Biochemistry

Volume 11, Number 2 January 18, 1972

A Nuclear Magnetic Resonance Study of the Influence of Aqueous Sodium Perchlorate and Temperature on the Solution Conformations of Uracil Nucleosides and Nucleotides*

Thomas Schleich,† Barry J. Blackburn,‡ Roy D. Lapper,§ and Ian C. P. Smith¶

ABSTRACT: Complete high-resolution proton magnetic spectral analyses for uridine, uridine 3′-phosphate, β-pseudouridine, β-pseudouridine 3′-phosphate, and deoxyuridine indicate that changes in temperature and/or the addition of sodium perchlorate, a potent biopolymer conformation destabilizing salt, result only in minimal changes in the solution conformations. The conformations of the above nucleosides and nucleotides are all characterized by: (a) a sugar–base torsional angle corresponding to an anti conformation; (b) a ribose ring which is a rapid time-average blend of conformers; (c) an exocyclic CH_2OH group which favors the gauche,gauche rotamer, except for deoxyuridine where the rotamers gauche,-gauche and gauche,trans have approximately equal probabilities; (d) in the 3′ nucleotides, an angle of approximately 50° between $H_{3'}C_{3'}O_{3'}$ and $C_{3'}O_{3'}P$ (the cis conformation is assigned to zero angle) as judged from the phosphorus–hydrogen coupling constants. With the exception of the dynamic processes in b, c, and d, the conformations in solution follow the general preferences indicated by X-ray studies of single crystals of similar compounds. No correlation of the small changes in conformation could be established with alterations in water structure due to thermal and solute-induced perturbations. The fact that the nucleoside and nucleotide conformations are insensitive to thermal and solute perturbation suggests that the site for the destabilization of polynucleotide conformation is at the level of base–base stacking and interbase hydrogen-bonding interactions. Furthermore the conclusions of this study and our interpretation of the nmr data of Kreishman and Chan (*Biopolymers 10*, 159 (1971)) for polyuridylic acid and uridine 5′-phosphate suggest that the relatively large characteristic ratio for polyuridylic acid originates in the rotational restrictions found in the monomers themselves.

Polynucleotide structures, like the fibrous and globular proteins, are susceptible to an enhanced ease of thermal unfolding or disruption of the "native" conformation in the presence of neutral salt denaturants in aqueous solution. The salt-induced depressions of the transition temperatures for unfolding of collagen, ribonuclease, and calf thymus DNA are approximately the same (the midpoints of the thermal transition span a 70° range), having a value of approximately 10°/mole of added sodium perchlorate. These observations suggest that the salt denaturation phenomenon transcends the chemical composition details of a particular biopolymer and that a common mechanism of action exists. Conceptually, the effects of salt on biopolymer conformational stability, which usually but not always follow the traditional ionic hierarchy, the Hofmeister series, can be viewed as arising in two ways: (a) by a "direct" mechanism, *i.e.*, preferential binding of the ionic component to a portion of the macromolecule; (b) by an "indirect" mechanism, *i.e.*, mediated by ion-induced changes in water structure which in turn modulate the energetics of some macromolecular stabilizing interaction such as hydrophobic interaction of apolar moieties. The phenomenology and interpretations of neutral salt effects on biopolymer structures have been reviewed (Tanford, 1968, 1970; von Hippel and Schleich, 1969a,b).

The unique molecular interactions (apart from the backbone electrostatic free-energy contribution) which serve to define and stabilize polynucleotide structures are the base–base stacking and hydrogen-bonding interactions and the

* From the Biochemistry Laboratory, National Research Council of Canada, Ottawa, Canada K1A OR6. *Received July 8, 1971.* NRCC Publication Number 12347.

† Visiting National Research Council Summer Scientist, 1970. Permanent address: Division of Natural Sciences, University of California, Santa Cruz, Calif. 95060. This investigation was supported, in part, by Research Grant GB-19503 from the National Science Foundation (to T. S.).

‡ NRCC Postdoctoral Fellow, 1968–1970. Present address: Department of Chemistry, University of Winnipeg, Winnipeg, Can.

§ NRCC Postdoctoral Fellow, 1970–1972.

¶ To whom to address correspondence.

nonbonded interactions of the ribose ring. A number of workers have shown (Donohue and Trueblood, 1960; Haschemeyer and Rich, 1967; Sundaralingam, 1969; Lakshminarayanan and Sasisekharan, 1969a) that the torsional angle which defines the orientation of the nearly planar purine or pyrimidine ring system relative to the furanose ring is crucially dependent on the type and degree of sugar ring puckering. Sundaralingam (1969) has collected evidence which shows that in the solid state the conformation of the furanose ring system in both the monomer and the polymer are remarkably similar (see also, Arnott, 1970). Recently Kreishman and Chan (1971) have extracted the nuclear magnetic resonance (nmr) parameters from the 220-MHz spectrum of polyuridylic acid. Although they did not point this out, the data show that the ribose conformation of polyuridylic acid is remarkably similar to those of 5′- and 3′-uridylic acid. Six torsional angles (of restricted values) define the conformation of the polynucleotide backbone chain and these are controlled in large part by the prevailing nonbonded interactions in the furanose ring system.[1] The importance of restricted rotations about the bonds defining the polynucleotide backbone has support from both conformational energy calculations and X-ray structure determinations, as well as from experimental polymer conformation measurements. The latter has emerged from the light-scattering studies of Felsenfeld and coworkers (Eisenberg and Felsenfeld, 1967; Inners and Felsenfeld, 1970) on synthetic polynucleotides in aqueous solutions at the so-called θ point (ideal polymer solution conditions). In particular, these workers have shown that the conformational dimensions of the polynucleotide backbone are identical under comparable conditions of stacking for different polynucleotides, thereby suggesting that the nature of the base is relatively unimportant in defining the conformational features of the polynucleotide backbone. Even in the absence of stacking the data are indicative of a highly extended but conformationally restricted polymer chain. Polynucleotide dimensions also appear to be essentially independent of the nature of the salt used to attain ideal solution properties ($LiClO_4$ *vs.* NaCl) (G. Felsenfeld, personal communication) when compared at the same extent of stacking.

Prestegard and Chan (1969) have interpreted their nmr data to indicate that *both* the sugar–base torsion angle and the nature of ring puckering of uridine and 5′-UMP are sensitive in a Hofmeister fashion to neutral salt denaturants, but that 3′-UMP and deoxyuridine furanose ring systems were sufficiently rigid to withstand the action of these additives. The sugar–base torsional angles of the latter two still remained sensitive to the presence of salt.

