in the ring compounds. It may be that pseudorotation (Westheimer, 1968) plays some part in these reactions.

References

Ashani, Y., Wins, P., and Wilson, I. B. (1972), *Biochim. Biophys. Acta* (in press).

Ellman, G. L., Courtney, K. D., Andres, V., and Featherstone, R. M. (1961), *Biochem. Pharmacol. 7*, 88.

Fukuto, T. R., and Metcalf, R. L. (1965), *J. Med. Chem. 8*, 759.

Hovanec, J. W., and Lieske, C. N. (1972), *Biochemistry 11*, 1051.

Kahan, S. A., and Kirby, A. J. (1970), *J. Chem. Soc. B*, 1172.

Lanham, W. M. (1959), U. S. Patent 2,892,862.

Main, A. R. (1969), *J. Bil. Chem. 244*, 829.

Slater, L. J. (1960), Confluent Hypergeometric Functions, London, Cambridge University Press.

Westheimer, F. H. (1968), *Account Chem. Res. 1*, 70.

Wilson, I. B., Hatch, M. N., and Ginsburg, S. (1960), *J. Biol. Chem. 235*, 2312.

Nuclear Magnetic Resonance and Infrared Identification of the N_1–H and the N_3–H Groups of Pseudouridine†

Achilles Dugaiczyk,*‡ Jasibe P. Cruz,§ and Macario Martinez§

ABSTRACT: From infrared and nuclear magnetic resonance (nmr) measurements on 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine in $CDCl_3$ solution, the tautomeric keto form is established for the two oxygen functions of the uracil ring. In nmr, the two NH signals of the ribose-substituted pseudouridine were identified on the basis of spin decoupling, the N_1–H signal being found at slightly higher fields and being coupled (J = 5.0 Hz) to C_6–H. The chemical shift of both NH groups is concentration dependent. Upon mixing with 5′-*O*-acetyl-2′,3′-*O*-isopropylidenadenosine the two NH resonances of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine are displaced downfield but one (N_3–H) more than the other. In the infrared, the monomeric stretching absorption of N_1–H of the pseudouridine derivative was identified at 3420 cm^{-1}, that of N_3–H at 3390 cm^{-1}. This assignment is based on comparison with N_1- and N_3-substituted uracil derivatives. The molar absorption coefficient of N_1–H (ϵ 314) is twice as large as that of N_3–H (ϵ 154), indicating a greater change in the dipole moment of the molecule caused by the N_1–H stretching vibration. Both NH groups are involved in strong self-association of pseudouridine by hydrogen bonds.

In the structure of tRNAs there are several odd nucleosides, and a search for their function remains one of the more interesting problems in nucleic acid biochemistry. Pseudouridine is one of these. It is present in the loops of the cloverleaf model of tRNA, in tyrosine tRNA it is present in the anticodon (Madison *et al.*, 1966), and very often it is found as a terminal $\psi \cdot A$[1] pair in double-stranded regions at the beginning of a loop. We have recently reinterpreted the ultraviolet (uv) spectrum of 5′-ψMP and established the anti conformation of this nucleotide by nuclear magnetic resonance (nmr) spectroscopy (Dugaiczyk, 1970). The anti conformation of the parent nucleoside has been independently established by Hruska *et al.* (1970).

The most characteristic and odd situation in pseudouridine arises from the two unsubstituted NH functions, both of which have a potential for a biochemical role. These two free NH groups have never been directly observed, let alone distinguished, although their presence was correctly inferred from uv spectroscopy and from the fact that pseudouridine is an isomer of uridine that can be N-substituted by methyls in two different positions (Scannell *et al.*, 1959). It would be highly desirable to be able to observe these two NH groups if one attempted a search for their biochemical function. In the present work we show that they can be experimentally observed and distinguished by nmr and infrared (ir) spectroscopy. Although the present results are obtained on a pseudouridine that has been substituted in the ribose moiety, the conclusions about the parent nucleoside appear to be a logical extension of the conclusions about the derivative.

