is then attributable to incomplete folding of DMSCT in state A. In the future more attention will have to be given to the possibility that specific chemical modification of single protein groups affects catalytic parameters indirectly through conformational alterations of this type. It is possible that the peculiar patterns of catalytic behavior distinguishing amide and peptide substrates from ester substrates, as, for example, is found with carboxypeptidases A[11] and B,[12] or large substrates from small substrates may be attributable to such changes in folding.

Complete details of the experiment and interpretation will be published soon.

(11) B. Vallee, J. Riordan, and J. Coleman. *Proc. Natl. Acad. Sci. U. S.*, **49**, 109 (1963).
(12) J. Folk, E. Wolff, E. Schirmer, and J. Cornfeld, *J. Biol. Chem.*, **237**, 3105 (1962).

Rodney L. Biltonen, Rufus Lumry
Laboratory for Biophysical Chemistry
Department of Chemistry, University of Minnesota
Minneapolis, Minnesota
Received July 23, 1965

Selective Chemical Modifications of Uridine and Pseudouridine in Polynucleotides and Their Effect on the Specificities of Ribonuclease and Phosphodiesterases

Sir:

1-Cyclohexyl-3-(2-morpholinyl-(4)-ethyl)carbodiimide metho-*p*-toluenesulfonate (CMC) has been shown to add specifically to uridine and guanosine components of ribonucleic acid (RNA) such that the modified uridine bases become resistant to the action of pancreatic ribonuclease.[1] As a result digestion of the modified RNA with this enzyme produces oligonucleotides which terminate with cytidine only.[2] It is now found that, when amino acid acceptor RNA from yeast is treated with CMC and hydrolyzed with ribonuclease, no pseudouridine phosphate, uridine phosphate, or their corresponding cyclic phosphates are produced. Thus it appears that pseudouridine is also blocked under the conditions necessary for the blocking of uridine and guanosine. Furthermore, if the modified RNA is treated with dilute ammonia to remove the blocking groups and then hydrolyzed with ribonuclease, the products obtained are similar to those obtained by the enzyme digestion of untreated RNA except that, in the former case, no pseudouridine phosphate or cyclic phosphate is formed. From these results it is implied that the pseudouridine bases in RNA form stable adducts with CMC and that the resistance of these adducts to ribonuclease hydrolysis results in the pseudouridine components being left in internal positions of the oligonucleotides that remain after the enzyme digestion.

These conclusions are confirmed by a study of the chemical blocking of pseudouridine itself. On reaction with CMC the nucleoside gives a mixture of two positively charged derivatives which can be separated by electrophoresis at pH 7. One of these has the electrophoretic mobility of a derivative containing one positive charge and is tentatively assigned the structure of the 3 adduct on the basis of its ultraviolet spectrum: λ_{max} 266 mμ at pH 7 and 292 mμ at pH 10. A structurally similar compound, 3-methyluracil, has λ_{max} 259 mμ at pH 7 and 282 mμ at pH 10, while 1-methyluracil has λ_{max} 268 mμ at pH 7 with no shift to longer wave lengths at higher pH values.[3] This assignment is supported by the fact that the 3 adduct is structurally analogous to the adduct obtained from uridine[1] and the fact that both of these adducts are resistant to ribonuclease when they are located in polynucleotide chains. The other derivative which must contain two positive charges in view of its electrophoretic mobility has λ_{max} 265 mμ at pH 7 and 9 (1,3-dimethyluracil has λ_{max} 266 mμ at these pH values[3]) and is assigned the structure of an adduct containing 2 molecules of CMC at the 1,3 positions. Its initial λ_{max} of 265 mμ at pH 9 changes to 292 mμ after 2 hr. at 25°, the shift presumably arising from the hydrolysis of the CMC group at the 1 position with the concomitant formation of a dissociable hydrogen atom. The 1,3 adduct can be converted to the 3 adduct by treatment with cold dilute ammonia, while the removal of the remaining blocking group from the latter requires hydrolysis with hot concentrated ammonia. Thus for sequence analysis studies it is now possible to selectively block guanosine, uridine, and pseudouridine in polynucleotides, imparting resistance to ribonuclease action at the positions occupied by the two pyrimidines. Further, by subsequent treatment with dilute ammonia a ribonuclease-resistant block can be produced at the pseudouridine positions only.

In order to study the effects of blocked nucleotides on the action of phosphodiesterases a number of dinucleoside phosphates were synthesized. A group of 2′,5′-diacetylnucleoside 3′-phosphates and 2′,3′-diacetylnucleosides were prepared, and appropriate combinations were condensed with dicyclohexylcarbodiimide by the method described by Rammler, *et al.*[4] The acetyl groups were then removed by dilute ammonia treatment and portions of the dinucleoside phosphates were treated with CMC under conditions previously described.[1] In the case of dinucleoside phosphates containing pseudouridine the products from the CMC reaction were subsequently treated with dilute ammonia to leave the pseudouridine moieties with one blocking group.