In order to improve our understanding of the mechanism of salt-induced polynucleotide denaturation, we initiated a similar series of experiments, confining our study to uridine, uridine 3′-phosphate, β-pseudouridine, β-pseudouridine 3′-phosphate, and deoxyuridine.[2] In view of the extensive data obtained by Prestegard and Chan (1969), only one salt and concentration, 2 M sodium perchlorate was used. Solution temperature is introduced as an additional variable. Our conclusions alter the previously established picture of how neutral salts affect nucleotide, nucleoside, and polynucleotide conformations.

Experimental Section

U, 3′-UMP, β-ψ, 3′-β-ψMP, and dU were the best grades commercially available and were used as received. Sodium perchlorate (anhydrous) was obtained from G. F. Smith. The two internal references employed in this work, DSS and TSP were obtained from E. Merck, Germany, and Merck, Sharpe and Dohme, Canada, respectively.

Nucleosides (and -tides) were made up in D_2O, or in 2 M $NaClO_4$ solutions in D_2O, at a concentration of 0.1 M, containing either 0.1 M DSS or TSP as an internal reference. The pD was adjusted to 6.8 ± 0.1 (nucleosides) or 7.2 ± 0.1 (nucleotides), and the solutions were lyophilized three times from D_2O. Spectra were obtained using the Varian HA-100, XL-100, HR-220 (Canadian 220 MHz NMR Center, Ontario Research Foundation, Sheridan Park) nmr spectrometers. Line positions were measured relative to the internal standard following customary procedures. Proton spin-decoupling and INDOR experiments were performed on the XL-100; ^{31}P decoupling was performed on the HA-100 using an NMR Specialties decoupler.

Results and Discussion

Spectral Assignment and Interpretation. Complete proton magnetic resonance spectra were taken at 100 and 220 MHz. Assignments were made by references to earlier work (Blackburn *et al.*, 1970; Hruska *et al.*, 1970a,b), by ^1H–^1H decoupling, and by ^1H–^{31}P decoupling. The $H_{2'}$ resonance of dU, due to the hydrogen cis to the OH at 3′, is assigned to the low-field peaks in accord with the deshielding effect of a cis hydroxyl group (see Hruska *et al.*, 1970b).

Spectral analysis was performed using standard methods (Pople *et al.*, 1959) and the computer programmes LAOCOON II and LAOCNPLT. To avoid the possibility of false solutions spectra were run at two different frequencies and the parameters extracted from spectra at one frequency were used to simulate spectra at the other frequency. Figures 1 and 2 show the experimental and calculated nmr spectra for the ribose region of 3′-β-ψMP and deoxyuridine. Uridine is not shown but may be found in Blackburn *et al.* (1970). Spectra taken at high temperatures (*ca.* 80°) and in 2 M salt resembled superficially those in D_2O alone at ambient temperatures, and thus no problems were encountered in analyzing "perturbed" spectra once the basic spectral parameters were determined. Table I contains the results of our spectral analysis for uracil nucleosides and nucleotides under a variety of conditions. The implications of these results will be discussed in subsequent sections.

Sugar–Base Torsion Angle. The sugar–base torsion angle (the dihedral angle formed by the intersection of a pyrimidine base plane with the plane defined by $O_{1'}$, $C_{1'}$, and N_1 of β-pyrimidine glycosides) typically falls in the range of −10 to −90° (Sundaralingam, 1969); this conformation is referred to as "anti" in the Donohue–Trueblood convention, and has the 5 and 6 positions of uracil over the ribose ring. The estimation of sugar–base torsional angles for nucleosides and 3′-substituted nucleotides by nmr is somewhat more indirect than for 5′ nucleotides where phosphate anion electrostatic effects on the base proton resonances offer a convenient measure. One procedure for nucleosides and 3′-substituted nucleo-

[1] These six angles define the magnitude of rotation about: P–$O_{5'}$, $O_{5'}$–$C_{5'}$, $C_{5'}$–$C_{4'}$, $C_{4'}$–$C_{3'}$, $C_{3'}$–$O_{3'}$, and $O_{3'}$–P.

[2] Abbreviations used are: U, uridine; dU, deoxyuridine; 3′-UMP, uridine 3′-phosphate; β-ψ, β-pseudouridine, 3′-β-ψMP, β-pseudouridine 3′-phosphate; poly(rA), polyadenylic acid; poly(rU), polyuridylic acid; UpU, uridylyl-3′,5′-uridine; ApA, adenylyl-3′,5′-adenosine; DSS, sodium 3-trimethylsilylpropanesulfonate; TSP, sodium 3-trimethylsilylpropane carboxylate-d_6; D_2O, deuterium oxide; HDO, hydrogen deuterium oxide.

tides is based on the following argument. The uracil H_6 resonance sustains a 0.3-ppm downfield shift when bound to a D-ribose moiety in a β-glycosidic linkage (Prestegard and Chan, 1969). This alteration in chemical shift has been rationalized as arising from the combined effect of magnetic anisotropy and electric field gradients due to the proximity of the furanose ether oxygen ($O_{1'}$) to the H_6 proton of the uracil moiety. Inspection of molecular models suggests that as the sugar–base torsion angle is made more negative, the H_6 proton swings away from the ribose ring ether oxygen. The calculations of Prestegard and Chan reveal that at a torsional angle of $-60°$ *both* electric field and magnetic anisotropic effects become negligible. Thus *if the aforementioned reasoning is correct* we must associate upfield shifts in the H_6 pyrimidine resonance with increasingly negative sugar–base torsional angles. Such effects have been demonstrated by Smith *et al.* (1969) for U, 5′-UMP, UpU, and UpUpU with increasing temperature. The data contained in Table I reveal that perturbation of an aqueous sample of U, β-ψ, 3′-UMP, 3′-β-ψMP, or deoxyuridine by either increasing temperature or by the addition of electrolyte denaturant ($NaClO_4$) results in an upfield shift in the H_6 resonance, suggesting that perturbation by either temperature or salt increases, in a negative direction, the sugar–base torsional angle. The magnitude of the change cannot be estimated quantitatively. Temperature, however, seems to be more potent in this regard than 2 M $NaClO_4$. We must emphasize, however, that conformational calculations and space-filling molecular model studies both indicate that the sugar–base torsional angle is crucially dependent on the mode and nature of the furanose ring buckling, which may also be temperature dependent.

Recently it has been pointed out that the chemical shifts of the ribose hydrogens are sensitive to the sugar–base torsion angle in pyrimidine nucleosides (Blackburn *et al.*, 1970; Dugas *et al.*, 1971).[3] This is due to the considerable magnetic anisotropy of the keto group at the 2 position in the pyrimidine base. This effect also depends on the nature of the ribose ring puckering, which can alter the distance between the ribose hydrogens and the 2-keto group. Because the ribose ring spin–spin coupling constants (see next section) indicate that the compounds studied here all have essentially the same ribose conformations, any effect of the keto group anisotropy could be clearly inferred from the chemical shift data. The absence of any large changes in ribose hydrogen chemical shifts induced by salt or temperature, plus the comparatively small changes in the shifts of the H_6 resonances, suggests that only a very slight change in the sugar–base torsion angles, within the range of the anti conformation, has occurred. Due to the various approximations involved in this argument, the magnitudes of the changes cannot be estimated.

