It is also worth noting that the photodimerization reaction at $\lambda > 265$ mμ was accompanied by formation of only traces of UdR—that is, photohydration was practically absent under these conditions. This, together with the absence of an O_2 effect at 254 mμ, and the pronounced influence of O_2 at $\lambda > 265$ mμ, suggests that the difference in photochemical reactions may be due to formation of different excited states, at least in aqueous medium. In a polynucleotide chain, where the EtU residues are suitably oriented for dimerization, both photohydration (with concomitant reversal to hydrated uracil) and dimerization would be expected as competing reactions, and results of preliminary experiments with synthetic polynucleotides are in agreement with this idea. The values for the quantum yields of the two reactions are also qualitatively in agreement with the unchanged sensitivity to radiation of phage T_3 containing incorporated EtU.

These results are of additional interest in relation to the aqueous-solution photochemistry of thymidine. The relative radiation resistance of thymine in aqueous medium at $\lambda = 254$ mμ has been variously interpreted in terms of formation of the photohydrate, which is unstable because of its rapid reversion to the parent compound (*3*, *7*, *8*). However, Nofre and Ogier (*9*), who reported the chemical synthesis of hydrated thymine, claimed that its half-life at neutral *p*H and room temperature is of the order of several hours, apparently in contradiction to the above hypothesis (*9*).

Preliminary trials with a view to demonstrating the possible photohydration and successive photoelimination of methanol from irradiated thymidine have so far been unsuccessful. On the other hand we have found that about 20 percent of the product (or products) of irradiation of thymidine in solution at $\lambda = 254$ mμ can be dark-reverted to one having an absorption maximum at about 265 mμ, but the nature of this product has not yet been elucidated.

Experiments with other 5-alkyl uracil nucleosides have shown some similarity in behavior to EtUdR, one of the final products being UdR. Further studies of these photochemical reactions should shed additional light on the photochemistry of thymine nucleosides and of thymine residues in DNA (*10*).

IRENA PIETRZYKOWSKA, D. SHUGAR
Institute of Biochemistry and Biophysics, Academy of Sciences, and *Department of Biophysics, University of Warsaw, Warsaw, Poland*

References and Notes

1. M. Piechowska and D. Shugar, *Biochem. Biophys. Res. Commun.* **20**, 768 (1965).
2. Pietrzykowska and D. Shugar, *ibid.* **25**, 567 (1966).
3. D. Shugar, in *The Nucleic Acids*, E. Chargaff and J. N. Davidson, Eds. (Academic Press, New York, 1960), vol. 3.
4. R. Bonischen, in *Methods of Enzymatic Analysis*, H. U. Bergmayer, Ed. (Academic Press, New York, 1963), p. 285.
5. A. Smietanowska and D. Shugar, *Bull. Acad. Polon. Sci. Classe II* **11**, 375 (1961).
6. E. Sztumpf-Kulikowska, D. Shugar, J. W. Boag, *Photochem. Photobiol.* **6**, 41 (1967).
7. S. Y. Wang, *Nature* **184**, 184 (1959).
8. E. Fahr, R. Kleber, F. Boebinger, *Z. Naturforsch.* **21b**, 219 (1966).
9. C. Nofre and M.-H. Ogier [*Compt. Rend. Acad. Sci. Paris* **263**, 1401 (1966)] claim to have shown that their synthetic 5,6-dihydro-6-hydroxythymine is the *trans* isomer. It is of course possible, if photohydration of thymine does occur, that the *cis* isomer is formed preferentially and that this is the more labile.
10. We thank Maria Zylonis for technical assistance, and the World Health Organization, the International Atomic Energy Agency, and the Wellcome Trust for support.

4 June 1968

High-Resolution Nuclear Magnetic Resonance Spectra of Selectively Deuterated Staphylococcal Nuclease

Abstract. *An analog of staphylococcal nuclease has been prepared in which all amino acids, except the six following, are fully deuterated: tryptophan; methionine; tyrosine, in ring positions 2 and 6; histidine, in ring position 2; aspartic acid and asparagine, β-methylene; and glutamic acid and glutamine, γ-methylene. The analog has a much simpler high-resolution nuclear magnetic resonance spectrum than the fully protonated enzyme. The effects of calcium ion and of the inhibitor 3′,5′-thymidine diphosphate on the spectrum of the analog were readily detected.*

High-resolution nuclear magnetic resonance (NMR) spectra of proteins have thus far yielded rather little information because of the extensive overlap (envelopes) of the resonance lines of the constituent amino acids. Only the very few lines outside these envelopes —the histidine C2-H (protons on the carbon in the 2-position) resonances, which can be seen downfield from the region of the aromatic spectrum (*1*, *2*) and the high-field peaks seen in some proteins upfield from the methyl region (*3*, *4*)—have proved amenable to detailed interpretation. Nevertheless, these few studies do demonstrate that unique information is provided by NMR spectra of proteins. To take advantage of this information, procedures must be devised for simplification of the spectra.

