69, 52%; and band 4, 0.87, 9%. The compounds in bands 2 and 4 were identified as adenosine and unreacted IPA, respectively, by their ultraviolet absorption spectra and by paper chromatography. The products in bands 1 and 3 both had ultraviolet absorption spectra corresponding to those of an N^6-(alkyl-substituted)adenosine. The material in band 1 was not investigated further and is listed in Scheme I as VII. The nucleoside in band III appears to have

SCHEME I



the structure VI and was purified and characterized as follows.

The water eluate of band 3 contained 130 mg of VI (spectrophotometric analysis, ϵ 20,000). The aqueous solution was evaporated to dryness and the residue was crystallized from a mixture of ethanol (4 ml) and ether (10 ml). The sample was dried over P_2O_5 in a vacuum desiccator for 2 days; yield 100 mg, mp 120–121°. *Anal.* Calcd for $C_{15}H_{23}N_5O_6 \cdot 1.5H_2O$: C, 45.45; H, 6.61; N, 17.67. Found: C, 45.07; H, 6.74; N, 18.09. The ultraviolet absorption spectra of VI are similar to those of IPA (Table III).

The mass spectrum of this compound (Figure 4) shows peaks at *m/e* of 369 and 372. The molecular ion for VI ($C_{15}H_{23}N_5O_6$) would have an *m/e* of 369. Since the molecular ion could not be an even number the peak at *m/e* of 372, if not an artifact, suggests that the molecular ion could be 387. Thus, 387 − 18 (H_2O) = 369 and 387 − 15 (CH_3) = 372. Assuming, however, that the molecular ion is 369 the following *m/e* assignments can be made: 310 (−59), loss of $(CH_3)_2$-COH, 280 (−89), loss of $(CH_3)_2CH(OH)CHOH$, and the base less a major sugar fragment; 266 (−103), loss of side chain, *i.e.*, adenosine-H and base +30 (sugar fragment). This fragmentation pattern suggests ready loss of the side chain to give adenosine-H (266) and its further fragmentation would account for a major peak at *m/e* of 178 which is known as a prominent peak in the fragmentation pattern of adenosine (Biemann and McCloskey, 1962). These authors have also pointed out that the sugar portion of ribonucleosides undergoes fragmentation in such a manner as to give two major fragments represented by the molecular ion minus 89 and minus 103. For VI these fragments correspond to *m/e* of 280 and 266, respectively. The analogous fragments appear in the mass spectrum of IPA (Figure 1a) at *m/e* of 246 and 232, respectively. The nmr spectra of VI were obtained in D_2O and DMSO-d_6 (Table II).

Reaction of IPA with Iodine. Iodine reacts with IPA in an aqueous solution of potassium iodine or in ethanol to yield a primary product which in turn degrades to other products. The following preparation was carried out in ethanol. A solution of 400 mg of iodine (1.58 mmoles) in 100 ml of 95% ethanol was added dropwise at 25° with stirring to a solution of 530 mg (1.58 mmoles) of IPA in 100 ml of 95% of ethanol over a 10-min period. The solution was stirred for a further 20 min and then evaporated to dryness *in vacuo*. The reaction products were resolved by partition chromatography on a column (solvent H) 2.54 (diameter) × 60 cm according to the general method of Hall (1962). The major product was eluted between the 1000- and 2000-ml mark and this fraction of the eluate was concentrated *in vacuo* to 2 ml. The addition of 10 ml of anhydrous ether yielded 0.14 g of white