Materials and Methods

5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine was obtained as described earlier (Dugaiczyk, 1970). The same derivatives of uridine and adenosine were purchased from Sigma Chemical Co., St Louis. $CDCl_3$ (99.8%, NMR Specialties Inc., New Kensington) contains some exchangeable deuterium, which is evident in ir studies by the appearance of peaks around 3600 and 3675 cm^{-1}. For quantitative ir measurements the chloroform-*d* was used immediately after

† From the Chemistry Department, Center for Research and Advanced Studies, National Polytechnic Institute, México 14, D. F., México. *Received December 13, 1971.* We thank the Instituto Nacional de la Investigacion Cientifica for scholarships to J. P. C. and to M. M.

‡ Present address: Department of Biochemistry and Biophysics, University of California School of Medicine, San Francisco, Calif. 94122.

§ Taken in parts from thesis work of J. P. C. and M. M.

[1] Abbreviations used are: ψ, pseudouridine; A, adenosine; 5′-ψMP, pseudouridine 5′-monophosphate.

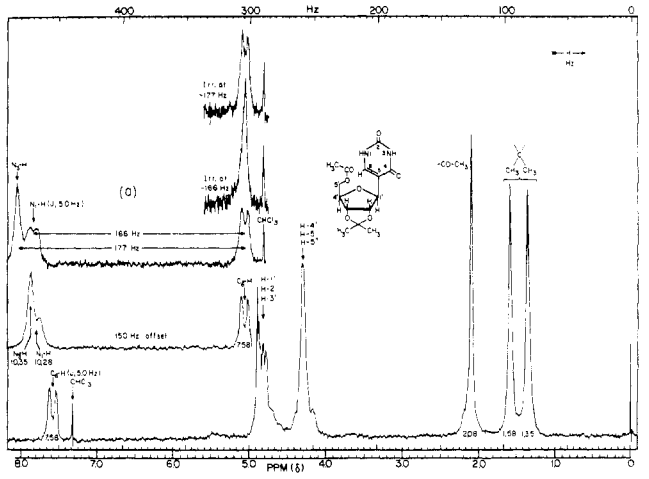

FIGURE 1: A 60-MHz nmr spectrum of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine in $CDCl_3$ solution (0.2 M) measured from internal $(CH_3)_4Si$ reference. Insert a represents part of a spectrum of 0.19 M 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine in the presence of 0.01 M 5′-*O*-acetyl-2′,3′-*O*-isopropylidenadenosine, showing the downfield shift and separation of the two NH resonances, and also the coupling of one of them to the C_6–H proton; temperature 32–33°.

