important question is whether, under equilibrium conditions, any structures with intermediate oxygen affinity are populated significantly. Recent kinetic investigations of the cooperative oxygen binding process suggest that intermediate states do not play a significant role (Van Driel *et al.*, 1974). Calculations of Wyman (1969) showed that in systems with a high number of binding sites intermediate conformations may be destabilized as compared to both extreme states. We have tried to trap the protein when it binds oxygen cooperatively, in possible intermediate states by cross-linking partially oxygenated hemocyanin. As shown in Figure 4, the oxygen affinity of cross-linked hemocyanin increased with increasing oxygen saturation at which cross-linking was carried out. The Hill coefficient was slightly higher than 1.0.

If the system can be described by a simple two-state model, it should be possible to describe the oxygen binding curves of cross-linked hemocyanin as mixtures of DSI-deoxy-Hc and DSI-oxy-Hc. Using the hypothetical ratios given in Table II, the fit between the calculated and observed binding curves is surprisingly good (Figure 7). This supports the two-state hypothesis for cooperative oxygen binding, although, it might be that intermediate states are not cross-linked, for instance because DSI might react more rapidly with both extreme states in equilibrium with the intermediates. The possibility to fix hemocyanin in states with different affinities may facilitate investigations of the structural changes that underlie the process of cooperative ligand binding by these giant proteins.

Acknowledgments

The authors thank Miss M. van Essen and Mr. J. Alserda for very capable technical assistance, and Drs. R. N. Campagne, M. Brouwer, H. A. Kuiper, R. J. Siezen, and B. Witholt for discussions.

References

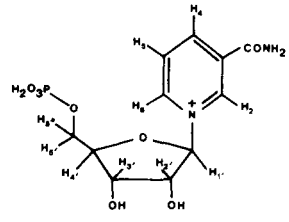
Bates, R. C. (1954), Electrometric pH Determinations, New York, N.Y., Wiley, pp 91–125.
Brouwer, M., and Kuiper, H. A. (1973), *Eur. J. Biochem. 35,* 428–435.
Davies, G. E., and Stark, G. R. (1970), *Proc. Nat. Acad. Sci. U. S. 66,* 651–656.
Dijk, J., Brouwer, M., Coert, A., and Gruber, M. (1970), *Biochim. Biophys. Acta 221,* 467–479.
Er-el, Z., Shaklai, N., and Daniel, E. (1972), *J. Mol. Biol. 64,* 341–352.
Habeeb, A. F. S. A. (1966), *Anal. Biochem. 14,* 328–336.
Heirwegh, K., Borginon, H., and Lontie, R. (1961), *Biochim. Biophys. Acta 48,* 517–526.
Hunter, M. J., and Ludwig, M. L. (1962), *J. Amer. Chem. Soc. 84,* 3491–3505.
Konings, W. N., Van Driel, R., Van Bruggen, E. F. J., and Gruber, M. (1969a), *Biochim. Biophys. Acta 194,* 55–66.
Konings, W. N., Siezen, R. J., and Gruber, M. (1969b), *Biochim. Biophys. Acta 194,* 376–385.
Monod, J., Wyman, J., and Changeux, J. P. (1965), *J. Mol. Biol. 12,* 88–118.
Van Driel, R. (1973), *Biochemistry 12,* 2696–2698.
Van Driel, R., Brunori, M., and Antonini, E. (1974), *J. Mol. Biol. 89,* 103–112.
Wold, F. (1967), *Methods Enzymol. 11,* 617–640.
Wyman, J. (1969), *J. Mol. Biol. 39,* 523–538.

# The Solution Conformation of Nicotinamide Mononucleotide: A Quantitative Application of the Nuclear Overhauser Effect†

William Egan,* Sture Forsén,* and John Jacobus*

ABSTRACT: Torsion about the glycosidic linkage in nicotinamide mononucleotide has been investigated by quantitative application of the nuclear Overhauser effect. These measurements show that the syn ($\chi \simeq 20°$) and anti ($\chi \simeq 200°$) conformers of the title compound are isoenergetic, or nearly so, and interconverting rapidly. The syn/anti partition is not measurably affected by either changes in pH or temperature.

Extensive studies of the pyridine nucleotides, directed toward the elucidation of their solution conformation, have been carried out (for a leading reference, see Blumenstein and Raftery, 1973). The intended goal of these studies has been to relate molecular geometry to biological function. Of the various pyridine nucleotides, nicotinamide mononucleotide ($NMN^+$) has received considerable attention (see Sarma and Mynott, 1973b, and references cited therein), its



$NMN^+$

† From the Division of Physical Chemistry, The Lund Institute of Technology, 220 07 Lund 7, Sweden (W.E. and S.F.), and the Department of Chemistry, Clemson University, Clemson, South Carolina 29631 (J.J). *Received April 19, 1974.*

Table I: Nuclear Overhauser Effect Enhancements for *ca.* 0.25 M $NMN^+$ in $D_2O$ (pD 4.8) at 35°.