Furanose-Ring Conformation. The furanose-ring conformation is defined by three dihedral angles between the planes containing atoms: $C_{1'}C_{2'}H_{1'}$ and $C_{1'}C_{2'}H_{2'}$; $C_{2'}C_{3'}H_{2'}$ and $C_{2'}C_{3'}H_{3'}$; $C_{3'}C_{4'}H_{3'}$ and $C_{3'}C_{4'}H_{4'}$. Other workers, however, have relied on the dihedral angle specified by planes $C_{1'}C_{2'}H_{1'}$ and $C_{1'}C_{2'}H_{2'}$, and as we shall see later reliance on this torsional angle as a measure of conformation for furanose-ring structures often leads to erroneous conclusions.

[3] For example, the chemical shift differences in parts per million for the corresponding ribose hydrogens of uridine and α-*N*-(β-cyanuric acid)-D-ribofuranoside were $H_{1'}$, -0.20; $H_{2'}$, -0.37; $H_{3'}$, -0.14; $H_{4'}$, $+0.18$; $H_{5'}$, $+0.06$; $H_{5''}$, $+0.08$. The $H_{1'}$ resonance of uridine was 0.20 ppm to higher field from the corresponding resonance in α-*N*-(β-cyanuric acid)-D-ribofuranoside.

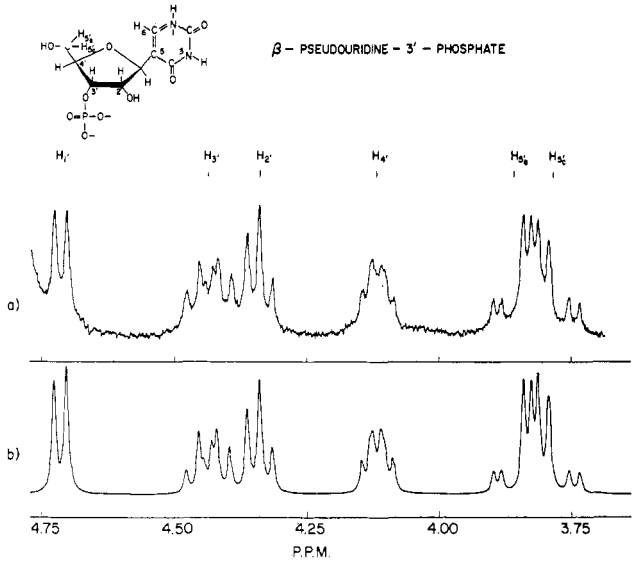


FIGURE 1: (a) 220-MHz nmr spectrum of the ribose hydrogens of 0.1 M 3′-β-ψMP in D_2O, pD 7.2, 23°, 0.1 M DSS. (b) Simulation of the experimental spectrum using the parameters from Table I.

The total number of conformational ring candidates, taking into consideration all possible combinations of bucklings, is 20 (Smith and Jardetzky, 1968). We will, however, confine our interest to the four principal conformations, those which reflect structures commonly found in crystals of nucleosides (and -tides) and in the fiber structures of DNA.

Knowledge of the proton–proton coupling constants allows an estimation of the relevant dihedral angle by the use of the Karplus relationship (Karplus, 1959, 1963)

$$J_{ij} = J_0 \cos^2 \theta - B \text{ (Hz)} \qquad (1)$$

where θ is the dihedral angle between the two coupled protons with coupling constant J_{ij}, and J_0 and B are empirical constants. We have selected the constants suggested by Abraham *et al.* (1962) as best representing the situation for a furanose ring. These constants are $J_0 = 9.27$ ($0° < \theta < 90°$), $J_0 = 10.36$ ($90° < \theta < 180°$), and $B = 0.28$ Hz.

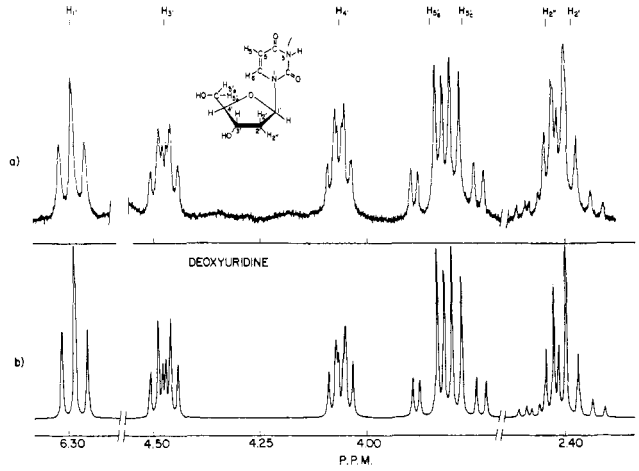


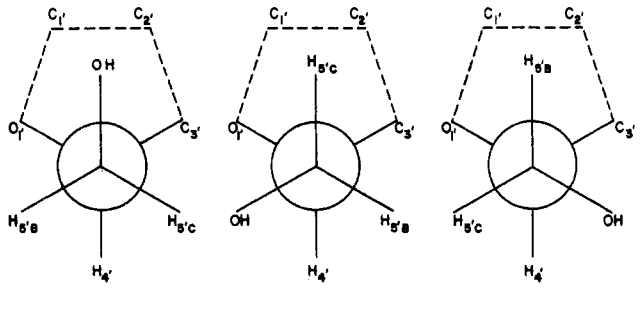
FIGURE 2: (a) 220-MHz nmr spectrum of the ribose hydrogens of 0.1 M deoxyuridine in D_2O, pD 6.8, 23°, 0.1 M TSP. (b) Simulation of the experimental spectrum using the parameters from Table I.

TABLE I: Chemical Shifts[a] and Coupling Constants for Uracil Nucleosides and Nucleotides.