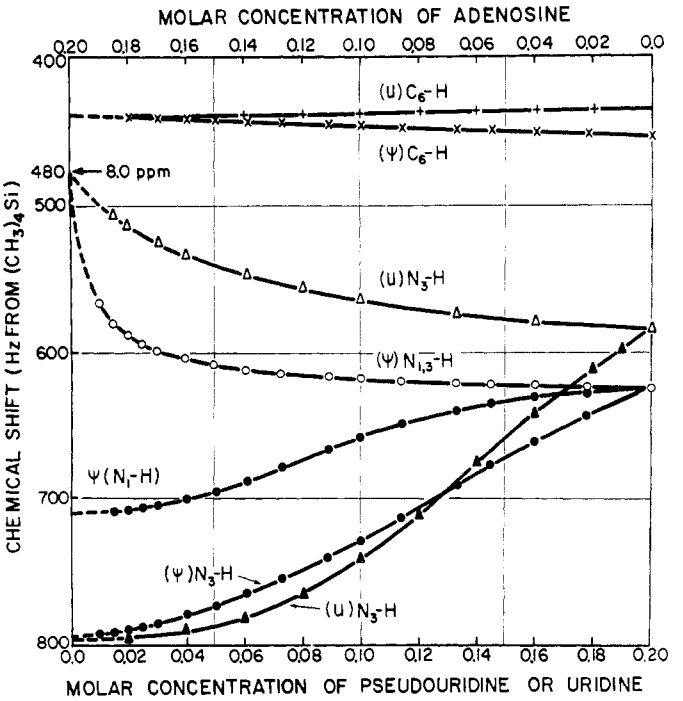

FIGURE 2: Concentration dependence of the chemical shift of the N_1–H and the N_3–H proton of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine in $CDCl_3$ in the absence (○) and in the presence of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenadenosine (●). For comparison, the same is shown for the N_3–H proton of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenuridine in the absence (△) and in the presence of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenadenosine (▲). Also, the chemical shift of the C_6–H protons of both uridine (+) and pseudouridine (×) derivatives is shown in the presence of the substituted adenosine. In the mixing experiments total nucleoside concentration (Ψ + A, or U + A) is constant at 0.2 M; temperature 33–34°.

passing it several times through a silica gel column. In fresh solutions of so purified $CDCl_3$ these peaks are negligible or disappear.

Infrared spectra were recorded with a Perkin-Elmer Model 421 grating spectrophotometer, using cells of 0.2, 1.0, and 5.0 mm. In the region of monomeric NH absorption (3420–3390 cm^{-1}) the slit width was 145–148 μ, respectively. This corresponds to a 2-cm^{-1} spectral slit width at 3400 cm^{-1}. Nuclear magnetic resonance spectra were recorded on A-60 Varian Associates spectrometer, using a sweep width of 500 Hz and sweep times of 250 and 500 sec. Chemical shifts were measured in $CDCl_3$ solutions from internal $(CH_3)_4Si$, with a probe temperature of 32–33°.

Results and Discussion

Nuclear Magnetic Resonance Studies. The 60-MHz spectrum, including spin-decoupling data, of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine in $CDCl_3$ solution are shown in Figure 1. The isopropylidene and acetyl methyl resonances are readily recognized. The peak at 2.08 ppm is in good agreement with the data of Cushley *et al.* (1967), who reported three acetyl resonances at 2.16, 2.14, and 2.10 ppm for 1-(tri-*O*-acetyl-β-D-ribofuranosyl)thymine in $CDCl_3$ solution. As a result of acetylation of the 5′-hydroxyl, the methylene resonance is shifted about 0.55 ppm downfield and overlaps now with the H-4′ signal of the ribosyl moiety. Consequently, all of the aliphatic signals overlap even more than in the spectrum of unsubstituted pseudouridine and cannot be resolved sufficiently on a 60-MHz instrument. However, the two NH signals are sufficiently removed downfield to permit their observation. In solutions of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine alone they overlap partially (Figure 1, main spectrum), being separated by about 0.07 ppm, which is not enough to be resolved on a 60-MHz instrument considering the width of NH signals. Consequently, the graph of their concentration-dependent chemical shift, shown in Figure 2, represents both the N_1–H and N_3–H shifts, as measured at the maximum of their common resonance peak. However, upon addition of even small amounts of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenadenosine to a solution of the same pseudouridine derivative, both the NH resonances are shifted downfield, but one more than the other, which causes their complete separation. Insert a in Figure 1 shows such a situation.

The two NH signals were identified in a double-resonance experiment, in which the C_6–H 5.0-Hz doublet[2] at 7.58 ppm could be made to collapse to a singlet by simultaneously irradiating downfield from C_6–H at one of the two NH frequencies. Irradiating at the other NH frequency was without effect on the splitting of the C_6–H signal (insert a in Figure 1). This permits assignment of the signal at the higher field to N_1–H because of its coupling to the neighboring C_6–H, leaving the further downfield shifted signal to N_3–H. The chemical shifts of both NH resonances are concentration dependent, being shifted upfield upon dilution. It seems interesting to note that the 5.0-Hz coupling between C_6–H and N_1–H can be observed only in solution of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine alone or in the presence of small amounts of 5′-*O*-acetyl-2′,3′-*O*-isopropylideneadenosine. It disappears upon further addition of the substituted adenosine; both the N_1–H doublet (proton participating in hydrogen bonding) and the C_6–H doublet (proton not participating

