Harada, F., Kimura, F., and Nishimura, S. (1971), *Biochemistry 10,* 3269–3277.
Hayatsu, H., and Inoue, M. (1971), *J. Amer. Chem. Soc. 93,* 2301–2306.
Hayatsu, H., Wataya, Y., and Kai, K. (1970a), *J. Amer. Chem. Soc. 92,* 724–726.
Hayatsu, H., Wataya, Y., Kai, K., and Iida, S. (1970b), *Biochemistry 9,* 2858–2865.
Kelmers, A. D., Novelli, G. D., and Stulberg, M. P. (1965), *J. Biol. Chem. 240,* 3979–3983.
Killer, E. B. (1964), *Biochem. Biophys. Res. Commun. 17,* 412–415.
Kim, S. H., Quigley, G. J., Suddath, F. L., McPherson, A., Sneden, D., Kim, J. J., Weinzierl, J., and Rich, A. (1973), *Science 179,* 285–288.
Kućan, Z., and Chambers, R. W. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 25,* 156–158.
Kućan, Z., Freude, K. A., Kućan, I., and Chambers, R. W. (1971), *Nature (London), New Biol. 232,* 177–179.
Lapointe, J., and Söll, S. (1972), *J. Biol. Chem. 247,* 4966–4975.
Levitt, M. (1969), *Nature (London) 224,* 759–763.
Litt, M. (1971), *Biochemistry 10,* 2223–2227.
Litt, M., and Greenspan, C. M. (1972), *Biochemistry 11,* 1437–1442.
Nishimura, S. (1971), *Proc. Nucl. Acid Res. 2,* 542–564.
Ohashi, Z., Harada, F., and Nishimura, S. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 20,* 239–241.
Pal, B. C., Shugart, L. R., Isham, K. R., and Stulberg, M. P. (1972), *Arch. Biochem. Biophys. 150,* 86–90.
Rubin, I. R., Kelmers, A D., and Goldstein, G. (1967), *Anal. Biochem. 20,* 533–544.
Rubin, I. R., Mitchell, T. J., and Goldstein, G. (1971), *Anal. Chem. 43,* 717–721.
Saneyoshi, M., and Nishimura, S. (1970), *Biochim. Biophys. Acta 204* 389–399.
Saneyoshi, M., and Nishimura, S. (1971), *Biochim. Biophys. Acta 246,* 123–131.
Schimmel, P. R., Uhlenbeck, O. C., Lewis, J. B., Dickson, L. A., Eldred, E., and Schreier, A. A. (1972), *Biochemistry 11,* 642–646.
Schulman, L. H., and Goddard, J. P. (1973), *J. Biol. Chem. 248,* 1341–1345.
Shapiro, R., Cohen, B. I., and Servis, R. E. (1970), *Nature (London) 227,* 1047–1048.
Singhal, R. P. (1971), *J. Biol. Chem. 43,* 245–252.
Singhal, R. P. (1972), *Arch. Biochem. Biophys. 152,* 800–810.
Singhal, R. P. (1973), *Biochim. Biophys. Acta 319,* 11–24.
Singhal, R. P. (1974), *Eur. J. Biochem. 43,* 245–252.
Singhal, R. P., and Best, A. N. (1973), *Biochim. Biophys. Acta 331,* 357–368.
Singhal, R. P., and Cohn, W. E. (1972), *Anal. Biochem. 45,* 585–599.
Singhal, R. P., and Cohn, W. E. (1973), *Biochemistry 12,* 1532–1537.
Uhlenbeck, O. C., Baller, J., and Doty, P. (1970), *Nature (London) 225,* 508–510.
Walker, R. T., and Rajbhandary, U. L. (1972), *J. Biol. Chem. 247,* 4879–4892.
Weeren, H. O., Ryon, A. D., Heatherly, D. E., and Kelmers, A. D. (1970), *Biotechnol. Bioeng. 12,* 889–912.
Willick, G. E., and Kay, C. M. (1971), *Biochemistry 10,* 2216–2222.

3-(3-Amino-3-carboxy-*n*-propyl)uridine. The Structure of the Nucleoside in *Escherichia coli* Transfer Ribonucleic Acid That Reacts with Phenoxyacetoxysuccinimide†

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ABSTRACT: The nucleoside that reacts with the *N*-hydroxysuccinimide ester of phenoxyacetic acid in *Escherichia coli* tRNA has been isolated and its structure has been determined. The phenoxyacetylated trinucleotide containing this nucleoside has been purified from combined pancreatic and T_1 ribonuclease digests of crude *E. coli* tRNA. It was found to occur in the sequence m^7GXC and is probably identical with the X nucleoside found in *E. coli* $tRNA^{Arg}$, $tRNA^{Ile}$, $tRNA^{Phe}$, $tRNA^{Met}_m$, and $tRNA^{Val}_{2A\&B}$. The structure has been determined by its nuclear magnetic resonance spectrum and high-resolution mass spectrum to be 3-(3-amino-3-carboxy-*n*-propyl)uridine.