In principle, the NMR spectral envelope of proteins can be simplified (i) by increasing the strength of the applied field, thereby increasing the separation between component lines, or (ii) by reducing the number of proton resonance lines through selective deuteration of the protein. In practice, however, the best available spectrometer (220 Mhz) does not give sufficient resolution to fully decipher the information contained in the spectrum (*3*). The chemical shifts of numerous amino acid peaks so overlap that many of the relatively broad peaks in proteins would not be separated even at frequencies of 1000 Mhz or more. Furthermore, the task of assigning the multitude of peaks would still remain. Jardetzky has indicated (*5*) that selective deuteration of proteins provides the method of choice in protein NMR spectroscopy. We now report the experimental realization of this proposal. Similar experiments have also been performed by Crespi, Rosenberg, and Katz (*6*).

The criteria for selecting staphylococcal nuclease as the object of our studies have been outlined (*5*). The nuclease and its selectively deuterated analog have been isolated from a pure culture of the Foggi strain of *Staphylococcus aureus* by a modification of the method of Fuchs, Cuatrecasas, and Anfinsen (*7*). In the preparation of the normal, protonated nuclease, the culture was grown on a complex medium containing casamino acids and yeast hydrolyzate. A chemically defined, synthetic medium containing a mixture of deuterated and protonated amino acids was used in the preparation of the selectively deuterated nuclease (*8*). The deuterated amino acid mixture used to prepare the selectively deuterated enzyme was obtained by the hydrolysis of protein isolated from algae (*Scenedesmus obliquus*) grown in 99 percent D_2O by Merck Sharp & Dohme of Canada. A portion of the mixture was separated by ion-exchange column chromatography, and the isotopic composition of the individual amino acids was determined by proton magnetic resonance spectroscopy. Proton resonance lines were un-

detectable except for the following: (i) the 2-position and the 6-position in the tyrosine ring were approximately 80-percent protonated; (ii) the 2-position of the histidine was fully protonated; and (iii) the β-positions of aspartic acid and the γ-positions of glutamic acid were approximately 50- and 30-percent protonated, respectively. Exchange at these sites occurs under the conditions for hydrolysis of the deuterated protein (*2, 9, 11*).

We chose tryptophan and methionine to be the only fully protonated amino acids added in the preparation of this selectively deuterated derivative of staphylococcal nuclease. These two amino acids are present in the deuterated amino acid mixture in low concentration. Thus the presence of the deuterated amino acid could be easily masked by an excess of protonated amino acid. Since there are only four methonine residues and one tryptophan residue in staphylococcal nuclease, we expected a spectrum simple enough for easy interpretation. Furthermore, the methionine-methyl peaks should show up easily since they are singlets of intensity equal to three protons.

The aromatic regions of the proton magnetic resonance spectrum of the nuclease and the deuterated nuclease are shown in Fig. 1, A and B. Peak assignments are indicated by letters above the peaks, and a bar graph indicates the intensities predicted for the peaks in the selectively deuterated analog on the basis of the amino acid composition of the growth medium.

The only positively identified peaks in the nuclease spectrum (Fig. 1A) are the four peaks (S1–S4) corresponding to the C2 protons of the four histidine residues (*1*). The position of these peaks is a sensitive indicator of the conformational integrity of the enzyme molecule (*11*), and the fact that the peak positions of the C2 protons of the histidines of the selectively deuterated enzyme (Fig. 1B) correspond to those of the protonated enzyme suggests that the two are conformationally identical. Since the tyrosine residues are proton-