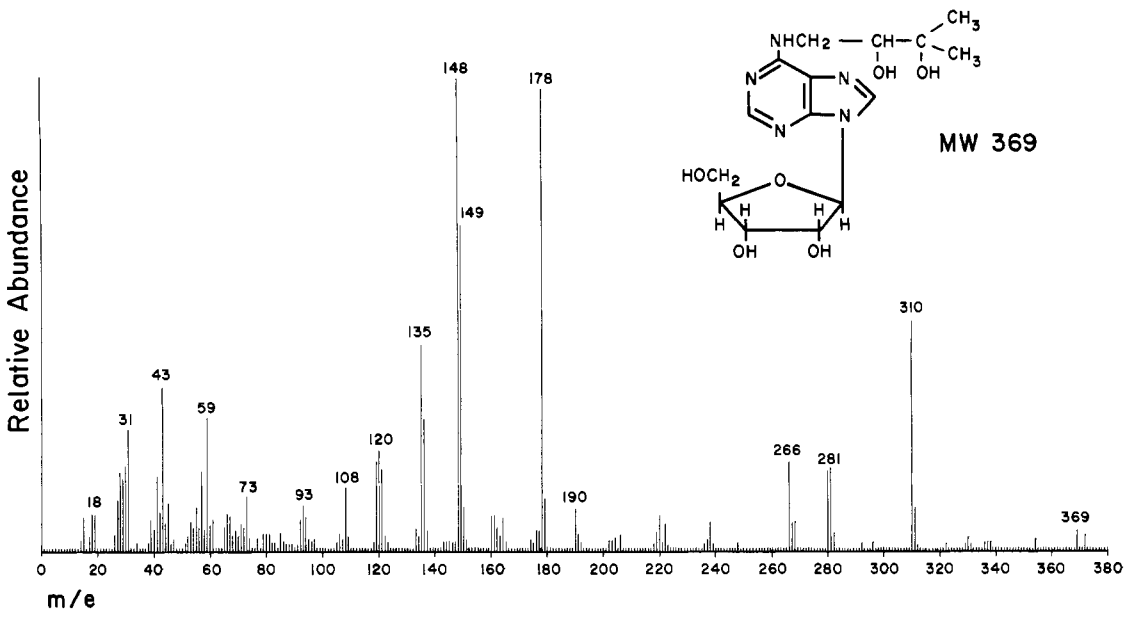
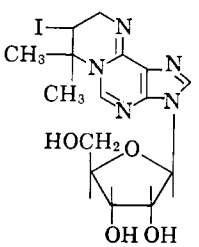


FIGURE 4: Mass spectrum of VI obtained by permanganate oxidation of IPA; direct inlet, 70 ev. See text for discussion of the spectrum.

solid. The product was dissolved in 0.5 ml of absolute ethanol and precipitated with anhydrous ether to give a material labeled IPA-I (decomposed at 145°). *Anal.* Calcd for $C_{15}H_{20}IN_5O_4$: C, 39.03; H, 4.35. Found: C, 38.69; H, 4.39.

IPA-I was unstable and lost iodine readily to give a series of compounds separable on paper chromatography in solvent D. Although IPA-I chromatographed as a single ultraviolet-absorbing spot in solvents C and D (R_F values 0.53 and 0.70, respectively) it smeared in a neutral solvent (such as E) which suggests that the sample was not homogeneous. The nmr spectrum obtained in DMSO-d_6 indicates that IPA-I consisted of two compounds. The salient feature of the spectrum is that two single peaks appear at δ 1.68 and 1.84 (ratio of 60:40) which together integrate for six protons, assuming that a singlet at δ 8.57 represents the C-2 and C-8 protons. The ultraviolet absorption spectra of IPA-I (Table III) are similar to those of N^1,N^6-dimethyladenosine (Broom *et al.*, 1964), and these data together with the analytical data suggest that IPA-I consists of two closely related compounds (perhaps isomeric) with structures of a type represented by VIII.



VIII

Reaction of IPA with Concentrated Ammonium Hydroxide. A solution of 5 mg of IPA in 1 ml of concentrated ammonium hydroxide was heated at 100° in a sealed tube for 2 hr. Chromatography of the reaction mixture in solvent E showed that adenosine (22%) and unchanged IPA (78%) were present. No other ultraviolet-absorbing product was detected.