| Proton Saturated | Proton Observed | | | | |
|---|---|---|---|---|---|
| | $H_2$ | $H_4$ | $H_5$ | $H_6$ | $H_{1'}$ |
| $H_2$ | | | | | 0.15 |
| $H_4$ | | | | | 0.0 |
| $H_5$ | | | | | 0.0 |
| $H_6$ | | | | | 0.16 |
| $H_{1'}$ | 0.22 | 0.0 | 0.0 | 0.12 | |
| $H_{2'}$ | 0.08 | 0.0 | 0.0 | 0.08 | |
| $H_{3'}$ | 0.06 | | | 0.05 | |
| $H_{5'+5''}$ | 0.0 | | | 0.0 | |

relative simplicity making it a logical starting point for the analysis of the more complicated and, from a biological point of view, more interesting dinucleotides (for a review of the chemistry of the pyridine nucleotides, see Kaplan, 1960).

The conformation of $NMN^+$ may be conveniently delineated in terms of the three dihedral angles $\chi$, $\psi$, and $\phi$ in the nomenclature of Sundaralingam (1969), and the general disposition of the furanose ring, describable in terms of an amplitude, $\tau_m$, and a phase angle, $P$, to pseudorotation (Altona and Sundaralingam, 1972).

Nuclear magnetic resonance (nmr) spectroscopy provides a convenient experimental technique for the solution conformational analysis of $NMN^+$ in terms of these parameters. Thus, in principle, torsion about the sugar–base linkage may be probed through nuclear Overhauser effect studies, and the remaining parameters by application of suitable Karplus relationships to measured spin–spin coupling constants.

This study is concerned with the determination of the nicotinamide-ribose torsion angle, $\chi$, through use of the nuclear Overhauser effect (a preliminary report of this work has appeared; Egan *et al.*, 1973); the results and conclusions are given in the following sections.

## Calculations and Results

In keeping with current terminology, the nuclear Overhauser effect is defined as the fractional enhancement in the integrated intensity of spin $d$ which occurs upon saturation of spin (s) $s$; eq 1 (Schirmer *et al.*, 1972). For a specified molecular geometry, subject to certain conditions and approximations, NOE enhancements are readily calculated (the methods and conditions applying to this study are given in the Experimental Section). Because $f_d(s)$ is a function of internuclear distance, comparison of theoretical calculations with observed enhancements can furnish information about molecular geometry and internal motions (for a discussion of the theory and application of the NOE, see Noggle and Schirmer, 1971; *cf.* Bachers and Schaefer, 1971; Bell and Saunders, 1973).

$$f_d(s) = \frac{\text{area of } d \text{ when } s \text{ is saturated} - \text{equilibrium area of } d}{\text{equilibrium area of } d} \quad (1)$$

The experimental NOE's for $NMN^+$ (pD 4.8, 0.25 M in $D_2O$) are given in Table I. In order to calculate the comparative theoretical Overhauser effects for $NMN^+$ as a function of sugar–base torsion angle, it is necessary to have a model for $NMN^+$ in terms of $\psi$, $\tau_m$, and $P$ (since the magnetogyric ratios for phosphorus, nitrogen, and deuterium are considerably lower than that for hydrogen, it is not necessary to consider dipolar relaxation contributions from these nuclei, and hence the geometry of groups containing only these and spin zero nuclei). We chose a model for $NMN^+$ having a planar furanose ring and the gauche–gauche ($\psi = 60°$) conformation about the $C_{4'}$–$C_{5'}$ linkage (for further details, see the Experimental Section).

The gauche–gauche conformation about the $C_{4'}$–$C_{5'}$ bond was chosen as it has been shown, from application of the Karplus relationship to observed spin–spin coupling constants, to dominate the equilibrium about this bond (Sarma and Mynott, 1973a,b). A quantitative description of the pseudorotational equilibrium of the ribose ring, however, is lacking. Due to uncertainties in the coefficients used in the Karplus equation (a different set of coefficients is, in principle, necessary for *each* four atom fragment) as well as to a lack of information concerning the limiting ribose geometries, a quantitative description of the ribose ring is presently not deducible from nmr measurements of coupling constants; alternate methods to supply this information are unfortunately not yet available. However, even if the details of the ribose pseudorotation were known, the theoretical NOE calculations would additionally require a knowledge of the correlation between the base torsion and ring puckering motions (several theoretical and experimental studies have addressed themselves to this correlation problem; for a recent study, see Saran *et al.*, 1973). In the absence of further information, and considering that Schirmer *et al.* (1972) have noted, during an NOE investigation of several nucleosides, that fits to glycosidic torsion angles are not overly sensitive to ribose conformation, we considered a planar ribose to represent a reasonable and convenient alternative (additionally, see below).