	U 23°	U* 23° $NaClO_4$	U 80°	U* 80° $NaClO_4$	β-ψ 30°	β-ψ 30° $NaClO_4$	β-ψ 70°	β-ψ 70° $NaClO_4$	dU* 23°
δ									
1′	5.901	5.934	5.901	5.934	4.674	4.729	4.668	4.714	6.285
2′	4.340	4.369	4.348	4.373	4.279	4.264	4.260	4.254	2.379
2″									2.437
3′	4.221	4.238	4.222	4.240	4.141	4.140	4.128	4.135	4.474
4′	4.127	4.155	4.122	4.146	4.009	4.026	3.993	4.017	4.060
5′	3.907	3.944	3.890	3.920	3.840	3.863	3.835	3.850	3.846
5″	3.803	3.835	3.798	3.825	3.726	3.744	3.719	3.737	3.771
5	5.883	5.925	5.918	5.961					5.888
6	7.886	7.859	7.838	7.817	7.660	7.629	7.627	7.612	7.870
J									
1′2′	4.8	4.3	5.1	4.6	5.0	4.9	5.2	5.1	6.5
1′2″									6.7
2′2″									−13.9
2′3′	5.2	5.5	5.2	5.7	5.0	5.0	5.0	5.0	6.8
2″3′									3.5
3′4′	5.4	5.9	5.1	5.6	5.2	5.6	5.2	5.6	4.0
4′5′	2.9	3.0	3.3	3.3	3.2	3.0	3.4	3.0	3.4
4′5″	4.4	4.7	4.6	5.1	4.6	5.3	4.8	5.5	5.1
5′5″	−12.7	−12.8	−12.7	−12.6	−12.7	−12.6	−12.4	−12.4	−12.6
1′6	*b*	*b*	*b*	*b*	0.8	0.9	0.8	0.9	*b*
56	8.1	8.0	8.1	8.1					8.0
3′P									
Frequency of nmr experiment (MHz)	220	220	220	220	100	100	100	100	220

[a] Chemical shifts are expressed relative to internal 0.1 M DSS, except those marked * which are relative to 0.1 M TSP. (To convert data relative to TSP to be relative to DSS, subtract 0.030 ppm.) [b] Not measurable <1 Hz. [c] Analyzable spectra could not

Assembled in Table II is a listing of four static ring structures of the type commonly encountered in nucleic acids and their constituents, along with the relevant dihedral angles and coupling constants calculated using eq 1. There are pitfalls inherent in such an approach; one arises as a matter of course in the use of Dreiding stick models, and another is of a fundamental physical nature.[4] However, the angles and the predicted coupling constants determined in this manner are still quite useful for they allow conclusions of a general nature



I (GAUCHE-GAUCHE) II (GAUCHE-TRANS) III (TRANS-GAUCHE)

FIGURE 3: Classical 60° staggered rotamers for the 5′-CH_2OH group of a nucleoside.

[4] The dihedral angles were obtained in the following manner: the particular conformation was generated by first creating a completely planar furanose ring followed by buckling either the $C_{2'}$ or $C_{3'}$ carbon atom out of the plane (exo or endo), keeping the other four atoms fixed in place, and then projecting the image of the model onto a surface which allowed easy measurement of the dihedral angle with a protractor. Done in this way reproducibility to within ±5° could be achieved. This is at best an artificial procedure, since no allowance is made for deviations from normal bond angles and lengths. Such deviations have in fact been noted (Sundaralingam, 1969). The second criticism of such an approach is that a "static" angle is recorded making no allowance for a range of angles, each of which lie within the accessible region of a potential energy well. Thus the physically real quantity is the *average* coupling constant which is defined by the following equation

$$\langle J_{ij} \rangle = \langle J_0 \cos^2 \theta - 0.28 \rangle = \frac{\sum_\theta (J_0 \cos^2 \theta - 0.28) \exp(-E(\theta)/RT)}{\sum_\theta \exp(-E(\theta)/RT)}$$

Such an approach would demand that the conformational energy as a function of the angle under consideration be known, which is clearly a formidable computational task.

dU[c]* 10° $NaClO_4$	dU* 80°	dU* 80° $NaClO_4$	3′-UMP 23°	3′-UMP[c] 10° $NaClO_4$	3′-UMP 88°	3′-UMP 85° $NaClO_4$	3′-β-ψMP 23°	3′-β-ψMP 23° $NaClO_4$	3′-β-ψMP 75°	3′-β-ψMP 75° $NaClO_4$
6.295	6.228	6.235	5.927	5.927	5.906	5.922	4.715	4.727	4.714	4.708
2.357	2.383	2.364	4.400	4.425	4.411	4.415	4.341	4.344	4.334	4.314
2.418	2.446	2.420								
4.475	4.469	4.448	4.496	4.510	4.541	4.544	4.433	4.434	4.455	4.451
4.079	4.062	4.039	4.228	4.255	4.229	4.243	4.116	4.122	4.111	4.104
3.864	3.848	3.825	3.898	3.918	3.905	3.911	3.850	3.861	3.853	3.845
3.779	3.775	3.748	3.852	3.865	3.855	3.851	3.785	3.786	3.785	3.772
5.939	5.880	5.896	5.896	5.936	5.903	5.945				
7.841	7.785	7.760	7.869	7.881	7.805	7.785	7.694	7.687	7.675	7.650
6.3	6.7	6.7	4.8	5.1	5.0	5.1	5.3	5.4	5.5	5.5
6.2	6.7	6.6								
−14.1	−14.4	−14.3								
6.5	6.9	6.9	5.2	5.0	5.3	5.3	5.3	5.4	5.5	5.6
3.7	4.0	4.1								
3.9	4.2	4.0	5.5	4.7	5.5	5.3	5.5	5.6	5.6	5.5
3.5	3.8	3.9	3.0	3.4	3.6	3.6	3.0	3.3	3.5	3.8
5.6	5.5	5.7	4.0	4.7	4.5	4.7	4.6	4.5	4.7	5.1
−12.5	−12.3	−12.3	−13.0	−13.8	−12.9	−12.6	−12.7	−12.6	−12.5	−12.4
b	*b*	*b*	*b*	*b*	*b*	*b*	0.75	0.8	0.8	0.8
8.1	8.0	8.1	8.0	8.5	8.1	8.1				
			7.7	7.9	7.6	7.6	7.8	7.8	7.6	7.7
220	220	100	220	220	220	220	220	220	220	220

be obtained at 23° due to unfavorable overlap of the HDO and ribose resonances.

to be made regarding the conformational features of the furanose ring.

The furanose ring conformations of both U and β-ψ have been shown by nmr techniques to be rapid time averages of various conformers, with the eclipsed form as a transient intermediate (Hruska *et al.*, 1970a,b; Blackburn *et al.*, 1970). A similar conclusion has been reached for α-ψ (Grey *et al.*, 1971), dihydrouridine (Deslauriers *et al.*, 1971), and β-cyanuric acid riboside (Dugas *et al.*, 1971). Consideration of $J_{1'2'}$ $J_{2'3'}$ $J_{3'4'}$, and $J_{1'2''}$ (for deoxyuridine) and comparison with Table II leads to the inescapable conclusion that the sugar ring conformations at 23° for 3′-UMP, 3′-β-ψMP, and dU are likewise such a rapid time-average blend of conformers (relative to the nmr experiment time scale) with an intermediate transitory eclipsed state. A previous study (Prestegard and Chan, 1969) based on an *apparent* $J_{1'2'}$ value concluded that both 3′-UMP and dU were in fact "rigid" and hence not susceptible to aqueous electrolyte perturbation.