Preparation of II and III. Compound IV (2.15 g) was added to 150 ml of boiling 1 N hydrochloric acid and the clear solution was refluxed for 15 min. The solution was cooled rapidly under running cold water and evaporated to dryness in a rotating flash evaporator in which the condensing flask was cooled in liquid nitrogen (vacuum at 2.0 mm). The residue was stored at room temperature in a vacuum desiccator over potassium hydroxide pellets and phosphorus pentoxide. Analysis by means of paper chromatography in solvent E showed that the residue consisted of a mixture of II and III and about 2% of the starting material.

These products were separated by means of partition chromatography on a column. The sample was dissolved in a small quantity of water and neutralized by concentrated ammonium hydroxide (2 ml). The solution was evaporated to dryness *in vacuo* and the residue was dissolved in 22 ml of the lower phase of solvent E. This solution was mixed with 45 g of Celite 545 and 1.75 g of Microcel-E and the mixture was charged on top of the column containing 760 g of Celite 545 and 30 g of Microcel-E mixed with 395 ml of lower phase of solvent E (Figure 5).

6-N-(3-Methyl-3-hydroxybutylamino)purine (II). The fraction containing this compound (Figure 5) was evaporated to dryness and the residue was crystallized from 3 ml of ethanol (charcoal). The product (0.8 g) was recrystallized from 25 ml of acetonitrile–ethanol

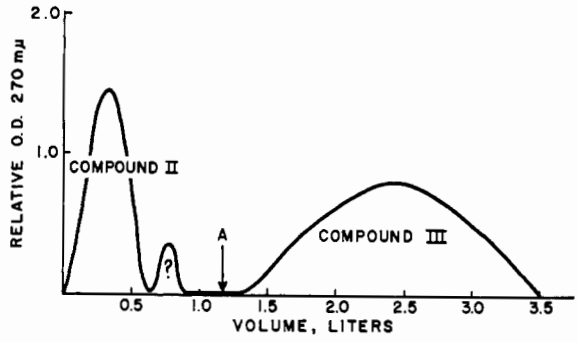


FIGURE 5: Chromatographic separation of II and III obtained from the acid treatment of 2.15 g of N^6-(Δ^2-isopentenyl)adenine (IV). Partition chromatography on a column, 5.08 (diameter) × 80 cm, solvent system E, flow rate 600 ml/hr. At point A the solvent was changed to the upper phase of solvent G.

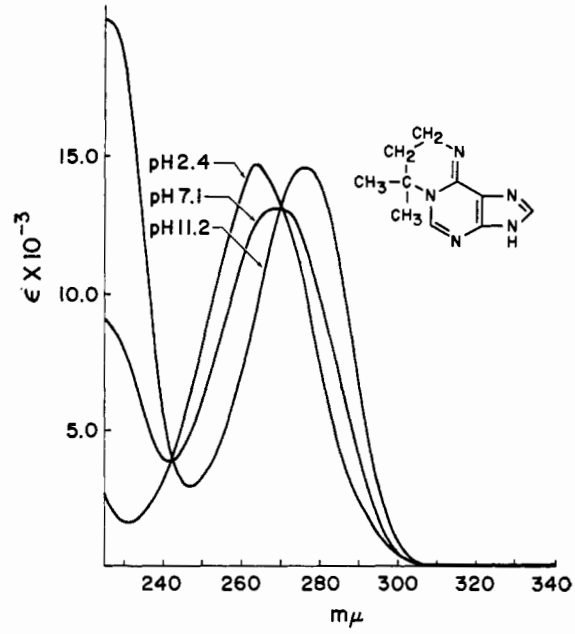


FIGURE 6: Ultraviolet absorption spectra of III.