Attempts were first made to interpret the observed enhancements in terms of a single torsional conformer about the $C_{1'}$–N bond. The angle $\chi$ is defined as zero when the nicotinamide $N_1$–$C_6$ bond is cis planar to the ribose $C_{1'}$–$O_{1'}$ linkage; $\chi$ is considered positive for a right-handed rotation and when looking along the C–N linkage, the far bond rotates relative to the near bond. Plots of theoretical enhancements for $f_{1'}(2)$, $f_{1'}(6)$, $f_2(1')$, and $f_6(1')$ as a function of $\chi$ are presented in Figure 1. From inspection of these curves, it is apparent that neither a pure anti ($\chi \simeq 0$–70°) nor a pure syn ($\chi \simeq 210$–260°) conformer will serve to account for the observed enhancements. While the equality of $f_{1'}(2)$ and $f_{1'}(6)$ can be accommodated by the torsional angles $\chi = 160$ or 340°, the magnitude of the theoretical enhancements at both these angles is considerably *lower* than observation. In addition, the remaining theoretical enhancements, particularly $f_2(2')$ and $f_6(2')$ (Figure 1), show little agreement with observation at these two angles. Further efforts to find a single conformation which agreed with observation were not successful.

As this lack of agreement might have arisen from the choice of a planar ribose ring, the theoretical Overhauser effects were recalculated using the 3′-endo, 2′-endo, and 3′-exo ribose geometries (see Altona and Sundaralingam (1972) for the relationship between exo–endo and $\tau_m$–$P$ nomenclatures). These particular conformations were chosen as they account for the ribose geometry of virtually all studied nucleosides and nucleotides (see Altona and Sundaralingam (1972) and references cited therein); indeed, the overwhelming majority of cases are accommodated by the 3′-endo and 2′-endo conformations.

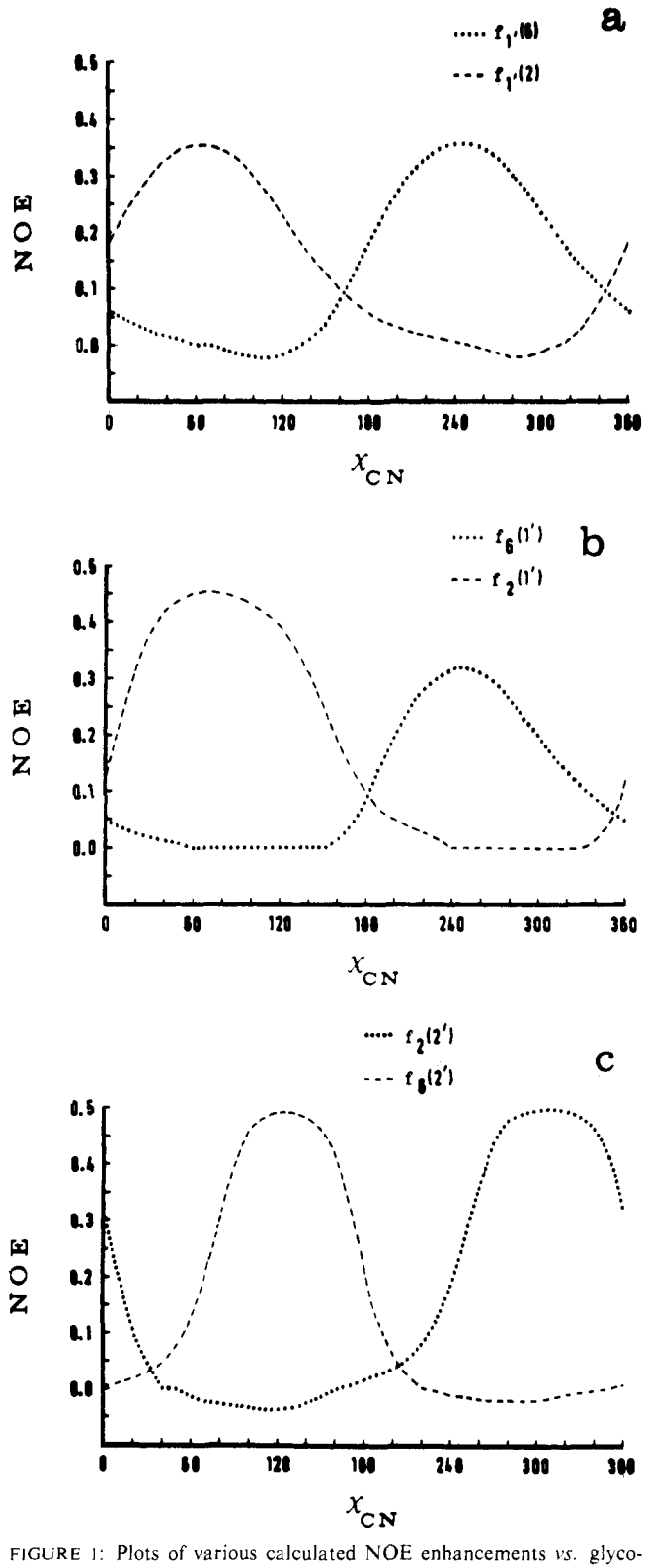



FIGURE 1: Plots of various calculated NOE enhancements *vs.* glycosidic torsion angle, $\chi$.