[2] It is really a pair of doublets, the other small (0.8 Hz) allylic coupling to H-1′ was better shown by Hruska *et al.* (1970) on a 100-MHz instrument, under conditions (D_2O) where no coupling to the N_1–H proton could be observed because of exchange of the latter.

in hydrogen bonding) collapse to singlets and there is no broadening of either resonance. The magnitude of this coupling to C_6–H allows location of one of the two dissociable protons in the neighboring N_1 position, our pertinent infrared data allow location also of the other proton in the N_3 position, establishing thus the correct tautomeric structure as the keto form for both carbonyl functions of pseudouridine.

On mixing with 5′-*O*-acetyl-2′,3′-*O*-isopropylidenadenosine, the two NH resonances of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine are displaced downfield but one more than the other (Figure 2). At above tenfold molar excess of the adenosine derivative, the N_3–H resonance of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine reaches the same plateau at about 796 Hz (13.27 ppm) as does the N_3–H resonance of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenuridine when completely hydrogen bonded to 5′-*O*-acetyl-2′,3′-*O*-isopropylidenadenosine. Thus, for the N_3–H resonance of the derivatives of uridine and pseudouridine, the chemical shift difference ($\Delta\delta$) between the pyrimidine monomer and its complex with adenosine is the same for both isomeric nucleosides and equals about 316 Hz (5.27 ppm). However, the N_1–H resonance of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine is displaced downfield only to about 712 Hz (10.87 ppm) when completely hydrogen bonded to the adenosine derivative, giving a chemical shift difference of 232 Hz (3.87 ppm).

With respect to the aromatic C–H protons, the following may be noted. In 5′-*O*-acetyl-2′,3′-*O*-isopropylidenuridine, C_6–H, being far removed from the hydrogen bond at N_3–H, shows almost no change as a result of complexing with the substituted adenosine, while the same C_6–H proton in 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine is shifted upfield by 14 Hz (0.22 ppm) under the same conditions (Figure 2). This reflects the involvement of the neighboring N_1–H (as a donor) of pseudouridine in a hydrogen bond. Due to partial withdrawal of this N_1–H proton from the uracil ring, the electron density is increased at N_1 and the vicinal C_6–H, shielding the latter proton. A complementary situation with respect to alteration in ring currents prevails in 5′-*O*-acetyl-2′,3′-*O*-isopropylideneadenosine and can be observed at C_8–H (not shown). On complexing with the pseudouridine derivative, this proton is also shifted 14 Hz (0.22 ppm), but downfield, reflecting the involvement of the neighboring N_7 nitrogen (as an acceptor) in a hydrogen bond. This would tend to decrease the electron density at N_7 and the vicinal C_8–H, deshielding the latter proton. It is interesting to note that these changes of charge distribution in the pyrimidine and purine bases are quantitatively equal, as observed by nmr, but opposite. Similar effects are not observed at C_2–H of the adenine ring, despite the fact that the neighboring N_1 nitrogen acts also as an acceptor in hydrogen-bond formation. This is probably because the pyrimidine ring of adenine acts both as a hydrogen-bond donor and acceptor, cancelling each other's effect on the ring current.