(1:1), yield 0.5 g, mp 173–174°. This product is very soluble in ethanol and acetone and sparingly soluble in acetonitrile. The analytical sample was dried *in vacuo* for 5 hr at 56°. There was no change in the melting point under these conditions. When a sample of the product, however, was dried *in vacuo* at 115° for 3 hr the melting point changed to 166–171° (soft mp 150°). *Anal.* Calcd for $C_{10}H_{15}N_5O$: C, 54.28; H, 6.83; N, 31.66. Found: C, 54.71; H, 6.77; N, 31.24. The ultraviolet absorption spectra of this compound (Figure 3) are typical of an N^6-(alkyl-substituted)-adenine and the fragmentation pattern of the mass spectrum (Figure 1b) shows a major peak at *m/e* 162 which corresponds to a loss of $(CH_3)_2COH$. The peaks in the nmr spectrum are tabulated in Table II. It is of some interest that the R_F values of II and those of zeatin,[4] 6-*N*-(*trans*-4-hydroxy-3-methylbut-2-enylamino)purine essentially coincide in solvent systems A–E. They separate better in the system pyridine–ethanol–water (2:7:1); R_F values of II, 0.71 and zeatin, 0.65.

3-H-7,7-Dimethyl-7,8,9-trihydropyrimido[2,1-i]-purine (III). The fraction (Figure 5) containing this material was evaporated to dryness *in vacuo* and the residue was crystallized from 20 ml of ethanol (charcoal). The product (250 mg) was recrystallized from 12 ml of ethanol, yield 142 mg (decomposed at 303°). This sample was crystallized from 8 ml of acetonitrile, to yield a product which melted at 290–292° (dried 115°, 4 hr, 2 mm). *Anal.* Calcd for $C_{10}H_{13}N_5$: C, 59.09; H, 6.45; N, 34.46. Found: C, 58.95; H, 6.58; N, 34.60. In our original report on this compound Hall *et al.* (1966) the melting point was erroneously reported as 200–202°. (Leonard *et al.*, 1966, report a melting point of 280.5–282° for this compound.) The ultraviolet absorption spectra of this product (Figure 6) are similar to those of N^1,N^6-dimethyladenine (Broom *et al.*, 1964). The nmr spectrum (Table II) shows a singlet at δ 1.82 integrating for six protons which establishes the linkage to the side chain at carbon 3. The C-2 and C-8 assignments for the cyclized product III are less certain, since the effect of substitution at the N′ position is unknown. In the case of 1-methylinosine the peak for the C-2 proton is moved 0.15-ppm downfield compared to its position for inosine whereas the chemical shift for the C-8 proton remains unchanged (M. P. Schweizer, private communication).

When a solution of IPA or its free base in 5 N perchloric acid was heated for 1 hr at 100° only III was obtained. Compound II can be converted into III by prolonged heating in hydrochloric acid or by the perchloric acid treatment. Compound III is degraded in alkali. In one experiment a solution of 100 μg of III in 100 μl of 0.2 N sodium hydroxide was heated for 2 hr at 100°. Paper chromatographic analysis in solvent D showed that 70% of II had been converted to a new product; $\lambda_{max}^{pH\ 1.0}$ 266 mμ, $\lambda_{max}^{pH\ 6.0}$ 266 mμ, and $\lambda_{max}^{pH\ 12.5}$ 275 mμ. These spectra are related to those of a 4-amino-5-imidazolecarboxamide. This compound was not investigated further.

Detection of II and III in an Acid Hydrolysate of Yeast tRNA. The tRNA (870 mg) was dissolved in 7.0 ml of water and the pH was adjusted to 4.0 with hydrochloric acid. The solution was warmed to 89° and 2.6 ml of 5 N hydrochloric acid was added. The solution was heated for 15 min at 100° and then cooled rapidly to room temperature, after which it was evaporated to dryness (vacuum at 2 mm) in a rotating evaporator using liquid nitrogen for cooling. The residue was dissolved in methanol and the solution was evaporated. This process was repeated several

[4] We are indebted to Dr. Shaw for sending us a sample of his synthetic compound (Shaw *et al.*, 1966).