Use of these nonplanar ribose geometries resulted in Overhauser effect curves between $H_{1'}$ and the nicotinamide ring (in both directions) which were quite similar in shape and magnitude to those obtained using a planar ribose;[1] as an example, $f_{1'}(2)$ for the various ribose conformations is depicted in Figure 2. Comparison of the theoretical curves for each ribose conformation with observation led to the same conclusion, namely, that no single conformer suffices to account for the observed NOE data. Since furanose pseudorotation would serve simply to produce Overhauser effects between $H_{1'}$ and the nicotinamide ring whose magnitudes are properly weighted averages of those for the pure conformers, inclusion of pseudorotational equilibria in the theoretical calculations will likewise not lead to a fitting of the data. Accordingly, we next sought to account for the NOE's by permitting exchange between torsional conformers about the glycosidic linkage.

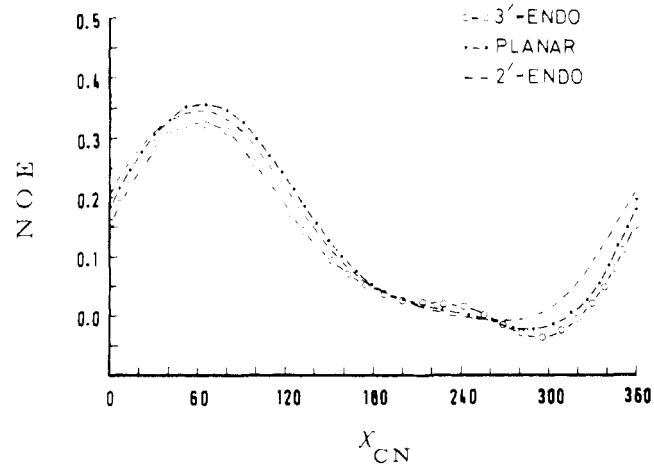



FIGURE 2: Plot of $f_{1'}(2)$ *vs.* glycosidic torsion angle, $\chi$, as a function of ribose geometry.

Two variables thus become necessary to describe the sugar–base torsion: the allowed angles and the probabilities at these angles. Calculations were begun using a two-site, $\chi$ and $\chi + 180°$, distribution. Theoretical calculations of various nucleosides and nucleotides have indicated that a two-site distribution is most probable (see, for examples, Haschemeyer and Rich, 1967; Lakshminarayanan and Sasisekharan, 1969, 1970; Jordan and Pullman, 1968). We initially assumed that the two sites were equally populated.

Under these conditions, one can note from inspection of Figure 3a and b that distributions centered about $\chi$ = 20/200 (the notation 20/200 indicates that the two torsional conformers at the angles $\chi$ = 20° and $\chi$ = 200° are exchanging) and 70/250 are in accord with the experimental values for $f_2(1')$ and $f_6(1')$ and 20/200 and 100/280 distributions with $f_{1'}(2)$ and $f_{1'}(6)$. The theoretical enhancements for $f_2(2')$ and $f_6(2')$ are presented in Figure 3c; while the use of a planar geometry will surely tend to overestimate both the steepness and maximum values of these curves, it is nevertheless apparent that only distributions lying between *ca.* $\chi$ = 20/200 and 60/240 will come into agreement with the low observed values for these enhancements. A $\chi$ = 20/200 geometry thus seems to provide an excellent match of calculated to observed enhancements.

The remaining experimental enhancements were, for the greatest part, in accord with this 20/200 distribution; the exceptions were $f_2(3')$ and $f_6(3')$ which were both calculated as being *ca.* 0.0 and $f_2(5' + 5'')$ and $f_6(5' + 5'')$ which were calculated as being *ca.* 0.06 and 0.04, respectively. A theoretical underestimation of $f_2(3')$ and $f_6(3')$ arises from the restriction of the furanose ring to planarity, *i.e.*, not allowing the 3'-endo conformer to be populated; this accounts, at least partially, for the discrepancy between the

[1] The theoretical Overhauser effects between $H_{2'}$ or $H_{3'}$ and the nicotinamide ring do, however, show a significant variation with ribose conformation; in suitable cases, it seems likely that the Overhauser effect might be exploited for obtaining information on ribose geometry.