Infrared Studies. The ir spectrum of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine is shown in Figure 3; its uv spectrum was earlier shown to be essentially the same as the uv spectrum of unsubstituted pseudouridine (Dugaiczyk, 1970).[3] The present demonstration of a lack of an alcoholic OH signal

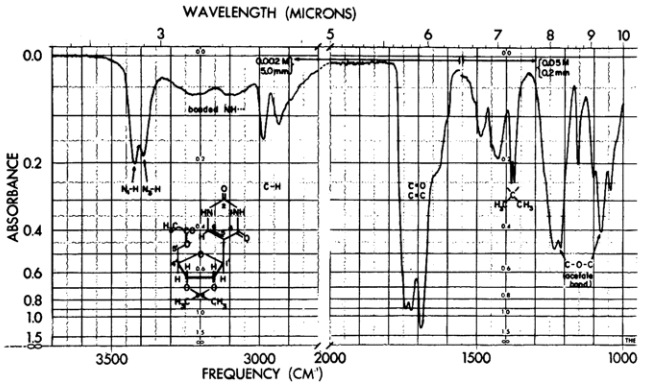

FIGURE 3: Infrared spectrum of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine in $CDCl_3$ solution at concentrations and path lengths as indicated; temperature 30°.

in the infrared 3620- to 3660-cm^{-1} region and the nmr spectrum (Figure 1) further confirm the assigned structure of the derivative on which our earlier claim was based (Dugaiczyk, 1970), that it is not the 5′-hydroxyl and an intramolecular 4–5′ hydrogen bond in free pseudouridine that is responsible for its uv spectum (pH 12), as proposed by Chambers (1966).

The more acidic a hydroxyl, the longer the O–H bond, the lower its stretching vibration, and consequently the longer the wavelength of its infrared absorption. Carboxylic or enolic (monomeric) hydroxyls absorb at a wavelength as long as 2.82 μ (3550 cm^{-1}) but not any longer (Nakanishi, 1966). The two bands (Figure 3) at 2.92 μ (3420 cm^{-1}) and 2.95 μ (3390 cm^{-1}) thus indicate that the two dissociable protons are on both nitrogens and not oxygens of the uracil moiety. Together with our present nmr data, this establishes the keto form for pseudouridine.

In the present study we will be concerned only with the two NH signals seen at 3420 cm^{-1} (N_1–H) and at 3390 cm^{-1} (N_3–H). This assignment is based on spectra in $CDCl_3$ of 1-cyclohexyluracil and of 5′-*O*-acetyl-2′,3′-*O*-isopropylideneuridine, both of which exhibit only one signal at 3390 cm^{-1} (N_3–H). 3-Methyluracil, on the other hand, shows only the peak at 3420 cm^{-1} (N_1–H) in $CDCl_3$ solution. It has been earlier observed (Shugar and Fox, 1952) on 1- and 3-methyluracils that a proton dissociates easier from N_3 than from the N_1 position of the uracil ring. The present finding of the lower stretching vibration of the N_3–H bond (longer and weaker) accounts well for the observed difference in dissociation.

Figure 4 shows the concentration dependence of the spectrum of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine in the region of free and hydrogen-bonded NH absorption, demonstrating the increasing self-association. Without prior correction for the mutual overlapping of the absorption bands, the apparent association constants (K_{app}) and the apparent molar extinction coefficient (ϵ_{app}) (Table I) have been computed from the slopes and intercepts, obtained by plotting E *vs.* C_0/E, as outlined by Kyogoku *et al.* (1967). Although their equations have been developed for dimerization, it is assumed here that at the low concentrations of 10^{-2}–10^{-3} M this restriction is not too seriously violated in our present measurements.

The infrared spectra show that the NH absorption bands are not completely resolved and do augment each other. Consequently, the apparent molar extinction coefficients are obviously higher from the true coefficients. The easiest to correct is the influence of the broad band at 3220 cm^{-1} on the free

[3] There are two printing errors in the above work. In Table I, the isosbestic point for pseudouridine (221.5) should read 211.5, and that for isopropylidenepseudouridine (221) should read 211, as correctly presented in Figures 1 and 2. Also, the unit for ϵ in Figures 1, 2, 3, and 6 is incorrectly given as $mmole^{-1}\ cm^{-1}$, instead of l. $mmole^{-1}\ cm^{-1}$.