We have previously reported that several species of *Escherichia coli* B and rat liver tRNA react with the *N*-hydroxysuccinimide ester of phenoxyacetic acid (HSP)[1] to form a phenoxyacetyl-tRNA adduct. The phenoxyacetylated species of tRNA can be identified by their altered chromatographic properties on BD-cellulose. The reactive rat liver tRNA species are $tRNA^{Tyr}$, $tRNA^{Thr}$, $tRNA^{Ile}$, $tRNA^{Asn}$, and $tRNA^{Cys}$ (Friedman, 1972). The reactive *E. coli* tRNAs are $tRNA^{Arg}$,

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[1] Abbreviations used are: HSP, phenoxyacetoxysuccinimide; BD-cellulose, benzoylated-DEAE; A_{260} unit, that quantity of material which, when dissolved in 1 ml of water, has an absorbance of 1.0 at 260 nm in a 1-cm light path; X = an unknown nucleoside.

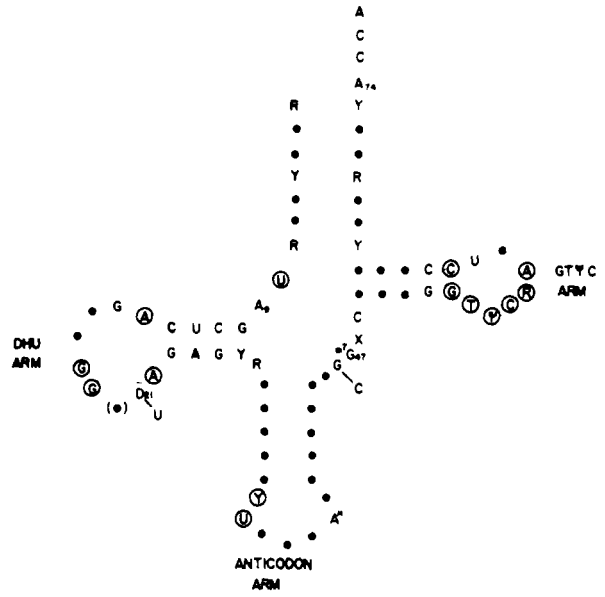


FIGURE 1: Common sequence of six *E. coli* tRNAs containing the nucleoside X. Encircled nucleosides are common to all *E. coli* tRNAs involved in protein synthesis. Abbreviations used are: Y, pyrimidine; R, purine nucleosides; A*, adenosine or modified adenosine. The diagram and enumeration are based on Levitt (1969). The parentheses in the DHU loop indicate that some tRNAs have an extra nucleoside in this position.

tRNA^{Ile}, tRNA^{Lys}, tRNA^{Phe}, tRNA$^{Met}_{m}$,[2] and minor species of tRNA^{Val} (Friedman, 1973). We had noted (Friedman, 1973) that all the *E. coli* tRNAs of known structure that react with HSP contain the unknown nucleoside N in the sequence m^{7}GXC (Figure 1) (Murao *et al.*, 1972; Yarus and Barrell, 1971; Barrell and Sanger, 1969; Cory *et al.*, 1968; Yaniv and Barrell, 1971). Since this is the only region in these *E. coli* tRNAs in which m^{7}G occurs, the isolation of this phenoxyacetylated trinucleotide and the determination of the structure of X would provide proof that it is this unique nucleoside that reacts with HSP. In this paper we report the isolation of the phenoxyacetylated trinucleotide m^{7}GXC from crude *E. coli* B tRNA. The structure of X is proven to be 3-(3-amino-3-carboxy-*n*-propyl)uridine.

Experimental Section

Materials. E. coli B tRNA was purchased from General Biochemicals. BD-cellulose was obtained from Gallard-Schlesinger Chemical Manufacturing Corp. *E. coli* alkaline phosphatase and bovine pancreatic ribonuclease were obtained from P-L Biochemicals Inc. Ribonucleases T_2 and T_1 and snake venom phosphodiesterase were obtained from Sigma Chemical Co. Thin-layer plates coated with microcrystalline cellulose (F1440) were obtained from Schleicher & Schuell, Inc.

Preparation of [^{14}C]*HSP.* The method of Hayes and Branch (1943) was modified to prepare [^{14}C]phenoxyacetic acid. Monochloroacetic acid (250 mg), [1-^{14}C]monochloroacetic acid (1.12 mg, specific activity 41 Ci/mol), freshly distilled phenol (253 mg), and 10 N sodium hydroxide (0.55 ml) were mixed in a test tube and heated in a boiling-water bath for 5 hr. The mixture, diluted to 5.5 ml with water, was acidified and extracted three times with 4 ml of chloroform, and the chloroform extracts were combined and reextracted three times with 10 ml of 0.5 M sodium carbonate. The phenoxyacetic acid crystallized after acidification of the combined sodium carbonate extracts: yield 282 mg (71%); mp 98–102° (lit. mp 98–99°). The product was dried *in vacuo* over phosphorus pentoxide and used to synthesize HSP by the method of Gillam *et al.* (1968).