An increase in temperature of approximately 60° does not appreciably affect the nmr spectral parameters. The same rapid time-average blend is present at the higher temperatures as at 25°. Since the coupling constants do in fact change somewhat, the only reasonable interpretation is that the position of the equilibrium characterizing the blend is shifted. This conclusion holds for all the compounds studied in this paper.

The addition of electrolyte, in this study $NaClO_4$, likewise alters the various coupling constants of *all* the compounds studied. This effect is small, and the rapid time-average blend of conformers is still valid as an interpretation of the data. Again we conclude that the equilibrium position is shifted slightly, but certainly for 3′-UMP and dU it is incorrect to say that the conformation of the respective furanose rings is insensitive to the presence of salt. It should be noted that temperature and electrolyte perturbation do not always perturb the various furanose rings in the same direction. Thus for U temperature and salt change the various ring coupling constants in opposite directions, while for both 3′-UMP and dU some coupling constants change in the same direction ($J_{1'2'}$ for 3′-UMP, and $J_{2''3'}$ for dU) while others change in opposite directions ($J_{2'3'}$ of 3′-UMP and $J_{1'2'}$ and $J_{3'4'}$ of dU). The simultaneous perturbation by both temperature and $NaClO_4$ results in an additive effect; thus if temperature and salt shifts are in the same direction the cumulative effect is large, and *vice versa*. These observations suggest that the effect of temperature and $NaClO_4$ perturbation are not identical. The implications of this finding will be discussed in a subsequent section.

TABLE II: Dihedral Angles[a] Estimated from Stick Models of Various Ribose Conformations,[b] and the Expected Spin–Spin Coupling Constants.

Atoms	ϕ (deg)	J (Hz)
2′-Endo conformation		
1′,2″	35 (2)	5.9
1′,2′	155 (2)	8.3
2″,3′	86 (2)	0
2′,3′	35 (2)	5.9
3′,4′	101 (1)	0
3′-Endo conformation		
1′,2″	20 (2)	7.9
1′,2′	97 (3)	0
2″,3′	153 (3)	8.0
2′,3′	32 (1)	6.4
3′,4′	159 (4)	8.5
2′-Exo conformation		
1′,2″	35 (1)	5.9
1′,2′	83 (1)	0
2″,3′	157 (4)	8.5
2′,3′	35 (1)	5.9
3′,4′	143 (2)	6.2
3′-Exo conformation		
1′,2″	20 (1)	7.9
1′,2′	140 (1)	5.8
2″,3′	93 (2)	0
2′,3′	28 (3)	7.0
3′,4′	95 (1)	0

[a] Numbers in parentheses refer to average deviations of three measurements. [b] In the models, four atoms define a plane and the fifth atom is above (endo) or below (exo) this plane.

TABLE III: Calculated Populations of the Three Classical 60° Staggered Rotamers[a] of the 5′-CH_2OH Group.

Compound	P_I	P_{II}	P_{III}
U, 23°	0.60	0.29	0.11
23°, 2 M $NaClO_4$	0.55	0.33	0.12
80°	0.52	0.32	0.16
80°, 2 M $NaClO_4$	0.46	0.38	0.16
β-ψ, 30°	0.54	0.32	0.14
30°, 2 M $NaClO_4$	0.48	0.40	0.12
70°	0.49	0.34	0.17
70°, 2 M $NaClO_4$	0.45	0.43	0.12
dU, 23°	0.45	0.38	0.17
10°, 2 M $NaClO_4$	0.38	0.44	0.18
80°	0.35	0.43	0.22
80°, 2 M $NaClO_4$	0.31	0.44	0.23
3′-UMP, 23°	0.64	0.24	0.12
10°, 2 M $NaClO_4$	0.50	0.33	0.17
88°	0.50	0.31	0.19
85°, 2 M $NaClO_4$	0.48	0.33	0.19
3′-β-ψMP, 23°	0.56	0.32	0.12
23°, 2 M $NaClO_4$	0.54	0.30	0.16
75°	0.49	0.33	0.18
75°, 2 M $NaClO_4$	0.40	0.38	0.22

[a] Rotamer I is gauche,gauche, II is gauche,trans, III is trans,gauche (Figure 3). For a general discussion of the calculation, see Blackburn *et al.* (1970).

To summarize, no major changes in furanose ring conformation occur with changing temperature and $NaClO_4$ addition. The small changes in the coupling constants which are observed on temperature and salt addition are best explained by small shifts in the equilibrium population of the blend of time-averaged conformers.

Conformation of the Exocyclic CH_2OH Group. The polynucleotide backbone, which is comprised of 3′- and 5′-phosphodiester linkages, has a conformation determined by six dihedral angles, one of which is contained in the exocyclic 5′-CH_2OH moiety. It has been demonstrated that for β-ψ (Hruska *et al.*, 1970a,b), U (Blackburn *et al.*, 1970), α-*N*-(β-cyanuric acid)-D-ribofuranoside (Dugas *et al.*, 1971), α-ψ (Grey *et al.*, 1971), and dihydrouridine (Deslauriers *et al.*, 1971), that the exocyclic CH_2OH group is interconverting rapidly between the three principal conformational isomers, and that usually a preference for one of them is manifest (that is, the CH_2OH group spends a longer time in one of the isomeric conformational states).

A complete description of this method and its limitations is given in Blackburn *et al.* (1970). Application of the method with $J_0 = 9.27$ Hz ($0° < \theta < 90°$), $J_0 = 10.36$ Hz ($90° < \theta < 180°$), $B = 0.28$ Hz, and considering the classical 60° staggered rotational isomers (Figure 3) yields the rotamer populations in Table III.

For all the compounds considered here, the gauche,gauche rotamer is preferred at room temperature and low salt concentration. For dU the gauche,trans rotamer occurs to almost the same extent as the gauche,gauche rotamer. The addition of $NaClO_4$ or elevation of temperature shifts the conformational equilibrium toward a more uniform distribution of rotamers. In contrast to the influences on the conformations of the ribose rings, the effects of added salt and increasing temperature are in the same direction. In no case does the population of the trans,gauche rotamer become equal to those of the other two, indicating that even at high temperatures and high salt concentrations, steric effects make this rotamer energetically unfavorable. It should be emphasized however that the distinction between gauche,trans and trans,gauche rotamers depends on the assignment of the $H_{5'}$ and $H_{5''}$ resonances. Since this cannot be done with certainty, one can only say that one of the two is energetically less allowed. The conclusions that the gauche,gauche rotamer predominates under normal conditions, and that salt addition or temperature increase causes a shift toward equalization of rotamer populations, are independent of the assignments.