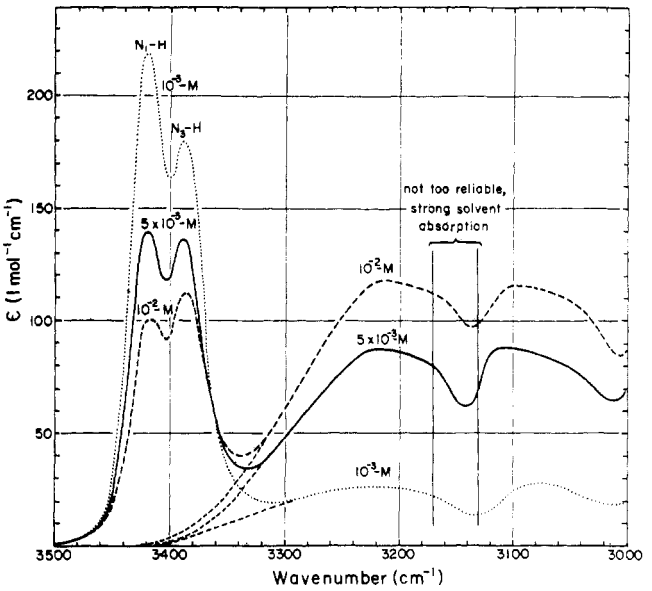

FIGURE 4: Concentration dependence of the infrared apparent molar extinction coefficient in the region of free and hydrogen-bonded NH-stretching absorptions of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine. Measurements in $CDCl_3$ solution at 30°.

TABLE I: Association Constants (l. mole^{-1}) and Molar Extinction Coefficients (l. mole^{-1} cm^{-1}) in $CDCl_3$ at 30°.

	5′-*O*-Acetyl-2′,3′-*O*-isopropylidene-pseudouridine at 3420 cm^{-1} (N_1–H)	3390 cm^{-1} (N_3–H)	5′-*O*-Acetyl-2′,3′-*O*-isopropyliden-uridine at 3390 cm^{-1} (N_3–H)	1-Cyclohexyl-uracil at 3390 cm^{-1} (N_3–H)
K_{app}	439	104		
ϵ_{app}	357	215		
K	472	71	12, 10.4[a]	4.9, 4.7[b]
ϵ	314	154	143, 150[a]	143, 135[b]

[a] Values from Krueger *et al.* (1968). [b] Values from Kyogoku *et al.* (1967).

N_3–H absorption at 3390 cm^{-1}. This has been done by extrapolation of the broad band as shown in Figure 4. It is further estimated that each NH absorption band (measured at its maximum) is augmented by its neighboring band by 20% of this neighboring band. This estimate is based on the following. In 1-cyclohexyluracil and in 5′-*O*-acetyl-2′,3′-*O*-isopropylideneuridine, neither of which has any interference of neighboring bands in the 3400-cm^{-1} region of their spectra, the molar extinction coefficient for N_3–H is found to be 143 (Table I). This value is assumed to be the same for N_3–H in pseudouridine, and so its apparent increase (ϵ_{app} 215) is due to the close N_1–H band at 3420 cm^{-1}, which is augmenting the measurements of the N_3–H band at 3390 cm^{-1}. This increase amounts to 215 − 143 = 72, which is 20.2% of 357, the apparent molar extinction coefficient of the neighboring N_1–H band (Table I). Secondly, the N_3–H absorption of 1-cyclohexyluracil or 5′-*O*-acetyl-2′,3′-*O*-isopropylidenuridine has a maximum at 3390 cm^{-1}, but a residual absorption of its shoulder at 3420 cm^{-1} (where the N_1–H maximum is found) represents again 20% of its maximum absorption at 3390 cm^{-1}. Therefore, at any given concentration of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine, the absorption of one NH peak has been diminished by 20% of the absorption of the other accompanying NH peak, and the so corrected values regraphed again as E *vs.* C_0/E. Table I summarizes the results of K and ϵ. It is estimated that they represent an accuracy of some ±15%, taking into account that quantitative infrared measurements are not very accurate and considering the corrections and assumptions that were made. Some caution is required considering the association constants of pseudouridine, calculated from the present data, because of the uncertainty about the acetyl group participating as hydrogen acceptor. More significant seems the twofold difference in the molar extinction coefficient between the two NH groups, implying a greater change in the dipole moment of the molecule caused by the N_1–H vibration.