Table I lists various dinucleoside phosphates and their blocked derivatives and shows a comparison of their rates of hydrolysis with snake venom and spleen diesterases.[5] Cpψ (cytidylyl-(3′→5′)-pseudouridine containing a blocking group on the pseudouridine) and CpU are both resistant to both of these enzymes. Thus it appears that a chemical block at uridine or pseudouridine in a polynucleotide chain will halt the normal hydrolytic progression of these exonucleases along the chain, a technique which is expected to be of some value in the determination of base sequences of nucleic acids. In addition, as mentioned above, ŪpA, ψpA, and ŪpC are resistant to hydrolysis by pancreatic ribonuclease.

The results listed in Table I also show an interesting difference in the effects of blocking groups on the actions

(1) P. T. Gilham, *J. Am. Chem. Soc.*, **84**, 687 (1962).
(2) J. C. Lee, N. W. Y. Ho, and P. T. Gilham, *Biochim. Biophys. Acta*, **95**, 503 (1965).
(3) D. Shugar and J. J. Fox, *ibid.*, **9**, 199 (1952).
(4) D. H. Rammler, Y. Lapidot, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 1989 (1963).
(5) Worthington Biochemical Corporation, Freehold, N. J.

Table I. Percentage Hydrolysis of Dinucleoside Phosphates[a]

	Snake venom diesterase	Spleen diesterase
UpA[b]	100 (U, pA)	94 (Up, A)
$\bar{U}$pA	25 ($\bar{U}$, pA)	47 ($\bar{U}$p, A)
ψpA	88 (ψ, pA)	69 (ψp, A)
$\bar{\psi}$pA	45 ($\bar{\psi}$, pA)	35 ($\bar{\psi}$p, A)
CpU	93 (C, pU)	61 (Cp, U)
Cp$\bar{U}$	0	0
Cpψ	53 (C, pψ)	23 (Cp, ψ)
Cp$\bar{\psi}$	0	0
UpC	94 (U, pC)	100 (Up, C)
$\bar{U}$pC	40 ($\bar{U}$, pC)	80 ($\bar{U}$p, C)

[a] Ten absorbancy units (260 mμ) of each dinucleoside phosphate was incubated with snake venom phosphodiesterase[5] (0.025 mg.) for 11 hr. at 37° in 0.02 *M* Tris acetate buffer (1.2 ml.) at pH 8.0 or with spleen phosphodiesterase[5] (0.15 unit) for 2 hr. at 37° in 0.02 *M* ammonium acetate (1.2 ml.) at pH 6.0. The products of the enzyme digests are included in parentheses. In the spleen diesterase digests adenosine is usually obtained as inosine due to the presence of the contaminant deaminase. [b] UpA = uridine-(3′→5′)-adenosine; $\bar{U}$pA = UpA with a blocking group on the uridine moiety.

of these two diesterases. Snake venom phosphodiesterase is known to degrade polynucleotides in an exonuclease fashion, removing nucleotides in a stepwise manner from the 3′-hydroxyl end of the chain. Thus, all the compounds containing an unblocked nucleoside on the right-hand side are hydrolyzed by this enzyme (although those compounds containing blocks at the left-hand side are hydrolyzed more slowly than the parent dinucleoside phosphates from which they were derived). Resistance to the enzyme occurs when the block is present on the 3′-hydroxyl terminal base (Cp$\bar{U}$ and Cp$\bar{\psi}$). The spleen diesterase is known to remove nucleotides in a stepwise fashion from the 5′-hydroxyl end of a polynucleotide chain. However, in contrast to the snake venom enzyme, this diesterase is capable of hydrolyzing dinucleoside phosphates where the block is located on the end at which the enzyme attacks but is unable to split those containing a block on the adjacent base.

Acknowledgment. This work was supported by the National Institutes of Health and the National Science Foundation.

R. Naylor, Nancy W. Y. Ho, P. T. Gilham
Department of Biological Sciences
Purdue University, Lafayette, Indiana
Received July 27, 1965

The Preparation and Isomerization of the Sulfur-Bonded Monothiocyanate Complex of Chromium(III)[1]

Sir:

It has been observed that thiocyanate ion is a much poorer bridging ligand than azide ion in many electron-transfer reactions.[2,3] This has been ascribed to the fact that, in contrast to N_3^-, the transfer of SCN^- in an inner-sphere reaction may yield an unstable isomer in which the "wrong end" of the thiocyanate is attached to the metal ion. For example, Ball and King[2] have proposed that the $CrNCS^{2+}$–Cr^{2+} exchange is slower than the CrN_3^{2+}–Cr^{2+} exchange because transfer of thiocyanate produces the relatively unstable $CrSCN^{2+}$, while transfer of azide produces a stable product. We have studied the oxidation of chromium(II) by iron(III) in the presence of thiocyanate ions[4] and have established that $CrNCS^{2+}$ and $CrSCN^{2+}$ are produced in this reaction. We have also determined the spectrum of $CrSCN^{2+}$ and some of its chemical properties.