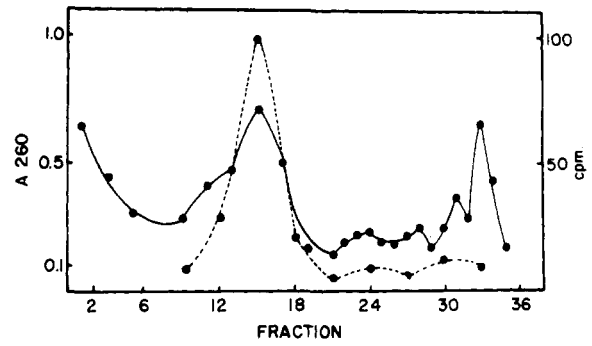


FIGURE 2: BD-cellulose chromatography of an aliquot of the pancreatic and T_1 ribonuclease digestion products of phenoxyacetylated tRNA. After loading the digest on the column, it was washed with 250 ml of 0.5 M NaCl. The radioactive nucleosides were eluted with a gradient of sodium chloride and ethanol as described in the Methods. Only the gradient elution is presented: (●—●) A_{260} units; (●- - -●) radioactivity.

Preparation of Phenoxyacetylated m^{7}GXC. In all the *E. coli* tRNAs of known structure that contain the trinucleotide m^{7}GXC, it is preceded by the nucleoside G or C. Since it has been reported that the XC nucleotide link was resistant to digestion by pancreatic and T_1 ribonuclease (Barrell and Sanger, 1969), it was recognized that the phenoxyacetylated trinucleotide could be prepared from crude *E. coli* tRNA by digestion with these two nucleases.

E. coli B tRNA (17,000 A_{260} units) was dissolved in 40 ml of 0.1 M triethanolamine-HCl containing 0.01 M magnesium chloride (pH 8.0) and maintained at 0° in an ice bath. HSP (145.9 mg; 9 nCi/μmol), dissolved in 8 ml of tetrahydrofuran, was added in two 4-ml portions to start the reaction, and again at 45 min. The pH was maintained at pH 8 by the addition of 5 M sodium hydroxide. After 90 min the reaction mixture was adjusted to pH 5 with 1 M acetic acid, extracted twice with 50 ml of ether, and the tRNA precipitated by the addition of 0.1 volume of 20% potassium acetate (pH 5) and 2 volumes of ethanol. The tRNA was collected by centrifugation and washed twice by solution in 2% potassium acetate and precipitation with ethanol. The precipitate was dissolved in 50 ml of water and dialyzed against three changes of 2 l. of water for 7 hr. The dialyzed tRNA was diluted to 95 ml with water and 5 ml of 1 M Tris-HCl buffer (pH 7.5) added. The tRNA was digested with 100,000 units of T_1 ribonuclease and 25 mg of pancreatic ribonuclease at 37° for 18 hr. The pH was maintained constant by the addition of 1 N sodium hydroxide. Aliquots of the digest (600 A_{260} units) were diluted in half with 1 M sodium chloride containing 0.05 M sodium acetate, the pH adjusted to 6.0, and loaded onto a 2 × 20 cm bd-cellulose column. The column was washed with 250 ml of 0.5 M sodium chloride containing 0.025 M sodium acetate (pH 6.0). The column was then eluted with a 500-ml gradient of 0.5–1.0 M sodium chloride, 0–40% ethanol containing 0.05 M sodium acetate (pH 6.0) at a flow rate of 0.5 ml/min. All the tRNA was processed in this way. The radioactive peaks (Figure 2) were combined, and the ethanol was removed by rotary evaporation. Desalting was accomplished by diluting the fractions threefold with water and absorbing 200 A_{260} units on a 1 × 20 cm BD-cellulose column. The column was then eluted with a 200-ml gradient 0.5–1.0 M ammonium formate, 0–60% ethanol at a flow rate of 0.5 ml/min. Only one uv-absorbing peak was eluted. The ammonium formate was removed by rotary evaporation.

The residue was dissolved in 10 ml of 0.1 M Tris-HCl (pH

[2] Unpublished observations.

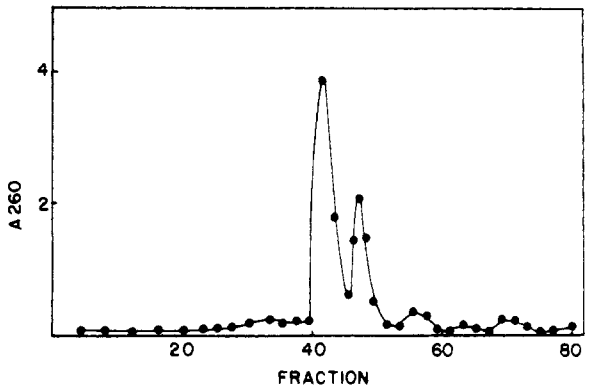


FIGURE 3: Elution profile of the combined material from the major radioactive peak from BD-cellulose chromatography (Figure 2) fractionated on a DEAE-cellulose column.