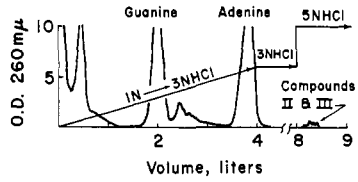


FIGURE 7: Elution profile of an acid hydrolysate of 870 mg of yeast tRNA chromatographed on an ion-exchange column, 1 (diameter) × 30 cm, of Dowex 50W-X8 [H^+] (200–400 mesh). The resin was equilibrated with a hydrochloric acid before introduction of the sample and the column was developed with a hydrochloric acid gradient as shown.

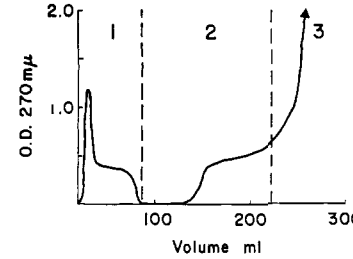


FIGURE 8: Elution profile of an enzymic hydrolysate of chick embryo tRNA consisting of 540 mg of mixed nucleosides chromatographed on a partition column of Celite 545, 2.54 (diameter) × 57 cm, solvent system E, flow rate 100 ml/hr. Major components of the fraction were: 1, IPA; 2, N^6,N^6-dimethyladenosine; and 3, 2′-*O*-methyladenosine and adenosine. This figure depicts only the initial portion of the elution pattern. About 98% of the digest still remains on the column.

times after which the residue was kept for 24 hr in a vacuum desiccator over potassium hydroxide pellets. The hydrolysate was dissolved in 10 ml of water and the products were separated on a column of the sulfonic acid resin, Dowex 50W-X8 (Cohn, 1949). The elution pattern is shown in Figure 7. The fraction containing II and III was evaporated to dryness *in vacuo* and the residue was chromatographed on Whatman No. 1 paper in solvent D. Six bands of ultraviolet-absorbing material were obtained. Band 6 (counting from the origin) contained II (30.2 μg) and band 5 contained III (24.7 μg). These two products were identified on the basis of their ultraviolet absorption spectra and cochromatography with the authentic markers. An additional 2 l. of 5 N hydrochloric acid was passed through the column but no additional ultraviolet-absorbing compounds were eluted.

The question of whether N^1-(Δ^2-isopentenyl)adenine might have been present but not detected was answered by a model experiment in which a standard mixture of guanine, adenine, II and III, and N^1-(Δ^2-isopentenyl)-adenine (Leonard and Fujii, 1964) was resolved under conditions identical with those described in Figure 7. Compounds II and III were eluted at a position in the elution pattern identical with that indicated in Figure 7. N^1-(Δ^2-Isopentenyl)adenine was eluted in a distinct peak 400 ml after elution of II and III. No N^1-(Δ^2-isopentenyl)adenine was detected in the corresponding fraction of the column analysis of the tRNA hydrolysate (Figure 2). In a model experiment to test whether N^1-(Δ^2-isopentenyl)adenine is destroyed by acid treatment a small sample was hydrolyzed in 0.5 N HCl for 15 min. About 90% of the sample was recovered unchanged along with a small amount of adenine.

RNA from Liver. Calf liver was obtained fresh from a local abbatoir. The human liver was obtained at autopsy within 4 hr of death of the patient, a 57-year-old woman with primary cancer of the breast. Histological examination of sections taken from the liver showed that the liver was free from disease. The tRNA and rRNA fractions were obtained from the livers using a method based on cell fractionation in the ultracentrifuge. Details of the procedure followed were described previously (Hall, 1964) with the exception that the initial buffer for homogenization consisted of 0.15 M sucrose, 0.025 M KCl, 0.01 M $MgCl_2$, and 0.035 M Tris (pH 7.8). The calf liver (450 g) yielded 550 mg of tRNA and 530 mg of rRNA. The human liver (400 g) yielded 150 mg of tRNA and 322 mg of rRNA.