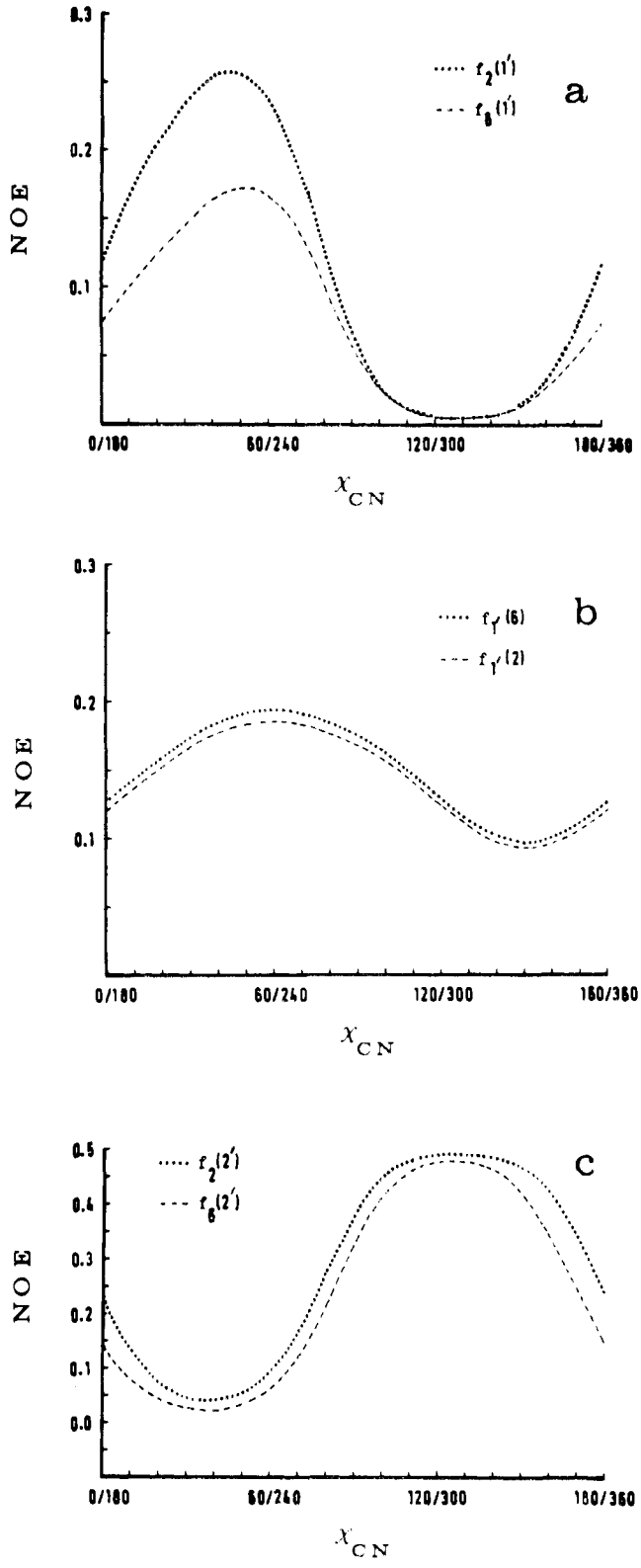



FIGURE 3: Plots of various calculated NOE enhancements *vs.* glycosidic torsion angle, $\chi$, considered as an equal probability, two-site exchange problem.

observed and calculated NOE enhancements. Although we can envision numerous factors which could affect the $f_2(5' + 5'')$ and $f_6(5' + 5'')$ enhancements, we cannot satisfactorily account for, in the sense that we would have predicted, the difference between experiment and calculation that is observed. We mention these discrepancies, which are relatively small and, for $f_2(3')$ and $f_6(3')$, in a direction that we anticipated, for the sake of completeness; they do not alter the preceding conclusion regarding the most probable value for $\chi$.

We next examined the sensitivity of this fit to variation in site distribution. Accordingly, theoretical NOE's as a function of $\chi$ were calculated for various site probabilities (50:50, 45:55, 40:60, and 20:80). The results for $f_2(1')$ and $f_6(1')$ for a 40:60 site distribution are shown in Figure 4a and the analogous curves for $f_{1'}(2)$ and $f_{1'}(6)$ in Figure 4b (the first angle of the set denotes the conformer with population 0.4, the second angle the conformer with population 0.6). Analysis of these curves reveals that, considered as a two-site exchange problem, the 40:60 site distribution does not provide torsion angles exactly in agreement with experimental observation. However, at $\chi$ approximately equal to 20/200 or 200/20, the difference between calculation and experiment is not overly large, *ca.* ±0.03. We therefore consider these 40:60 site distributions to represent acceptable fits to observed data. A 20:80 site distribution, however, appears to lie outside the range of probable error in the analysis. Thus, for example, when $\chi$ is in the range 185/005 to 200/20, $f_2(1')$ lies between 0.18 and 0.26, $f_6(1')$ between 0.05 and 0.07, $f_{1'}(6)$ between 0.06 and 0.08, and $f_{1'}(2)$ between 0.16 and 0.23. Alternatively, when $\chi$ is in the range 20/200 to 60/240, $f_2(1')$ lies between 0.18 and 0.25, $f_6(1')$ between 0.10 and 0.12, $f_{1'}(6)$ between 0.18 and 0.25, and $f_{1'}(2)$ remains constant at 0.08. We note in passing that the sensitivity of various Overhauser effects to changes in site distribution is itself a function of $\chi$; at 20/200 and 200/20, the NOE enhancements are relatively insensitive to changes in site distribution.