8.8) and digested with 13 units (Garen and Levinthal, 1960) of *E. coli* alkaline phosphatase at 37° for 6 hr. This solution was diluted to 100 ml with water and applied to a 0.5 × 50 cm DEAE-cellulose column. The column was eluted with a 300-ml gradient of 0.02–0.7 M ammonium formate (pH 5.5) at 0.28 ml/min. Fractions of 4.2 ml were collected. Two uv-absorbing peaks were obtained which were pooled separately and lyophilized (Figure 3).

Complete Digestion of Phenoxyacetylated m^{7}GXC. The first nucleotide peak eluting from DEAE-cellulose was digested to nucleosides by incubation of 25 A_{260} units with 500 units (Uchida and Egami, 1967) of ribonuclease T_2 in 0.4 ml of 0.1 M ammonium acetate (pH 4.7) at 37° for 16 hr. The digest was lyophilized, the residue was taken up in 0.1 M ammonium bicarbonate (pH 8.8), and 3 units of alkaline phosphatase was added. The mixture was incubated at 37° for 6 hr and lyophilized. The residue was dissolved in 0.2 M ammonium formate (pH 4.7) and the nucleosides were separated by chromatography on a 1 × 7 cm column of Dowex 50-X8 resin (200–400 mesh). The column was eluted with a 50-ml gradient of 0.2–1.0 M ammonium formate (pH 4.7), and finally with 130 ml of 1.0 M ammonium formate (pH 4.7).

Phenoxyacetyl-X-nucleoside and the alkaline degradation product of m^{7}G (N^6-ribosyl-$N^{5(6)}$-formyl-5,6-diaminoisocytosine) both appeared in the void volume (separated by paper chromatography, see subsequently), whereas C and m^{7}G were eluted, respectively, at 60 and 140 ml. The nucleosides were identified and quantitated by their spectra, and their purity was verified by chromatography in isobutyric acid–ammonia on microcrystalline cellulose thin-layer plates.

This complete digestion of the first peak eluted from DEAE-cellulose gave m^{7}G and its alkaline degradation product, phenoxyacetyl-X, and C, in a ratio of 1:1.1:1.

Similar treatment of the second peak isolated from the DEAE-cellulose column yielded the alkaline degradation product of m^{7}G, phenoxyacetyl-X, and C, in the ratio of 0.8:0.7:1. No m^{7}G was found in the digest of this peak.

Partial digestion of the first peak from DEAE-cellulose with snake venom phosphodiesterase (Harada *et al.*, 1971) followed by thin-layer electrophoresis in 0.1 M ammonium formate (pH 3.5) yielded m^{7}G as the nucleoside. It can therefore be concluded that two peaks are eluted from DEAE-cellulose because of partial alkaline hydrolysis of m^{7}G during isolation of the trinucleoside diphosphate, the structure of which is m^{7}G-phenoxyacetyl-XC.

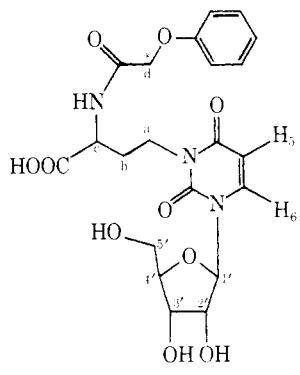
The overall recovery of trinucleoside diphosphate, calculated on the basis of 0.18 mol of X/mol of tRNA (Friedman, 1972), was 30%.

Purification of Phenoxyacetyl-X-Nucleoside. Phenoxyacetyl-X-nucleoside and m^{7}G alkaline degradation product contained in the void volume from the DEAE-cellulose column were separated by paper chromatography, Whatman No. 3MM, in isobutyric acid–ammonia–water–0.1 M EDTA (100:4.2:55.8:1.6, Krebs and Hems, 1952). The nucleosides were eluted with 0.1 N $(NH_4)HCO_3$ at room temperature, and the solutions were lyophilized.

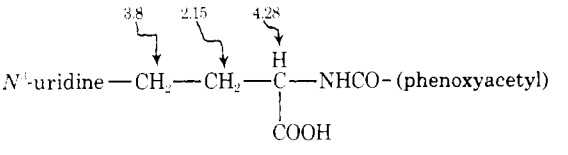
The residue from the phenoxyacetyl-X-nucleoside band was submitted to reverse-phase high-speed liquid chromatography (lc) in 10% *degassed* aqueous methanol on a 91 × 0.32 cm i.d. Poragel PN column. It was found that under these conditions, a satisfactory separation of pyrimidine nucleosides (uridine, cytidine) from purine nucleosides (adenosine, guanosine) could be achieved, the former group being eluted first.

The lc thus obtained is shown in Figure 4. The fastest moving fraction I only showed end absorption in the ultraviolet (uv) region and hence was discarded. Fraction II, which retained the radioactivity, had its uv maximum at 263 nm and minimum at 235 nm (Figure 5) in H_2O, and is thus the X-base fraction. Reinjection of fraction III showed a small lc peak between fractions I and II, and had its $\lambda_{max}(H_2O)$ at 260 nm but was discarded due to its minute quantity.