RNA from Chick Embryo. METHOD 1. Embryos from 24 eggs incubated for 19 days were blotted and frozen. The tissue (1.5 kg) was thawed and the RNA was extracted as described above; yield was 196 mg of tRNA, and 750 mg of rRNA.

METHOD 2. The method used by Brunngraber (1962) for extraction of tRNA from rat liver was followed. The yield of tRNA from 3.5 kg of embryos (19-day-old) was 748 mg.

Assay Procedure for IPA in RNA. This procedure is adapted from the general isolation procedure described above which essentially takes advantage of the much greater solubility of IPA than other nucleosides in organic solvents. The tRNA sample is hydrolyzed enzymically as described above and the hydrolysate after clarification by centrifugation is lyophilized. The following procedure is described in terms of the chick embryo tRNA. The lyophilized sample containing 540 mg of mixed nucleosides was stirred vigorously for 30 min with 20 ml of the lower phase of solvent E. The mixture was centrifuged at 10,000*g* for 5 min and the clear solution was mixed with 43 g of Celite 545. This mixture was packed on top of a column containing a mixture of 120 g of Celite 545 and 55 ml of the lower phase of solvent E. The elution profile of the column is shown in Figure 8.

Fraction 1, containing IPA and some impurities, was concentrated and the residue was chromatographed on Whatman No. 1 paper in solvent D. In addition to the major band of IPA, there were two bands which fluoresced in ultraviolet light and a band owing to *p*-hydroxybenzaldehyde. IPA was eluted with water and the amount, determined spectrophotometrically,

was 195 μg. This product possessed ultraviolet absorption spectra identical with those of synthetic IPA and it cochromatographed with the synthetic material in solvent systems A–E.

Solvent E was used rather than solvent F because N^6,N^6-dimethyladenosine is resolved as a separate band. This nucleoside was purified by paper chromatography in solvent D (yield 310 μg) and identified by cochromatography with an authentic sample and by its ultraviolet absorption spectra. The amounts of IPA isolated from the RNA samples by this technique are listed in Table IV.

TABLE IV: Amount of IPA Isolated from RNA.

Sample	Mole % of Total Nucleoside Residues in the RNA
Yeast tRNA	0.065 ± 0.005[a]
Calf liver tRNA	0.05
Human liver tRNA	0.05
Chick embryo tRNA	0.03
Human liver rRNA	Not detected
Chick embryo rRNA	Not detected

[a] The value of yeast tRNA is the average of six analyses, the data for the other sources were obtained on single samples.

Discussion

The level of IPA in yeast tRNA is equivalent to one nucleotide in about 1500 and if we assume that there is only one IPA residue in a given tRNA molecule this means that one tRNA molecule in about 20 contains an IPA residue. Thus, if the serine tRNA fraction constitutes approximately $^1/_{20}$ of the total tRNA, IPA may occur only in this fraction.

When IPA is hydrolyzed in dilute acid the free base is not obtained but rather the hydrated product, II is formed. On continued acid treatment the hydroxyl group is expelled forming, what is presumably, a carbonium ion intermediate that undergoes ring closure forming III. The formation of III has an analogy in the ready cyclization of N^6-substituted adenines to the N-1 position. N^6-(Glycyl)adenine, for example, undergoes facile cyclization with the loss of ammonia (Chheda and Hall, 1966) and N^6,N^6-bis(2-chloroethyl)adenine undergoes cyclization spontaneously with elimination of hydrogen chloride (Johnston *et al.*, 1962). Compound III, although stable to acid, degrades in alkaline solution to form a product that appears to be an imidazole derivative.

The ready hydration of the allylic double bond under relatively mild chemical conditions indicates that the N′ position of the purine residue actively promotes reaction of the side chain. Thus, the spatial arrangement of the allylic double bond in the side chain and the N′ position may well play an important role in the biological function of the IPA molecule. In preliminary studies on the metabolism of IPA in human subjects conducted by Drs. A. Mittelman and G. B. Chheda in this institute, a metabolic product identified as II was isolated (results to be published).