Conformation of the Phosphate Group. Molecular entities containing the system H–C–O–P display nuclear spin H–P couplings akin to those found for H–H in H–C–C–H systems. Trans couplings are estimated to be in the range 25 ± 3 Hz, and gauche couplings 3 ± 2 Hz (Kainosho *et al.*, 1969; Hall and Malcolm, 1968; Malcolm, 1969). The exact functional relationship connecting J_{HP} and the dihedral angle between the planes H–C–O and C–O–P has not been established with certitude; the functional form is apparently similar to that for proton–proton couplings. A graph with a few points has been presented which allows one to estimate dihedral angles from J_{HP} (Tsuboi *et al.*, 1969a,b; Malcolm, 1969). Using this graph a time-averaged dihedral angle of approximately 50 or 130° is obtained between the planes H–$C_{3'}$–O and $C_{3'}$–O–P of 3′-UMP and 3′-β-ψMP. The latter angle

can in all likelihood be discarded because in this case the atoms of the H–C–O–P subsystem are in an eclipsed conformation. However, two conformational possibilities exist for a H–C–O–P dihedral angle of 50°: (a) $C_{3'}$–$C_{2'}$ is gauche to $O_{3'}$–P and $C_{3'}$–$C_{4'}$ is trans to $O_{3'}$–P; (b) $C_{3'}$–$C_{2'}$ is trans to $O_{3'}$–P and $C_{3'}$–$C_{4'}$ is gauche to $O_{3'}$–P. In studies of ApA and UpU (Tsuboi *et al.*, 1969b) the H–$C_{3'}$–$O_{3'}$–P dihedral angle was found to be 50 or 130° in both cases; in order to make the bases stack (in ApA) with an anti sugar–base torsional angle conformation (b) had to be rejected as a possibility. Theoretical conformational calculations by Lakshminarayanan and Sasisekharan (1969b) indicate an H–C–O–P dihedral angular range of 80–180° with $C_{3'}$–$C_{2'}$ gauche to $O_{2'}$–P and $C_{3'}$–$C_{4'}$ trans to $O_{3'}$–P. We thus conclude that conformation (a) is the more likely for the phosphate groups of 3′-UMP and 3′-β-ψMP.[5]

It is of interest to observe that neither temperature increases nor electrolyte addition serve to alter appreciably J_{HP}, and hence the H–$C_{3'}$–$O_{3'}$–P dihedral angle of 3′-UMP and 3′-β-ψMP. This torsional angle is one of the determinants of the polynucleotide backbone conformation. In the ApA and UpU studies referred to above, nearly identical J_{HP} values were obtained, suggesting that in the dimers base–base stacking plays little role in establishing the "backbone" conformation. This observation is apparently at variance with the polymer case where the characteristic ratio (Flory, 1969; definition and further discussion given elsewhere) was found to be dependent on stacking in poly(rA); however, when corrected for the condition of no stacking this ratio matches the characteristic ratio for unstacked polyuridylic acid, thereby implying that the nature of the base does not influence the polynucleotide backbone conformation (Inners and Felsenfeld, 1970).

Role of Water Structure. Bernal and Fowler (1933) have developed the concept of a liquid structure temperature which relates ion-induced changes in water structure to those mediated by thermal changes. Thus in the present case 2 M $NaClO_4$ at 25° has the liquid structure temperature of water heated to 60° (based on the nmr data of Schleich and von Hippel, 1970). The role of ion-induced water structural changes *vs.* ion-binding effects in altering biopolymer conformational structure and stability has been the object of considerable study (von Hippel and Schleich, 1969b).

It was recently shown (Schleich and von Hippel, 1970) that the proton chemical shift of water in various ionic solutions bore no simple relationship to the midpoints of the thermally induced unfolding transitions of ribonuclease in these same solutions. This suggests that a definitive role cannot be established for the involvement of water structure in protein stability.

Hamaguchi and Geiduschek (1962) and Gordon *et al.* (1965) have suggested that water structure modification by ionic solutes was responsible for the destabilization of DNA structures in aqueous ionic solutions. More recently, however, Noelken (1970) demonstrated a positive correlation between the effectiveness of a salt in destabilizing the conformation of a modified bovine serum albumin and its tendency to interact preferentially with the protein. This correlation may not be valid for all types of biopolymers in view of the observation that the salt-induced unfolding of the poly-L-proline form II helix does not follow a trend such as that described for bovine serum albumin (T. Schleich, in preparation). Tanford (1969), on the other hand, has concluded from thermodynamic considerations that the unfolded form of a biopolymer binds neutral salt denaturants to a greater extent than the corresponding folded form. Such interactions of the so-called direct type are sufficient to cause the lowering in the observed midpoint of the thermal unfolding transition.

We will not be concerned with the ribose chemical shift changes induced by temperature and/or the addition of salt to the aqueous environment, inasmuch as these changes are all small and the interpretation of such changes is quite complicated. Instead, attention will be focussed on the changes which occur in the ribose ring coupling constants.

Perhaps the most striking fact to emerge from an analysis of the coupling constant data (in terms of the role of water structure in defining conformational stability) is that in an appreciable number of instances increasing temperature produces changes *opposite* to those recorded for increasing $NaClO_4$ concentration, thus revealing a lack of correspondence between temperature disruption of water structure and the disruption of water structure induced by the addition of neutral salt denaturant. If one believes in the validity of the liquid structure temperature concept (Bernal and Fowler, 1933), then the conclusion must be reached that *simple* water structure effects are not operating in the definition of nucleoside and nucleotide ribose conformations.

Implications for Polynucleotide Conformational Structure and Stability. The results of this study imply that perturbants which destabilize polynucleotide conformations do not operate in an appreciable manner on ribose ring conformations, the dihedral angle in H–$C_{3'}$–$O_{3'}$–P, or the sugar–base torsional angle. All of these conformations contribute toward the specification of the local short-range set of interactions which in turn dictate the overall conformational details of a polynucleotide chain. We conclude from the ribose proton coupling constant data presented for poly(rU) by Kreishman and Chan (1971) that a similar time-average blend of ribose ring conformers exists in the polymer as in the monomeric units. Thus, the most likely site of neutral salt effects in polynucleotides must be the base–base stacking interactions, and perhaps also the inter-base hydrogen bonds (Gruenwedel *et al.* (1971) have concluded that certain salts destabilize G·C base pairs relative to A·T pairs). Robinson and Grant (1966) found that neutral salts of the destabilizing variety all lowered the activity coefficient of these constituents. This observation suggests, but does not prove, that denaturing salts disrupt base–base stacking to some degree.

The above conclusion on the mode of polynucleotide destabilization receives considerable support from the experiments of Felsenfeld who showed that there was not more than a 10% difference in the unperturbed dimensions of poly(rA) in NaCl and $LiClO_4$ (salt concentration was between 1 and 2 M in both cases) at the same fraction of stacking.