Because of its relative simplicity, we should like to mention an additional considered torsional model for $NMN^+$, namely the free-rotation model in which all angles have equal probability.[2] Employing this model, $f_{1'}(2)$ and $f_{1'}(6)$ are both calculated as being *ca.* 0.15, and $f_2(1')$ and $f_6(1')$ as being *ca.* 0.03. This model is clearly not accommodated by observation (see tables).

At this point the experimental data could be fit to gaussian or similar distributions. Due to experimental, model, and theoretical uncertainties (the latter arising since libration about potential minima may be faster than the overall correlation time) we considered such a refinement to be unwarranted.

Upon lowering the sample temperature to 5°, $f_{1'}(2)$ and $f_{1'}(6)$ were found to be 0.14 and 0.15, respectively. Hence, there does not appear to be any appreciable change in site partition accompanying the change in temperature.

The above studies were carried out at pD 4.8. We also investigated $NMN^+$ at pD 7.0 (the $pK_a$ ($H_2O$) for the second ionization of the phosphate group has been shown to be *ca.* 5.8 (Sarma and Mynott, 1973b)); the results are given in Table II. Comparison of Tables I and II reveals little, if any, difference between the observed Overhauser effect for $NMN^+$ at the two pD values. The geometric disposition of $NMN^+$ does not seem, therefore, to be measurably altered

[2] Enhancements for a free-rotation model may be approximated by averaging $r_{ij}$ (see Schirmer *et al.*, 1972) over the circuit employing successively smaller intervals and noting the values that the individual enhancements approach. Equivalently, one may use average $r_{ij}^6$'s obtained by a numerical integration technique, for example, Simpson's rule (Courant, 1937). Strictly speaking, for a free rotor, where "$\tau_i$" < $\tau_c$ the equations used in this study are not applicable; the calculations thus refer to a model in which all angles have equal probability, but interconversions between these angles occur at a rate less than $1/\tau_c$.