The reactivity of the allylic double bond of IPA is also illustrated by the rapid oxidation by permanganate under very mild conditions to produce VI and by the facile addition of iodine. The iodine reaction is more complex than that of simple addition across a double bond and secondary reactions occur. The primary isolable reaction product has been identified tentatively as structure VIII. The fact that IPA reacts with iodine under conditions that no other known nucleoside (except sulfur-containing nucleosides) reacts, enables yeast seryl-tRNA to be modified selectively at the point of location of the IPA residue (Fittler and Hall, 1966). This structural modification does not affect the acceptance of serine residues but does interfere with the binding of seryl-tRNA to the ribosome–mRNA complex.

The nucleoside isolated from yeast tRNA was identified rigorously as IPA, I. A question that must be considered is whether this compound or the alternative N^1 isomer exists in native tRNA. This question has a parallel in tRNA structure since both N^1- and N^6-methyladenosine occur in the nucleic acids of various species (Dunn, 1961). The chemical properties of the N^1 isomer of IPA differ radically from that of the N^6 isomer, and further the N^1 isomer does not possess cytokinin activity (Leonard *et al.*, 1966). Thus the influence of the isopentenyl side chain on the biochemistry of the tRNA molecule would vary greatly depending on whether it is attached to the N-1 or N-6 position of an adenylic acid component. N^1-(Alkyl-substituted)adenines rearrange easily to the N^6 isomer (see, for example, Taylor and Loeffler 1960). Leonard *et al.* (1966) found that N^1-(Δ^2-isopentenyl)adenine rearranges to the N^6 isomer in aqueous solution at 100° at pH 7.5 and in our isolation procedure the nucleosides were exposed to conditions (pH 8.5 at 37° for 24 hr) which also promote this rearrangement.

In order to resolve this question an acid hydrolysate of tRNA was examined. Under mild acid conditions the purines are released from the RNA and N^6-(Δ^2-isopentenyl)adenine is converted to II and III, while N^1-(Δ^2-isopentenyl)adenine, if present, would remain unchanged. Analysis of the acid hydrolysate of tRNA by means of ion-exchange chromatography (Figure 7) showed the presence of II and III. The N^1 isomer was not detected. The question remains of whether the rearrangement occurs during extraction of the RNA but it would seem unlikely, that if this were the case, only the N^6 isomer would be isolated. N^1-Methyladenine can be obtained in good yield from the acid hydrolysate of tRNA (Dunn, 1961).

Dr. Srivastava of this institute tested the cytokinin activity of IPA in the tobacco callous tissue system (Linsmaier and Skoog, 1965) and found it to be as active as kinetin, 6-(furfurylamino)purine. Hamzi

and Skoog (1964) had tested synthetic N^6-(Δ^2-isopentenyl)adenine and found it to be about ten times as potent as kinetin in the tobacco callous system. IPA stimulates the germination of lettuce seeds to the same degree as kinetin (Table V). Dr. B. I. S. Srivastava (private communication) tested IPA in the leaf senescence test (Srivastava and Ware, 1965) and found it to be active. Drs. L. Engelbrecht and K. Mothes (private communication) confirmed the results in the leaf senescence test and also found that IPA is more active than zeatin or kinetin in the correlative inhibited bud test (Wickson and Thimann, 1958). IPA, therefore, appears to exhibit a spectrum of biological activity in plant systems similar to that exhibited by kinetin. With respect to the plant systems the free base (IV) is as active or more active than IPA, but on the other hand, in mammalian cell systems, IPA is much more active than the free base. Grace *et al.* (1967) found that IPA at 1 μg/ml inhibits certain mammalian cell lines grown in culture while the free base is much less active. Whether there is any relationship between the biological activity of IPA and its presence in the tRNA remains an interesting question.