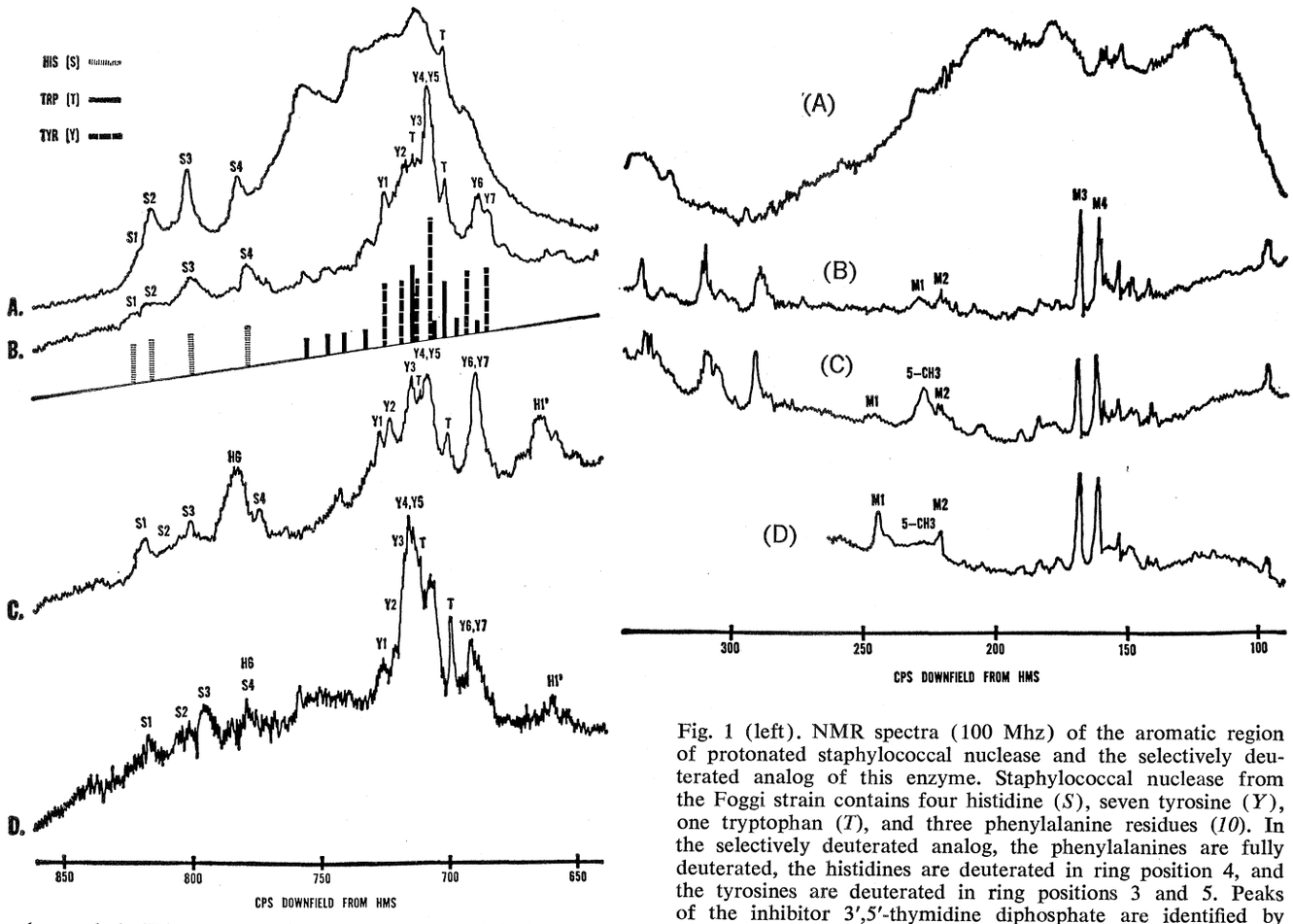

Fig. 1 (left). NMR spectra (100 Mhz) of the aromatic region of protonated staphylococcal nuclease and the selectively deuterated analog of this enzyme. Staphylococcal nuclease from the Foggi strain contains four histidine (*S*), seven tyrosine (*Y*), one tryptophan (*T*), and three phenylalanine residues (*10*). In the selectively deuterated analog, the phenylalanines are fully deuterated, the histidines are deuterated in ring position 4, and the tyrosines are deuterated in ring positions 3 and 5. Peaks of the inhibitor 3′,5′-thymidine diphosphate are identified by the symbols *H6* and *H1′*. All solutions contained 0.3*M* NaCl in 99.87 percent D_2O and were adjusted to *p*H 8.0 $\pm$ 0.1 (uncorrected glass electrode) at 32°C with NaOD or DCl. Spectra were obtained with a Varian HA-100 spectrometer at a sweep rate of 1 hz/sec. The probe temperature was 32°C. Spectra were averaged over the given number of scans with a Varian C-1024 computer of average transients. The external standard used was hexamethyldisiloxane (HMS). (A) Twenty percent solution of protonated staphylococcal nuclease; 60 scans. (B) Six percent solution of the selectively deuterated analog of nuclease; 129 scans. The bar graph indicates the predicted intensities of the spectral lines. (C) Six percent solution of the selectively deuterated analog of nuclease plus the inhibitor 3′,5′-thymidine diphosphate; the molar ratio of inhibitor to enzyme is approximately 3; 147 scans. (D) Six percent solution of the selectively deuterated nuclease analog plus 3′,5′-thymidine diphosphate (the molar ratio of inhibitor to enzyme is approximately 3) plus calcium ion (the molar ratio of Ca^{2+} to enzyme is approximately 10); 130 scans.