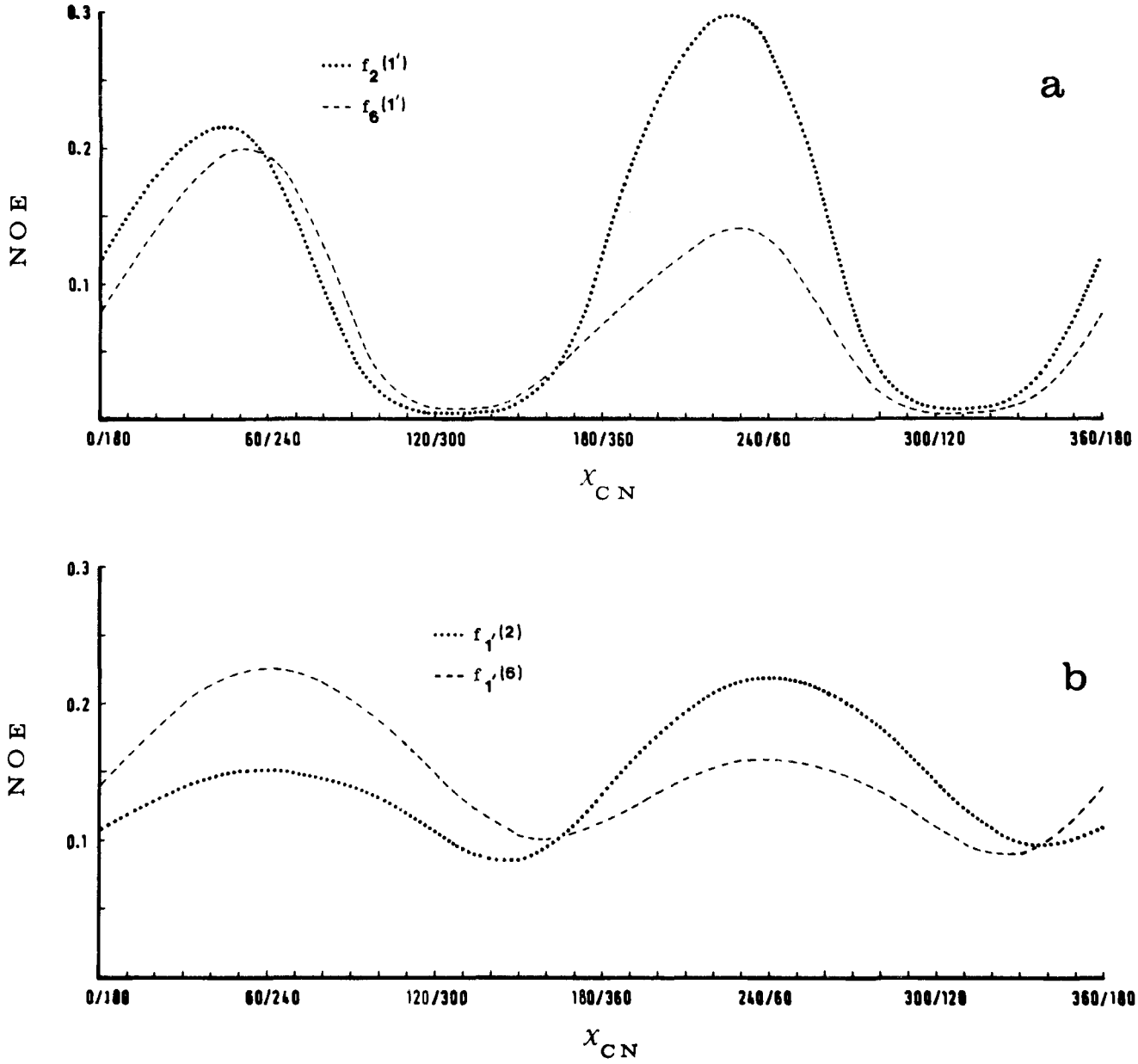



FIGURE 4: Plots of various calculated NOE enhancements *vs.* glycosidic torsion angle, $\chi$, considered as an unequal probability (40:60), two-site exchange problem.

Table II: Nuclear Overhauser Effect Enhancements for *ca.* 0.25 M $NMN^+$ in $D_2O$ (pD 7.0) at 35°.

| Proton Saturated | Proton Observed | | | | |
|---|---|---|---|---|---|
| | $H_2$ | $H_4$ | $H_5$ | $H_6$ | $H_{1'}$ |
| $H_2$ | | | | | 0.14 |
| $H_6$ | | | | | 0.17 |
| $H_{1'}$ | 0.21 | 0.0 | 0.0 | 0.15 | |
| $H_{2'}$ | 0.09 | | | 0.06 | |

by the change in phosphate ionization. This change in ionization may, however, be somewhat less than one expects on the basis of $pK_a$ and pD values due to the possible intervention of a deuterium isotope effect (Meadows, 1972).

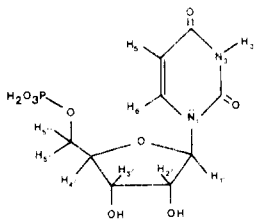
Having equated the observed enhancements to a set of parameters, the question of solution degeneracy naturally arises. Unfortunately, no investigations into this aspect of Overhauser effects are extant. For $NMN^+$, more complete solutions than the one offered here undoubtedly exist; that is, solutions which take into account rapid (relative to $\tau_c$) torsional oscillations about potential minima, the presence of and correlation of additional internal motions, population in ranges other than those considered, *etc.* The taking of these considerations into account should not, however, result in a model of $NMN^+$ that differs *in essence* from that already presented. Thus, the low observed values for $f_2(2')$ and $f_6(2')$ will, in any model, rule out *significant* population in the ranges centered about $\chi$ = 120 and 300°, while both the equality of $f_{1'}(2)$ and $f_{1'}(6)$ and the high values for $f_2(1')$ and $f_6(1')$ will, in any model, rule out *significant* deviation from equal population in the two ranges, $\chi$ *ca.* 0–60 and 180–240°. We emphasize, however, that the exact values for $\chi$ determined by this study for $NMN^+$ should be taken with due caution.

Son *et al.* (1972) have recently expressed the view that

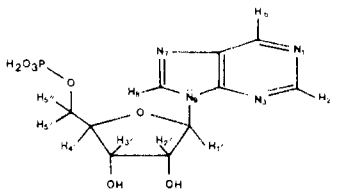
an elaborate fitting of observed Overhauser enhancements to theoretical calculations, such as those carried out by Schirmer *et al.* (1972) or here is not warranted, due to uncertainties in our present knowledge of the geometry and internal modes of motion of various nucleosides and nucleotides; they accordingly employed a simplified theoretical model for interpreting Overhauser enhancements. While we concur with these authors that our present knowledge of nucleoside and nucleotide geometry is limited, we maintain that it is only through a full theoretical treatment that the sensitivity of the measured Overhauser effects to the parameters of interest is determinable.

## Discussion

Comparison of our results with direct studies, that is, X-ray structural and theoretical investigations, is unfortunately not possible, these being not yet available for the free pyridine nucleotides. Our results, however, may be discussed in terms of the somewhat analogous pyrimidine and purine 5′-nucleotides, of basic structures **1** and **2**, respectively.



1



2

To date, the results of X-ray structural studies on 5′-nucleotides, with regard to their torsional distribution about the sugar-base linkage, may be summarized as follows: the glycosyl conformation is always in the anti range and the allowed range of $\chi$ is relatively small, being between *ca.* 0 and 70° (Sundaralingam, 1973; Yathindra and Sundaralingam, 1973). Moreover, the $\chi$ distribution appears to be bimodal, and correlatable with the furanose conformation (the two observed sugar conformations being the $C_{3'}$-endo and the $C_{2'}$-endo). The purine and pyrimidine nucleosides appear to be conformationally less rigid and both the syn and anti conformers have been noted (Sundaralingam, 1973); for these nucleosides, however, it is primarily the purine compounds which exhibit the syn conformation (Sundaralingam, 1973; Olsen, 1973). The preferred values of $\chi$ appear to be: 0–70° for pyrimidine nucleosides and 0–60° and 210–225° for purine nucleosides; a few exceptions to these ranges exist (Sundaralingam, 1969, 1973). The results of solution state studies of 5′-mononucleotides are conflicting (see, for examples, Son and Chachaty, 1973; Barry *et al.*, 1974); we hope to comment further on this point at a later time.