It seems unfortunate that the infrared and nmr data could not be correlated well; the self-association of pseudouridine could be observed only by infrared, whereas its association with adenosine only by nmr spectroscopy. The difference between N_1–H and N_3–H during self-association of pseudouridine is readily noted in the infrared 3400-cm^{-1} region when comparing spectra at two different concentrations (Figure 4). This difference in behavior could not be observed by nmr spectroscopy because the two NH resonances are too close (Figure 1; 150-Hz offset), and they never separate in solutions of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine throughout the entire concentration range studied. Furthermore, the infrared results were obtained at concentrations below 10^{-2} M, so they are in the extrapolated region of the chemical shift curves of Figure 2, where no more nmr measurements could be made. On the other hand, the difference between N_1–H and N_3–H during association of pseudouridine with adenosine is readily noted by nmr, whereas by infrared spectroscopy it could not be observed because the symmetric NH_2-stretching vibration of adenosine is found at 3415 cm^{-1} (Hamlin *et al.*, 1965), superimposed on both NH-stretching absorption of pseudouridine. In nmr, even a small addition of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenadenosine to a solution of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine causes immediate separation of the two NH resonances (insert a in Figure 1) because the N_3–H signal experiences a larger downfield shift upon mixing with the adenosine derivative.

Although data obtained by one spectroscopic method could not be confirmed by the other because of technical difficulties, the results obtained by the two methods are complementary, each showing the behavior of pseudouridine under different conditions. The different behavior of the two NH groups of pseudouridine during self-association and during interaction with adenosine can be explained by two factors: (1) differences in length and ionic character between the two N–H bonds; (2) different hydrogen-bond acceptors during self-association of pseudouridine (carbonyl oxygens of the uracil ring) and during association with adenosine (ring nitrogens of the adenine ring). This difference in electronic and/or geometric complementarity between the two aromatic rings of uracil and adenine must account for the specific interaction of the two N–H functions of pseudouridine.

The present identification of the infrared N_1–H and N_3–H absorptions and the assignment of their nmr resonances should be useful in future investigations concerning a bio-

chemical function of pseudouridine. Both of its NH groups are capable of hydrogen bonding with adenosine, but it remains an open question if their biochemical function in tRNA is the same as observed in the present study.

References

Chambers, R. W. (1966), *Progr. Nucl. Acid Res. Mol. Biol.* *5*, 349.

Cushley, R. J., Watanabe, K. A., and Fox, J. J. (1967), *J. Amer. Chem. Soc.* *89*, 394.

Dugaiczyk, A. (1970), *Biochemistry* *9*, 1557.

Hamlin, R. M., Jr., Lord, R. C., and Rich, A. (1965), *Science* *148*, 1734.

Hruska, F. E., Grey, A. A., and Smith, I. C. P. (1970), *J. Amer. Chem. Soc.* *92*, 4088.

Krueger, U., Breuer, H., Abdel Kerim, F. M., Perkampus, H.-H. and Scheit, K. H. (1968), *Z. Naturforsch. B* *23*, 1360.

Kyogoku, Y., Lord, R. C., and Rich, A. (1967), *J. Amer. Chem. Soc.* *89*, 496.

Madison, J. T., Everett, G. A., and Kung, H. (1966), *Science* *153*, 531.

Nakanishi, K. (1966), Infrared Absorption Spectroscopy, San Francisco, Calif., Holden-Day.