Fig. 2 (right). A portion of the aliphatic region of the NMR spectrum (100 Mhz) of staphylococcal nuclease and the selectively deuterated nuclease analog. Solutions and experimental conditions are the same as in Fig. 1, curves A–D. Tentative assignments of the *S*-methyl (*S-CH₃*) peaks (*M1–M4*) of the four methionine residues of the enzyme are given. The 5-methyl (*5-CH₃*) peak of the inhibitor 3′,5′-thymidine diphosphate is identified. (A) Protonated staphylococcal nuclease; 31 scans. (B) Selectively deuterated nuclease analog; 92 scans. (C) Selectively deuterated nuclease plus 3′,5′-thymidine diphosphate; 58 scans. (D) Selectively deuterated nuclease plus 3′,5′-thymidine diphosphate plus calcium ion; 56 scans.

ated in only the 2- and 6-positions of the ring they appear as singlets in the spectrum of the selectively deuterated staphylococcal nuclease. This simplification allows the peak positions of the individual tyrosines to be determined. The positions of the peaks corresponding to the single tryptophan residue were deduced from its unique splitting pattern.

The effect of a threefold excess of the inhibitor deoxythymidine-3′,5′-diphosphate (pdTp) on the spectrum of the selectively deuterated nuclease is shown in Fig. 1C. The most striking change in the spectrum of the nuclease occurs in the region of tyrosine peaks Y4 and Y5. The peak height diminishes by almost one-half in the presence of the pdTp indicating that one of these peaks is either broadened or shifted. Our interpretation favors broadening of this peak. Tyrosine peaks Y6 and Y7 are shifted downfield in the presence of pdTp and appear to become magnetically equivalent. Tyrosine peaks Y1 and Y2 shift downfield slightly. The tryptophan spectrum appears to remain unchanged.

The proton in the 6-position (H6) of the thymine ring of the inhibitor is shifted 35 hz upfield from its position in the absence of selectively deuterated staphylococcal nuclease and is broadened markedly. The proton of the ribose (H1′) is shifted 3 hz upfield and broadened only slightly in the presence of the deuterated nuclease. Similar changes in the pdTp spectrum have been observed in the presence of the protonated nuclease (*11*).

Further changes in the pdTp spectrum of the deuterated nuclease occur on the addition of calcium ion. The H6 peak and, to a lesser extent, the H1′ peak of pdTp are further broadened in the presence of Ca^{2+}. Tyrosine peaks Y6 and Y7 shift further downfield. Tyrosine peaks Y1, Y2, and Y3 are shifted upfield and become more nearly equivalent.

A portion of the aliphatic NMR spectral region of the protonated nuclease is shown in Fig. 2A. The simplified spectrum of the selectively deuterated nuclease is reproduced in Fig. 2B. The methyl peak of methionine monomer occurs at 247 hz. In this region, there appear two broad peaks (M1, M2), tentatively identified as methionine peaks. The sharp peaks at 169 and 162 hz have the proper intensity for methyl peaks and are tentatively labeled M3 and M4. The peaks in the region 350 to 275 hz probably represent peaks corresponding to the γ-CH_2 groups of the four methionine residues, β-CH_2 groups of the 14 aspartic and asparagine side chains and γ-CH_2 groups of the 18 glutamic and glutamine groups. The peak at 97 hz is a sideband from the HMS (hexamethyldisiloxane) peak used as an external standard. The peak identified as M1 is the only resonance line in this region, which appears to be affected by the addition of pdTp (Fig. 2C) and pdTp + Ca^{2+} (Fig. 2D). The 5-methyl peak of pdTp is broadened but not shifted in the presence of the selectively deuterated enzyme. It remains unshifted but broadens when calcium ion is added.