The amount of material thus purified by lc was unknown but later was estimated to be *ca.* 280 μg based on structure **1.** About one-sixth was left for mass spectral (ms) studies and the remainder was used for 100- and 220-MHz fast Fourier Trans-



1



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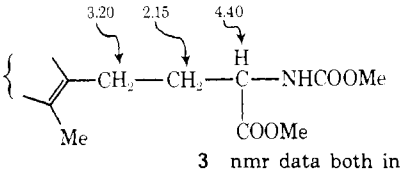
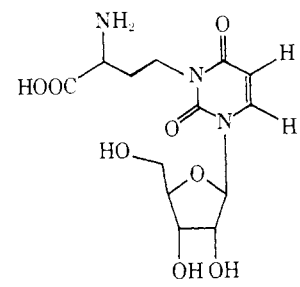


3 nmr data both in D_2O



4

form (FFT) nuclear magnetic resonance (nmr) measurements in D_2O.

Structural Studies

Nuclear Magnetic Resonance Results. About 250 μg of pure phenoxyacetylated X-nucleoside obtained as described above was submitted to nmr measurements.

By using the sealed nmr sampling technique described earlier (Hayashi *et al.*, 1973), it was possible to prepare a virtually 100% D_2O solution as shown by the very weak HDO peak (Figure 6) after 14,399 scans; the 1-ml ampoules of 100% D_2O were from Stohler Isotope Chemicals.

A detailed comparison of the nmr (Figure 6) with those of uridine and phenoxyacetic acid, in conjunction with the high-resolution ms data (Table I) and uv (Figure 5), established the structure of phenoxyacetyl-X-nucleoside as **1**. (There was no uv change between pH 2 and 12.)

Thus, the uv suggested the sample to be a N^3-substituted uridine (Hall, 1971) and in fact it had previously been pointed out that X base could be a modified uracil (Yarus and Barrell, 1971; Yaniv and Barrell, 1971; Friedman, 1973). The ms data (see below) showed that the molecular formula of X-nucleoside itself corresponded to $C_{13}H_{19}N_3O_8$; subtraction of a uridine moiety leaves $C_4H_8NO_2$ for a side chain (to which the phenoxyacetyl moiety is attached). Furthermore, since the ms showed that a free carboxyl group was present in the original sample (*m/e* 650 peak, Table I), the extra group is C_3H_7N and hence is saturated.

The nmr peaks at 7.35, 7.1, and 4.66 ppm (Figure 6) are those derived from the *N*-phenoxyacetyl group. The pair of doublets (J = 8.0 Hz) at 7.76 and 5.87 ppm correspond, respectively, to the 6-H (7.83 ppm) and 5-H (6.03 ppm) doublet of J = 7.8 Hz in uridine.

The 5.85-ppm doublet (J = 4.8 Hz) compares well with the anomeric 1′-H of uridine (5.89 ppm, J = (3.3 Hz). A direct comparison of the multiplet in the 3.6- to 4.4-ppm region ("ribose" region) with that of uridine showed that the multiplet at *ca.* 4.28 and the multiplet centered at *ca.* 3.8 ppm were due to the extra group in X base (see inset, Figure 6); furthermore, another multiplet is present at the high field of 2.15 ppm.

Significantly there is no methyl peak in the nmr. Hence HSP (Friedman, 1972) had reacted with an NH_2 group and not an N-Me group. The extra group C_3H_7N can now be reduced to C_3H_5, and these are the five protons which account for the *ca.* 4.28-, *ca.* 3.80-, and 2.15-ppm peaks. This leads to grouping **2**, the nmr data of which are in remarkably good agreement with those of moiety **3** present in the Y base of $tRNA^{Phe}$ (Nakanishi *et al.*, 1970; Funamizu *et al.*, 1971). The X base 3.80-ppm and Y base 3.20-ppm signals arising from CH_2's attached to the nuclei are the only ones which differ, but this can be ascribed to the attachment of the former to nitrogen as compared to that of the latter to an olefinic carbon.

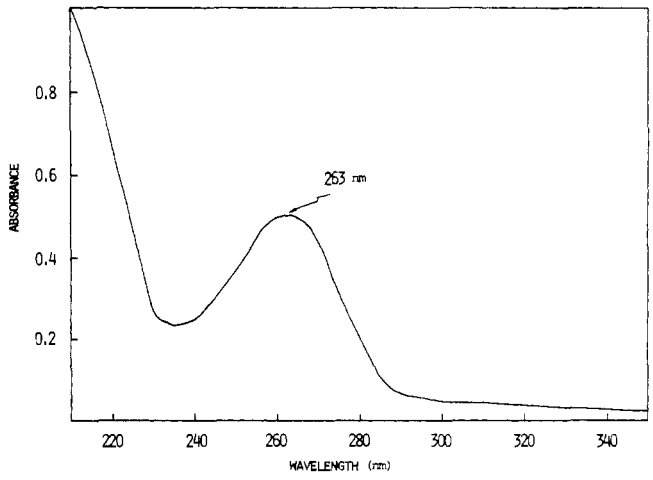


FIGURE 5: Ultraviolet absorption spectrum of **1** in H_2O. The spectrum is the same between pH 2 and 12.