The reactions were studied in 1 *M* perchloric acid at 25.0° on the flow apparatus which has been described previously.[5] The following concentration ranges were used: $(Cr^{2+}) = 5.0 \times 10^{-3}$ to 5.0×10^{-2} *M*, $[Fe(III)] = 5.0 \times 10^{-4}$ to 1.0×10^{-2} *M*, $(SCN^-) = 1.0 \times 10^{-4}$ to 5.0×10^{-3} *M*. On mixing a solution containing Fe(III) and SCN^- with one containing excess Cr^{2+}, three distinct changes in absorbance occur. These changes are most readily detected at 460 mμ (the maximum for $FeNCS^{2+}$) and in the range 260 to 350 mμ (where iron(III) and the chromium(III) thiocyanate complexes have high molar absorbancy indeces). The first change, which is due to the $FeNCS^{2+}$–Cr^{2+} reaction, is too rapid to be followed on the flow apparatus even at reactant concentrations of 5×10^{-5} *M* ($k \geq 2 \times 10^7$ M^{-1} sec.$^{-1}$). The second change in absorbance, of which only the tail is observed, is associated with the Fe^{3+}–Cr^{2+}, $FeOH^{2+}$–Cr^{2+},[6] and Fe^{3+}–Cr^{2+}–SCN^- reactions ($k = 2.3 \times 10^3$ M^{-1} sec.$^{-1}$, 3.3×10^6 M^{-1} sec.$^{-1}$, and $\sim 2 \times 10^5$ M^{-2} sec.$^{-1}$, respectively). The final absorbance change corresponds to the chromium(II)-catalyzed isomerization of the $CrSCN^{2+}$ produced in the first reaction ($k = 42 \pm 4$ M^{-1} sec.$^{-1}$).[7] The Fe^{3+}–Cr^{2+}–SCN^- reaction, which does not involve $FeNCS^{2+}$ as a reactant, was studied by mixing solutions containing Cr^{2+} and SCN^- with solutions containing iron(III). These studies demonstrated that the $CrSCN^{2+}$ was produced in the $FeNCS^{2+}$–Cr^{2+} reaction and not in the oxidation of a chromium(II)–thiocyanate complex by iron(III).[6] The latter reaction does, however, produce some $CrNCS^{2+}$.

The $FeNCS^{2+}$ is quantitatively converted into $CrNCS^{2+}$ and $CrSCN^{2+}$. This establishes that the $FeNCS^{2+}$–Cr^{2+} reaction proceeds *via* an inner-sphere mechanism. It is difficult to determine the yield of $CrSCN^{2+}$ exactly, but it appears to be about 35%. This estimate is based on two different analytical methods. The first involves reaction of $CrNCS^{2+}$ and $CrSCN^{2+}$ with chlorine; the isomers give Cr^{3+} and $CrCl^{2+}$, respectively. In the second method Hg^{2+} is used to convert $CrNCS^{2+}$ and $CrSCN^{2+}$ to $CrNCSHg^{4+}$ and Cr^{3+}, respectively. The products of these reactions were separated by chromatography on a Dowex 50-X8 column.

A solution containing $CrSCN^{2+}$ can be readily pre-

(1) Research performed under the auspices of the U. S. Atomic Energy Commission.
(2) D. L. Ball and E. L. King, *J. Am. Chem. Soc.*, **80**, 1091 (1958).
(3) J. H. Espenson, *Inorg. Chem.*, **4**, 121 (1965).
(4) The monothiocyanate complex of iron(III) is formulated as $FeNCS^{2+}$. This structure has been suggested by S. Fronaeus and R. Larsson, *Acta Chem. Scand.*, **16**, 1447 (1962), on the basis of infrared measurements, and also receives some support from the present studies.
(5) G. Dulz and N. Sutin, *Inorg. Chem.*, **2**, 917 (1963).
(6) G. Dulz and N. Sutin, *J. Am. Chem. Soc.*, **86**, 829 (1964).
(7) The rapidity of the chromium(II)-catalyzed isomerization is probably responsible for the lack of success of earlier attempts [R. L. Carlin and J. O. Edwards, *J. Inorg. Nucl. Chem.*, **6**, 217 (1958)] to prepare $CrSCN^{2+}$ by the reaction of Cr^{2+} with $Co(NH_3)_5NCS^{2+}$. It should be possible to prepare $CrSCN^{2+}$ using this reaction by gradually adding a dilute chromium(II) solution to an excess of a concentrated $Co(NH_3)_5NCS^{2+}$ solution. We have not yet attempted this.