The chemical shifts of amino acid side chain protons in peptides are affected only slightly by peptide bond formation, nearest neighbors (*12*), and helix formation (*13*). The large shifts observed in several peaks of the protonated side chains of the deuterated nuclease from their monomer positions, therefore, reflect the enivronment conferred on them by the tertiary structure of the enzyme. The NMR peaks of the single tryptophan residue of the deuterated enzyme lie 53 hz upfield from their positions in the dipeptide tryptophanylalanine. Shifts of this direction and magnitude are observed on stacking of aromatic rings. The expected chemical shift at *p*H 8.0 for deuterated tyrosine in which the 2- and 6-positions in the ring are protonated is 716 hz, or approximately the position of Y2 (Fig. 1B). Thus, in the deuterated nuclease, one tyrosine is shifted downfield (Y1) and five tyrosines are shifted upfield (Y3 to Y7) from their normal positions. It is the nonequivalence of the tyrosines which makes the aromatic region of the spectrum of protonated nuclease (Fig. 1A) virtually impossible to interpret. The ring protons of tyrosine give an AA′BB′ spectrum which is roughly a quartet. These overlapping quartets probably account for the relatively featureless spectral envelope of the fully protonated nuclease.

The 35-hz upfield shift of H6 of pdTp and the concurrent shifts in the tyrosine peaks of the deuterated enzyme indicate that the inhibitor is bound to the enzyme in the absence of Ca^{2+}. This result is at variance with the conclusions of Cuatrecasas, Fuchs, and Anfinsen (*14*). However, the binding constant in the absence of calcium may be so small that it is undetectable by the methods they used, or it may be altered by the relatively high ionic strength used in our experiments. The addition of Ca^{2+} to a mixture of selectively deuterated nuclease and pdTp does produce further striking changes in the spectrum. The broadening of the H6 peak of pdTp and the further shifting and broadening of the tyrosine peaks indicate the formation of a stronger complex. Even in the presence of Ca^{2+} the bound pdTp appears to be exchanging rapidly with free excess pdTp in solution since only a single broad H6 peak of pdTp is observed. Thus, one may calculate from the observed 35-hz shift in the H6 peak of the bound as compared to the unbound pdTp an upper limit of 5×10^{-3} second for the lifetime of the inhibitor-enzyme complex.

Calcium ion alone at similar concentrations shifts only the histidine C2-H peaks of the deuterated nuclease. Shifts of these C2-H peaks in staphylococcal nuclease in the presence of Ca^{2+} have been reported (*1*).

JOHN L. MARKLEY
IRVING PUTTER
OLEG JARDETZKY

Department of Biophysics and Pharmacology, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065

References and Notes

1. D. H. Meadows, J. L. Markley, J. S. Cohen, O. Jardetzky, *Proc. Nat. Acad. Sci. U.S.* **58**, 1307 (1967).
2. D. H. Meadows, O. Jardetzky, R. M. Epand, H. H. Rüterjans, H. A. Scheraga, *ibid.* **60**, 766 (1968).
3. C. C. McDonald and W. D. Phillips, *J. Amer. Chem. Soc.* **89**, 6332 (1967).
4. H. Sternlicht and D. Wilson, *Biochemistry* **6**, 2881 (1967); J. Cohen and O. Jardetzky, *Proc. Nat. Acad. Sci. U.S.* **60**, 92 (1968).
5. O. Jardetzky, *Proceedings of the International Symposium on Nuclear Magnetic Resonance*, Tokyo, Japan, N-3-14 (1965).
6. H. L. Crespi, R. M. Rosenberg, J. J. Katz, personal communication.
7. S. Fuchs, P. Cuatrecasas, C. B. Anfinsen, *J. Biol Chem.* **242**, 4768 (1967).
8. Description of the procedures used in the growth of the *S. aureus* on a deuterated and protonated amino acid mixture, and details concerning the isolation and characterization of the selectively deuterated staphylococcal nuclease are in preparation.
9. R. B. Martin and V. J. Morlino, *Science* **150**, 493 (1965).
10. J. N. Heins, J. R. Suriano, H. Taniuchi, C. B. Anfinsen, *J. Biol. Chem.* **242**, 1016 (1967).
11. J. L. Markley and O. Jardetzky, unpublished data.
12. A. Nakamura and O. Jardetzky, *Proc. Nat. Acad. Sci. U.S.* **58**, 2212 (1967); *Biochemistry* **7**, 1226 (1968).
13. J. L. Markley, D. H. Meadows, O. Jardetzky, *J. Mol. Biol.* **27**, 25 (1967).
14. P. Cuatrecasas, S. Fuchs, C. B. Anfinsen, *J. Biol. Chem.* **242**, 3063 (1967).
15. The assistance of B. Wilker and A. Barreto in growing the staphylococcus cultures, of M. Feil and C. Short in isolating the enzyme, and of J. S. Cohen in separating the deuterated amino acids, is acknowledged. The initial experiments on selective deuteration were carried out by O. Jardetzky in the laboratory of Professor E. F. Gale, University of Cambridge, whose counsel is appreciated.

24 July 1968