In their work on the conformation of poly(rU) in solution Inners and Felsenfeld (1970) were faced with accounting for the arge value of the characteristic ratio[6] in terms of restricted

[5] ADDED IN PROOF. Recent studies on three-bond ^{13}C–^{31}P couplings have confirmed that (a) is the preferred conformer for 3′-UMP (Mantsch and Smith, 1972).

[6] The dimension of a freely jointed chain polymer most widely used to characterize its spatial or configurational character is the unperturbed mean-square end-to-end distance ($\langle r^2 \rangle_0$). The characteristic ratio for a chain polymer is given by $\langle r^2 \rangle_0 / nl^2$, where n is the number of bonds in the backbone and l^2 is the mean-square bond length. This ratio is indicative of the degree of polymer backbone rotational restriction. Since the value of the characteristic ratio must be unity for a hypothetical linear freely jointed polymer (no restrictions in the backbone bond rotations) increased values of this ratio imply restrictions in the allowed bond angles defining the backbone conformation.

rotations about the six bonds which define the backbone conformation of a polynucleotide chain. They stated, "steric interference is not sufficient to account for the required restriction" and suggested several possibilities to account for such restriction. These were, residual base–base stacking, hydrogen bonding between the 2′-OH group and the phosphate backbone, and electrostatic interaction between negatively charged oxygens of neighboring phosphate groups. Of the six bonds which define the backbone conformation, high-resolution nmr allows us to "sense" rotation about all but the P–$O_{5'}$ and $O_{3'}$–P bonds. Our data, and our interpretation of the nmr data presented by Kreishman and Chan (1971), allow us to conclude that the bond rotation potentials (in terms of time-averaged dihedral angles) which are present in the monomer units are largely preserved in the polymer. It seems most unlikely that base–base stacking and 2′-OH hydrogen bonding would not influence the bond rotation potentials of the furanose ring; a large difference in $J_{1'2'}$, attributable to base–base stacking, has been observed between 5′-AMP and ApA (Hruska and Danyluk, 1968). Alteration of polymer rotation potentials by base–base stacking has been implied for poly(rA) by Eisenberg and Felsenfeld (1967). It also seems unlikely that if electrostatic interactions were present only bond rotation potentials about P–$O_{5'}$ and $O_{3'}$–P would be affected and no others, *e.g.*, O_5–$C_{5'}$, $C_{3'}$–$O_{3'}$. Thus, the problem of restricted rotation in polyuridylic acid reduces to an explanation of restricted rotation in the monomer units themselves.

Comparison with X-Ray Crystallographic Data. A large number of nucleosides and nucleotides have been studied by X-ray diffraction from single crystals. A survey of the structural data is given by Sundaralingam (1969). Often two molecules with different conformations are found in the asymmetric unit. The differences can exist in the mode of ribose ring pucker, or in the torsional angles about the glycosidic bond or the 4′–5′ bond. The significant point is, however, that the conformations observed by X-ray diffraction are static. Spectroscopic studies on a large number of nucleosides and nucleotides in water at physiological temperatures (Blackburn *et al.*, 1970; Deslauriers *et al.*, 1971; Dugas *et al.*, 1971; Grey *et al.*, 1971; Hruska *et al.*, 1970a,b) indicate that the molecules are in rapid equilibrium between the various conformations found in the solid state (except the syn and anti conformers, where an equilibrium has not been demonstrated), and that a preference may exist for some of these conformations, *i.e.*, the molecules spend more time in one conformation than in the others. In some cases the conformational preferences in solution correspond to the static structures found in the solid state, and in others they do not. For example, whereas 4-thiouridine has the syn conformation in the solid state (Saenger and Scheit, 1970) and the anti conformation in solution (Schweizer *et al.*, 1971), adenosine 5′-phosphate has the anti conformation in the solid state (Kraut and Jensen, 1963) and in solution (Schweizer *et al.*, 1968). Consideration of all the X-ray data assembled so far leads to the following generalizations. (a) For pyrimidine nucleosides and nucleotides the anti conformation of the base is preferred. (b) The $C_{2'}$-endo and $C_{3'}$-exo ring conformers occur with approximately equal frequency. (c) The conformational priority for the exocyclic CH_2OH rotamers is gauche,gauche > gauche,trans > trans,gauche. Of the compounds studied here, only deoxyuridine has been investigated by X-ray diffraction (Rahman and Wilson, 1971). In the solid state the ribose ring has the $C_{2'}$-endo conformation, the exocyclic CH_2OH exists as the trans,gauche rotamer, and the base is in the anti range.

The present nmr data indicate that in aqueous solution at 23° the ribose ring is in a rapid equilibrium between puckered conformers with very little preference for any one in particular, that the exocyclic CH_2OH group is rotating rapidly with a slight preference for the gauche,gauche rotamer (which can be altered to gauche,trans by addition of $NaClO_4$), and that the base has the anti conformation. The dynamic aspects of the conformations in solution are no doubt due to removal of intermolecular effects experienced in the solid state. With the exception of the dynamic aspect, the present nmr data are in rough agreement with the general scheme above. In large biological polynucleotides such as mRNA, tRNA, or DNA, strong intermolecular forces may come into play again, and the static conformations found in the X-ray studies may be more relevant than the equilibrium conformations found in solution.

Conclusion

The conformations of U, β-ψ, dU, 3′-UMP, and 3′-β-ψMP in aqueous solution are highly mobile; only time-averaged conformations are observable. With the exception of this dynamic character, the conformational preferences are similar to those generally found for such compounds in the solid state. The solution conformations are affected only slightly by elevation of temperature or addition of 2 M $NaClO_4$. In some cases the effects of temperature and salt are in the same direction, in other cases the opposite is true. This suggests that water structure is not a principal determinant of the molecular conformations of these compounds. The similarity between the present data and those for polyuridylic acid (Kreishman and Chan, 1971) lead to a similar conclusion for the polymer. The destabilizing effects of temperature and salt on polynucleotide conformations are apparently due to modification of base–base-stacking and hydrogen-bonding interactions.

References

Abraham, R. J., Hall, L. D., Hough, L., and McLauchlan, K. A. (1962), *J. Chem. Soc.*, 3699.

Arnott, S. (1970), *Progr. Biophys. Mol. Biol. 21*, 267.

Bernal, J. D., and Fowler, R. H. (1933), *J. Chem. Phys. 1*, 515.

Blackburn, B. J., Grey, A. A., Smith, I. C. P., and Hruska, F. E. (1970), *Can. J. Chem. 48*, 2866.

Deslauriers, R., Lapper, R. D., and Smith, I. C. P. (1971), *Can. J. Biochem.* (in press).

Donohue, J., and Trueblood, K. N. (1960), *J. Mol. Biol. 2*, 363.