TABLE V: Stimulation of the Germination of Lettuce Seeds (*Lactuca sativa*).[a]

Test Solutions	Number of Seeds Germinated at Given Times				
	14 hr	16 hr	18 hr	20 hr	24 hr
Distilled water	6	6	6	6	6
Kinetin (1 mg/l.)[b]	18	33	42	54	57
IPA (natural sample (1 mg/l.)	30	36	39	39	45

[a] This assay is based on that described by Skinner *et al.* (1956). Seeds, "Simpson Early Curved," were soaked in the test solution for 6 hr at room temperature, then placed in a petri dish on filter paper soaked in the test solutions. The seeds (75/dish) were incubated in the dark at 25°. Zero time is start of incubation. [b] Obtained from the Calbiochem Co.

Some workers, using the sensitive biological assays, have detected cytokinin activity in nucleic acid hydrolysates. Kovoor and Ruch (1964) observed cytokinin activity in an alkaline hydrolysate of the tRNA calf liver and Bellamy (1966) found cytokinin activity in the alkaline hydrolysates of rat and sheep liver RNA. Skoog *et al.* (1966) have also detected cytokinin activity in the acid hydrolysates of yeast, liver, and *Escherichia coli* tRNA. However, as initially reported (Hall *et al.*, 1966) and also described in this paper acid hydrolysis of tRNA containing IPA does not yield the free base IV but II and III. Therefore, any cytokinin activity in the acid hydrolysate of RNA cannot be ascribed to the free base of IPA, IV. In addition if the RNA sample should be contaminated by a trace of DNA the potent cytokinin, 6-furfurylaminopurine, will be formed by interaction of adenine and deoxyribose in the presence of acid (Hall and de Ropp, 1955). Thus, biological data with respect to the presence of cytokinins obtained on gross RNA hydrolysates must be interpreted with caution.

The data presented in Table IV show that IPA occurs in the tRNA fraction of yeast and mammalian tissue but not in the ribosomal fraction. IPA has been detected in the tRNA of peas, and spinach but not in the tRNA of sweet corn kernels (Hall *et al.*, 1967). The tRNA of each of these plant sources contains a hydroxylated derivative of IPA, 6-*N*-(*cis*-4-hydroxy-3-methylbut-2-enylamino)-9-β-D-ribofuranosylpurine (Hall *et al.*, 1967).

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Kethoxal—A Potentially Useful Reagent for the Determination of Nucleotide Sequences in Single-Stranded Regions of Transfer Ribonucleic Acid*

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ABSTRACT: β-Ethoxy-α-ketobutyraldehyde (kethoxal) is believed to react specifically with guanylic acid residues in ribonucleic acid (RNA). Evidence is presented that residues present in single-stranded regions of transfer ribonucleic acid (tRNA) react much more rapidly with kethoxal than those present in double-stranded regions. Extensive treatment with kethoxal does not cause substantial irreversible damage to tRNA. Pancreatic ribonuclease digests of kethoxal-treated RNA may be analyzed by a two-dimensional mapping procedure yielding information about nucleotide sequences containing modified guanylic acid residues.

It is well established that involvement of tRNA nucleotides in secondary structure decreases their reactivity toward chemical reagents (Penniston and Doty, 1963; Marciello and Zubay, 1964; Kisselev *et al.*, 1964; Augusti-Tocco and Brown, 1965; Hayatsu and Ukita, 1966; Knorre *et al.*, 1966). The results of these studies have been interpreted as showing that only residues which occur in nonhydrogen-bonded "loops" remain susceptible to reaction with the appropriate reagents. However, this knowledge has not yet been generally applied to the identification of specific nucleotide sequences present in looped regions of purified tRNAs. In this report, it will be shown that β-ethoxy-α-ketobutyraldehyde (kethoxal) shows considerable promise as a tool for this purpose.

It was shown by Staehelin (1959) that kethoxal reacts specifically with guanylic acid among the four major

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