Structure **1** is thus derived and is fully corroborated by the following ms data.

Mass Spectroscopic Results. The sample from **1c** (*ca.* 20 μg) was converted into the trimethylsilyl (Me_3Si) derivative by reacting it with 15 μg of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 4 μl of trimethylchlorosilane in 11 μl of pyridine at room temperature for 4 hr, and evaporation to dryness. The ms data obtained from a high-resolution instrument are summarized in Table I.

Mass spectral analysis of this Me_3Si derivative yielded evidence for the assigned structure even though the derivative had partially degraded to a tritrimethylsilyl derivative before examination. The high mass region was dominated by the ions at *m/e* 767 and 752 which were assigned to the characteristic m → m − CH_3 fragmentation commonly observed with Me_3Si derivatives (Sharkey *et al.*, 1957). Accurate mass data (Table

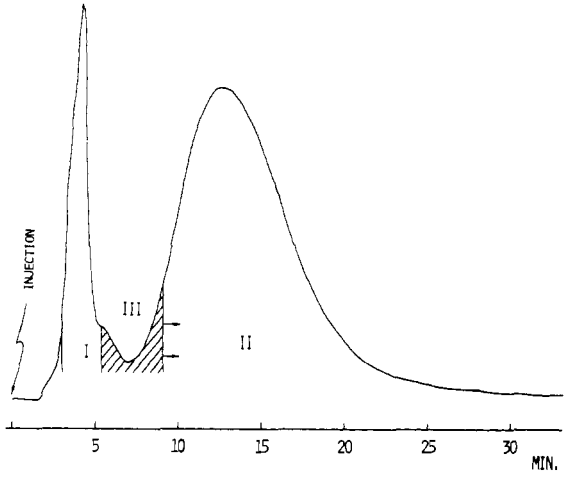


FIGURE 4: High-speed liquid chromatogram of phenoxylacetyl-X-nucleoside **1** (fraction II): Waters ALC 100, Poragel PN column, 91 × 0.32 cm i.d., 0.75-ml/min flow rate, 10% *degassed* aqueous MeOH, total sample *ca.* 300 μg; degassing was essential for removing air bubbles.

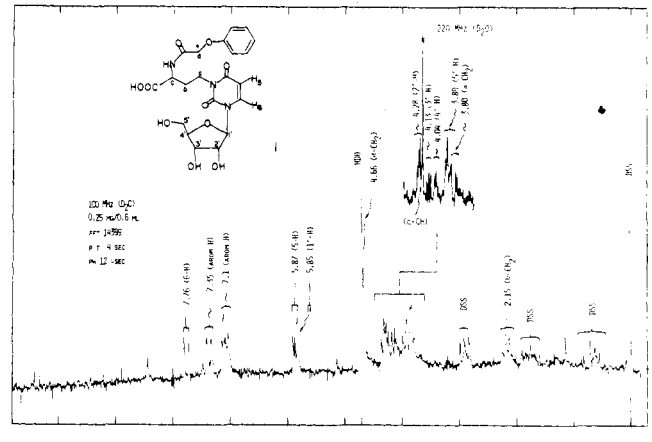
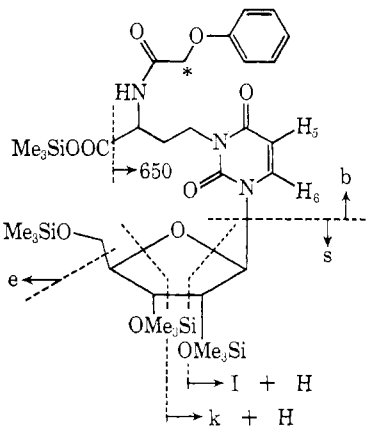


FIGURE 6: Nmr of **1** 0.25 mg/0.6 ml of D_2O (100% D atom): JEOL-PS-100, 100-MHz FFT system, 14,399 scans, repetition time 4 sec, pulse width 12 μsec, ppm from sodium 2,2-dimethyl-2-silopentane-5-sulfonate internal standard. The inset is partial spectrum of a 220-MHz run, Varian, HR-220.

TABLE I: Prominent Ions Observed in the Mass Spectra of Me_3Si Derivative of **1**.[a]

Ion	Composition	Obsd Mass	Calcd Mass	Rel Absorb (%)
m^+	$C_{33}H_{57}N_3O_{10}Si_4$	767.3078	767.3121	20
m − CH_3	$C_{32}H_{54}N_3O_{10}Si_4$	752.2882	752.2886	17
m − Me_3SiOCH_2		664		2
m − CO_2Me_3Si		650		10
k + H		550		7
I + H		534		4
b + HMe_3Si		492		33
b + CH_2O		448		18
b + 2H		420		5
S		349		5
S − Me_3SiOH		259		80
S − CH_2OMe_3Si − H		245		10
S − $2Me_3SiOH$		169		45
e		103		100



[a] The low resolution $m/\Delta m$ = 1000 mass spectrum was determined with an AEI MS-9 mass spectrometer at an ionizing voltage of 70 eV, and source temperature 220° above ambient. The exact mass measurements were carried out at a resolving power $m/\Delta m$ = 10,000 with PFK for reference ions.