Dugas, H., Blackburn, B. J., Robins, R. K., Deslauriers, R., and Smith, I. C. P. (1971), *J. Amer. Chem. Soc. 93*, 3468.

Eisenberg, H., and Felsenfeld, G. (1967), *J. Mol. Biol. 30*, 17.

Flory, P. J. (1969), Statistical Mechanics of Chain Molecules, New York, N. Y., Interscience, p 11.

Gordon, D. E., Curnutte, B., Jr., and Lark, K. (1965), *J. Mol. Biol. 13*, 571.

Grey, A. A., Smith, I. C. P., Hruska, F. E. (1971), *J. Amer. Chem. Soc. 93*, 1765.

Gruenwedel, D. W., Hsu, C. H., and Lu, D. S. (1971), *Biopolymers 10*, 47.

Hall, L. D., and Malcolm, R. B. (1968), *Chem. Ind. (London)*, 92.

Hamaguchi, K., and Geiduschek, E. P. (1962), *J. Amer. Chem. Soc. 84*, 1329.

Haschemeyer, A. E. V., and Rich, A. (1967), *J. Mol. Biol. 27*, 369.
Hruska, F. E., and Danyluk, S. S. (1968), *J. Amer. Chem. Soc. 90*, 3266.
Hruska, F. E., Grey, A. A., and Smith, I. C. P. (1970a), *J. Amer. Chem. Soc. 92*, 214.
Hruska, F. E., Grey, A. A., and Smith, I. C. P. (1970b), *J. Amer. Chem. Soc. 92*, 4088.
Inners, L. D., and Felsenfeld, G. (1970), *J. Mol. Biol. 50*, 373.
Kainosho, M., Nakamura, A., and Tsuboi, M. (1969), *Bull. Chem. Soc. Jap. 42*, 1713.
Karplus, M. (1959), *J. Chem. Phys. 30*, 11.
Karplus, M. (1963), *J. Amer. Chem. Soc. 85*, 2870.
Kraut, J., and Jensen, L. H. (1963), *Acta Crystallogr. 16*, 79.
Kreishman, G. P., and Chan, S. I. (1971), *Biopolymers 10*, 159.
Lakshminayanan, A. V., and Sasisekharan, V. (1969a), *Biopolymer 8*, 475.
Lakshminarayanan, A. V., and Sasisekharan, V. (1969b), *Biopolymers 8*, 489.
Malcolm, R. B. (1969), M.Sc. Thesis, University of British Columbia, Vancouver, B. C.
Mantsch, H. H., and Smith, I. C. P. (1972), *Biochem. Biophys. Res. Commun.* (in press).
Noelken, M. E. (1970), *Biochemistry 9*, 4122.
Pople, J. A., Schneider, W. G., and Bernstein, H. J. (1959), High-resolution Nuclear Magnetic Resonance, New York, N. Y., McGraw-Hill.
Prestegard, J. H., and Chan, S. (1969), *J. Amer. Chem. Soc. 91*, 2843.
Rahman, A., and Wilson, H. R. (1971), *Nature (London) 232*, 333.
Robinson, D. R., and Grant, M. E. (1966), *J. Biol. Chem. 241*, 4030.
Saenger, W., and Scheit, K. H. (1970), *J. Mol. Biol. 50*, 153.
Schleich, T., and von Hippel, P. H. (1970), *Biochemistry 9*, 1059.
Schweizer, M. P., Broom, A. D., Ts'O, P. O. P., and Hollis, D. P. (1968), *J. Amer. Chem. Soc. 90*, 1042.
Schweizer, M. P., Witkowski, J. T., and Robins, R. K. (1971), *J. Amer. Chem. Soc. 93*, 277.
Smith, I. C. P., Blackburn, B. J., and Yamane, T. (1969), *Can. J. Chem. 47*, 513.
Smith, M., and Jardetzky, C. D. (1968), *J. Mol. Spectrosc. 28*, 70.
Sundaralingam, M. (1969), *Biopolymers 7*, 821.
Tanford, C. (1968), *Advan. Protein Chem. 23*, 121.
Tanford, C. (1969), *J. Mol. Biol. 39*, 539.
Tanford, C. (1970), *Advan. Protein Chem. 24*, 1.
Tsuboi, M., Kainosho, M., and Nakamura, A. (1969a), *in* Recent Developments of Magnetic Resonance in Biological Systems, Fujiwara, S., and Piette, L. H., Ed., Tokyo, Hirokawa Publishing Co., p 43.
Tsuboi, M., Takahashi, S., Kyogoku, Y., Hayatsu, H., Ukita, T., and Kainosho, M. (1969b), *Science 166*, 1504.
von Hippel, P. H., and Schleich, T. (1969a), *in* Structure and Stability of Biological Macromolecules, Timasheff, S. N., and Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, p 417.
von Hippel, P. H., and Schleich, T. (1969b), *Accounts Chem. Res. 2*, 257.

Primary Structure of Cytochrome *c* from the Camel, *Camelus dromedarius**

Mordechai Sokolovsky† and Moshe Moldovan

ABSTRACT: The amino acid sequence of the cytochrome *c* from heart tissue of the camel, *Camelus dromedarius*, has been established from the structures of the chymotryptic and tryptic peptides. The camel protein consists of a polypeptide chain 104 residues in length with an acylated N-terminal residue. The most remarkable variation in the camel cytochrome *c* is the substitution of valine for the proline commonly present in most mammalian cytochrome *c* in position 44.

A study of the amino acid sequence of cytochrome *c* from camel heart (*Camelus dromedarius*) was undertaken to extend the knowledge of the comparative structures of cytochrome *c* of the "mammalian type" (Nolan and Margoliash, 1968; Smith, 1970) which are all homologous, both structurally and functionally. The isolation procedure, a number of physicochemical properties and the sequence of the heme peptide have been reported elsewhere (Schejter *et al.*, 1972).

Materials and Methods

Cytochrome *c* was prepared as previously described (Schejter *et al.*, 1972). All enzymes were obtained from Worthington. Trypsin was further purified from residual chymotryptic activity by affinity chromatography on Sepharose–ε-aminocaproyl-D-tryptophan methyl ester (Cuatrecasas *et al.*, 1968). Similarly, chymotrypsin was purified by chromatography on Sepharose–ovomucoid inhibitor (Feinstein, 1970). The chemicals used were of analytical grade when available.

Chymotryptic and Tryptic Hydrolysis of Cytochrome c. Cytochrome *c* (96 mg) in 0.2 M NH_4HCO_3 (pH 8.0) at a concentration of 6 mg/ml was digested with chymotrypsin (0.6

* From the Department of Biochemistry, Tel-Aviv University, Tel-Aviv, Israel. *Received August 3, 1971.*
† To whom to address correspondence.