Scannell, J. P., Crestfield, A. M., and Allen, F. W. (1959), *Biochim. Biophys. Acta* *32*, 406.

Shugar, D., and Fox, J. J. (1952), *Biochim. Biophys. Acta* *9*, 199.

Subunit Structure of Glucose Oxidase from *Aspergillus niger*†

James J. O'Malley* and Judy L. Weaver

ABSTRACT: Glucose oxidase from *Aspergillus niger* is a branched protein containing 16% carbohydrate and two flavin-adenine dinucleotide cofactors per molecule. The properties of the native enzyme have been well studied, but there is relatively little known about the denatured enzyme. In this study we examined the structural properties of the denatured enzyme, using the techniques of light scattering, sedimentation velocity, intrinsic viscosity, and amperometric titration to determine whether glucose oxidase is a single or multichain (subunit) enzyme. The results of these studies indicate that glucose oxidase is a subunit enzyme. The native enzyme has a molecular weight of 160,000 and light-scattering measurements in 6 M guanidine hydrochloride indicate the denatured enzyme has the same molecular weight as the native enzyme. However, chemical reduction with β-mercaptoethanol of the enzyme's two disulfide bonds results in the formation of molecular species with molecular weights of 80,000. Schlieren patterns taken during sedimentation velocity runs show single sharp sedimentation peaks for both the denatured and the denatured-reduced species. These data are consistent with a model for glucose oxidase in which the enzyme is composed of two polypeptide chains, equal in molecular size, which are covalently linked by disulfide bonds.

Glucose oxidase is a flavin containing glycoprotein which catalyzes the oxidation of glucose to gluconic acid (Müller, 1928). The enzyme isolated from *Aspergillus niger* contains approximately 16 wt % carbohydrate and these sugars are thought to be present as oligomeric polysaccharides covalently attached to the polypeptide chain *via* serine, glutamic acid, and aspartic acid residues (Pazur *et al.*, 1963, 1965). The native enzyme is also known (Franke and Deffner, 1939; Pazur and Kleppe, 1964) to contain two molecules of flavin-adenine dinucleotide (FAD).[1] These flavin cofactors are responsible for the oxidation–reduction properties of the enzyme and available evidence suggests they are firmly bound, but not covalently linked, to the polypeptide portion of the enzyme. Denaturation generally results in flavin release from the enzyme and, concomitantly, changes in the absorption and fluorescence emission spectra of the flavin are observed (Swoboda and Massey, 1966).

Many measurements of the molecular weight of the native enzyme have been made and, while the reported values range from 150,000 (Pazur and Kleppe, 1964) to 186,000 (Swoboda and Massey, 1965), most of the data fall in the range of 155,000 ± 5000. In view of the interest in glucose oxidase, it is surprising that not until recently (O'Malley and Weaver, 1971) was the molecular weight of the denatured enzyme measured and the possibility of subunit structure seriously considered. The present study, using glucose oxidase isolated from *A. niger* and techniques such as light scattering, sedimentation velocity, and intrinsic viscosity, points to a subunit structure for this enzyme. The data are consistent with a model in which the native enzyme is composed of two polypeptide chains equal in molecular size and covalently linked by disulfide linkages.

Experimental Section

Materials. Glucose oxidase (EC 1.1.3.4) from *A. niger* was obtained from Worthington Biochemicals as a highly purified, salt-free lyophilized powder with a specific activity of 136 IU/mg. The following materials were purchased from sources as indicated: Gdn·HCl and urea from Mann; guanidine thiocyanate from K & K; dithiothreitol from Pierce;

† From the Research Laboratories, Xerox Corporation, Webster, New York 14580. *Received April 12, 1972.* A preliminary report of this work was presented at the 162nd National American Chemical Society Meeting in Sept 1971 at Washington, D. C.

[1] Abbreviations used are: FAD, flavin-adenine dinucleotide; Gdn·HCl, guanidine hydrochloride; βME, β-mercaptoethanol.