I) for these ions indicated the molecular formula $C_{33}H_{57}N_3O_{10}Si_4$ for the Me_3Si derivative. The fragmentation pattern observed in the rest of the spectrum parallels that previously reported for Me_3Si derivatives of other similar nucleosides (McCloskey *et al.*, 1968). Ions at *m/e* 103, 169, 245, 259, and 349 arise from the ribose moiety (McCloskey *et al.*, 1968), while the ions observed at *m/e* 420, 448, 492, 534, 550, and 664 are accounted for by the base or base plus portions of the ribose unit.

Discussion

Gillam *et al.* (1969) had carried out acylation studies of aminoacyl-tRNA and were able to separate the acylated and nonacylated tRNAs on a BD-cellulose column. In the present case the radioactive [^{14}C]HSP-acylating agent reacts with 3-(3-amino-3-carboxy-*n*-propyl)uridine but we cannot conclude that all HSP-reactive *E. coli* tRNAs contain this nucleoside. Since our yield of the phenoxyacetylated nucleoside was 30% of that present in tRNA, it is possible that other modified nucleosides exist in tRNA that are capable of reacting with HSP.

Cedergen *et al.* (1973) have reported that the nucleoside 2-thio-5-(*N*-methylaminomethyl)uridine, present in the anticodon of *E. coli* $tRNA^{Glu}$, can be acylated by benzoic anhydride. This nucleoside may react with HSP as well; however, we had not detected any significant shift in the position of $tRNA^{Glu}$ on BD-cellulose chromatography after the reaction of crude *E. coli* tRNA with HSP (Friedman, 1973). Since 75% of the aminoacyl acceptor activity is lost on benzoylation of this nucleoside in *E. coli* $tRNA^{Glu}$, our methods may not have been sensitive enough to find the acylated product if there was only partial reaction of this rare nucleoside with HSP.

One of us had previously reported the isolation of a phenoxyacetylated nucleoside from both *E. coli* and rat liver tRNA (Friedman, 1972). The nucleoside had similar spectral and chromatographic properties to the one described in this paper and it is very likely that the nucleoside is identical with the present X-nucleoside. However, it did not migrate on thin-layer electrophoresis at pH 9.2, whereas the material isolated in this paper migrated toward the anode with a mobility of 1.2 compared to picric acid. The original samples of nucleoside used in the earlier work are no longer available for direct comparison with material described in this paper. One explanation for this discrepancy may be that the material described previously, which was isolated from phosphodiesterase and alkaline phosphatase digests of tRNA, was esterified on the carboxyl group. Precedents exist for the esterification of nucleosides containing carboxyl groups in yeast tRNA (Baczynskyj *et al.*, 1968; Gray and Lane, 1968; Chheda *et al.*, 1969; Nakanishi *et al.*, 1970) and in mammalian tRNA (Blobstein *et al.*, 1973).

In view of the specific reactivity of the X-base amino acid group with acylating reagents, it is clear that it can be used for other derivatization studies.

In the present studies, the X-nucleoside was isolated as its [^{14}C]HSP derivative **1** (*ca.* 280 μg) by reacting the crude tRNA mixture with [^{14}C]HSP, and structural studies were carried out on this derivative to give the structure **4** for X-nucleoside. On the other hand, Ohashi *et al.* (1974) have derived structure **4** through studies on the nucleoside itself, and have confirmed it by a synthesis.

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References

Baczynskyj, L., Biemann, K., and Hall, R. (1968), *Science 159*, 1481.

Barrell, B. G., and Sanger, F. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 3*, 275.

Blobstein, S. H., Grunberger, D., Weinstein, I. B., and Nakanishi, K. (1973), *Biochemistry 12*, 188.

Cedergen, R. J., Beauchemin, N., and Toupin, J. (1973), *Biochemistry 12*, 4566.

Chheda, G. B., Hall, R. H., Magrath, D. I., Mozejko, J., Schweizer, M. P., Stasiuk, L., and Taylor, P. T. (1969), *Biochemistry 8*, 3278.

Cory, S., Marcker, K. S., Dube, S. K., and Clark, B. F. C. (1968), *Nature (London) 220*, 1039.

Friedman, S. (1972), *Biochemistry 11*, 3435.

Friedman, S. (1973), *Nature (London), New Biol. 244,* 18.
Funamizu, M., Terahara, A., Feinberg, A., and Nakanishi, K. (1971), *J. Amer. Chem. Soc. 93,* 6706.
Garen, A., and Levinthal, C. (1960), *Biochim. Biophys. Acta 38,* 470.
Gillam, I., Blew, D., Warrington, R. C., von Tigerstrom, M., and Tener, G. M. (1968), *Biochemistry 7,* 3459.
Gray, M. W., and Lane, B. G. (1968), *Biochemistry 7,* 3441.
Hall, R. H. (1971), The Modified Nucleosides in Nucleic Acids, New York, N. Y., Columbia University Press, p 160.
Harada, F., Kimura, F., and Nishimura, S. (1971), *Biochemistry 10,* 3269.
Hayashi, H., Nakanishi, K., Brandon, C., and Marmur, J. (1973), *J. Amer. Chem. Soc. 95,* 8749.
Hayes, N. V., and Branch, C. E. K. (1943), *J. Amer. Chem. Soc. 65,* 1555.
Krebs, H. A., and Hems, R. (1952), *Biochim. Biophys. Acta 12,* 172.
Levitt, M. (1969), *Nature (London) 224,* 759.
McCloskey, J. A., Lawson, A. M., Tsuboyana, K., Krueger, P. M., and Stillwell, R. N. (1968), *J. Amer. Chem. Soc. 90,* 4183.
Murao, K., Tanabe, T., Ishii, F., Namiki, M., and Nishimura, S. (1972), *Biochem. Biophys. Res. Commun. 47,* 1332.
Nakanishi, K., Furutachi, N., Funamizu, M., Grunberger, D., and Weinstein, I. B. (1970), *J. Amer. Chem. Soc. 92,* 7617.
Ohashi, Z., Maeda, M., McCloskey, J. A., and Nishimura, S. (1974), Submitted for Publication.
Sharkey, A. G., Jr., Friedel, R. A., and Langer, S. H. (1957), *Anal. Chem. 29,* 770.
Uchida, T., and Egami, F. (1967), *Methods Enzymol. 12,* Part A, 239.
Yaniv, M., and Barrell, B. G. (1971), *Nature (London), New Biol. 233,* 113.
Yarus, M., and Barrell, B. G. (1971), *Biochem. Biophys. Res. Commun. 43,* 729.

Biophysical Studies on the Mechanism of Quinacrine Staining of Chromosomes†

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ABSTRACT: The fluorescence of quinacrine was measured in solution in the presence of interphase chromosomal material (chromatin) and in the presence of chromatin which had been fractionated into extended and condensed regions (euchromatin and heterochromatin). Quinacrine fluorescence is quenched most effectively by the euchromatin fraction, intermediately by unfractionated chromatin, and least effectively by the heterochromatin fractions. These differences are abolished when the fluorescence of quinacrine is measured in the presence of DNA isolated from chromatin and the chromatin fractions. Spectrophotometric titrations indicate that the association constants for quinacrine binding to the various chromatin fractions differ by only a factor of two, and that the number of sites per nucleotide available for quinacrine binding at saturation are nearly identical for all fractions. Circular dichroism spectroscopy suggested that the conformation of the DNA in the euchromatin fraction is most like that of protein-free DNA in aqueous solution ("B"-form DNA) while the DNA in the heterochromatin fractions is partially in the "C" conformation. These results suggest that protein–DNA interactions in chromatin are responsible for the fluorescence patterns observed and that chromosome banding with quinacrine might arise from differences in protein–DNA interactions (and DNA conformation) along the chromatids of metaphase chromosomes.

Caspersson *et al.* (1968) and others have shown that the fluorescent dye quinacrine mustard stains specific regions of chromosomes with a very brilliant intensity, leaving other areas of chromosomes relatively dark. It was originally thought that the linear differentiation of chromosomes into fluorescent bands and poorly staining interband regions was due to the specific alkylation of guanine residues by the mustard function of the dye. The finding that quinacrine itself produces identical banding patterns suggested that alkylation of guanine residues was not the primary mechanism of fluorescence staining of chromosomes (Caspersson *et al.*, 1969). Ellison and Barr (1972) have suggested that enhancement of quinacrine fluorescence might be a function of base ratio, with (A + T)-rich regions fluorescing brightly. Weisblum and de Haseth (1972) and Pachmann and Rigler (1972) have investigated quinacrine fluorescence *in vitro* with a series of natural and synthetic polynucleotides, and found that A·T base pairs are responsible for fluorescence enhancement. Guanine residues were shown to give rise to quenching of quinacrine fluorescence. These data, and several other lines of evidence (Schreck *et al.*, 1973; Lomholt and Mohr, 1971), suggested that the fluorescent bands observed with quinacrine-stained chromosomes are indeed (A + T) rich.

This investigation was undertaken to determine whether quinacrine flourescence in the presence of isolated chromosomal material is due solely to intrachromosomal differences in DNA base composition, or whether DNA–protein interactions in chromatin play a role in producing banding patterns. Chromatin has been fractionated into extended and condensed regions (euchromatin and heterochromatin) (Billing and Bonner, 1972; Bonner *et al.*, 1974; Gottesfeld *et al.*, 1974). It has been suggested that the extended chromatin fraction corresponds to

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