The values of the range of $\chi$ found for $NMN^+$ correspond to those generally found for purine and pyrimidine nucleosides and nucleotides. This general preference for the common syn and anti ranges appears to be more a reflection of the instability associated with the ranges centered about 120 and 300°, arising from the interaction of the ortho substituents of the base with the ribose $H_{2'}$ proton, than the special stability of the other ranges (see, for example, Lakshminarayanan and Sasisekharan, 1970); for $NMN^+$, the minimum $H_2$–$H_{2'}$ distance for the 2′-exo conformer is *ca.* 1.7 Å.

In contrast to the purine and pyrimidine 5′-nucleotides, $NMN^+$ appears to show no marked preference for either the syn or anti geometry. Although it is tempting to rationalize this lack of preference in terms of the two equivalent ortho substituents of the pyridine base (attributing thereby the observed behavior of the pyrimidine and purine nucleotides to some factor, apparently "steric repulsion," residing in their respective ortho substituents), too many factors, as yet not evaluated, contribute to the syn/anti partition to permit proposing such an explanation. Insight into the nature of the torsional process in $NMN^+$ and related compounds must therefore await the results of theoretical calculations and further experimental studies.

The present work may be compared with previous determinations of the syn/anti ratio in $NMN^+$ (Sarma and Kaplan, 1969; Kaplan and Sarma, 1970; Sarma and Mynott, 1973a,b). These determinations, based on the changes in the $H_2$ and $H_6$ $^1H$ nmr chemical shifts as a function of phosphate ionization, indicated that $NMN^+$ exists exclusively or overwhelmingly in the syn conformer. Numerous assumptions go into such a determination, most of which are unable to be evaluated;[3] hence the significance of conclusions drawn from these studies is uncertain (for a related criticism of such studies as applied to $NAD^+$ and NADH, see Jacobus, 1971). In contrast, the NOE possesses a simple relationship between observation and geometry; moreover, geometric models arising from NOE studies on a given system are readily evaluated for parameter sensitivity.

It is evident from this study that the syn and anti forms of $NMN^+$ are isoenergetic, or nearly so, and interconverting rapidly on the nmr chemical shift time scale; were the interconversion not rapid, resonance doubling of all or some of the ten diastereotopic hydrogens would be expected in its $^1H$ nmr spectrum (this argument may be further strengthened by noting that no resonance doubling is observed in the C-13 nmr spectrum of $NMN^+$; see, Blumenstein and Raftery, 1973). An ultrasonics study of Rhodes and Schimmel (1971) has indicated that the interconversion rate of the syn and anti forms of several nucleosides is on the order of $10^9$ $sec^{-1}$; one expects the same order of magnitude for $NMN^+$. As a result of the relatively small energy difference between the syn and anti forms, and their apparently low barrier to interconversion, the partition may be readily altered by comparatively weak interactions, such as those involved in enzyme binding.

## Experimental Section

Samples of $NMN^+$ were purchased from both Sigma and PL Biochemicals and used without further purification. Following lyophilization from $D_2O$ (3x), *ca.* 0.25 M solu-

[3] These assumptions include: (1) that the phosphate group does not influence $H_2$ or $H_6$ when they reside "exo" to the phosphate group; (2) that $H_2$ and $H_6$ are relatively shielded to the same extent when they are "endo" to the phosphate group; (3) that the syn/anti ratio, or any other mode of internal motion is not itself a function of medium pH; (4) that the syn and anti ranges differ by 180°. Additionally, one must distinguish between magnetic shielding and electrostatic field effects (T'so, 1970); the latter mechanism will, for equal geometric dispositions of the phosphate to the test proton, influence most strongly the most acidic proton. The 2 position of $NMN^+$ has been shown to be more acidic than the 6 position (Dubb *et al.*, 1958). Thus, the greater downfield shift of the $H_2$ proton of $NMN^+$ relative to the $H_6$ proton could have arisen from the greater sensitivity of $H_2$ to phosphate ionization rather than the proposed preference for the syn geometry. That the interpretation of $NMN^+$ chemical shifts is even more complex than noted above comes from the recent observation of the $^{13}C$ chemical shifts of $NMN^+$ as a function of pH (Blumenstein and Raftery, 1973).

tions of $NMN^+$ in $D_2O$ (at pD 4.8 and 7.0) were degassed by the usual freeze–thaw method (five cycles at $5 \times 10^{-4}$ Torr) and sealed under vacuum in thin-wall nmr tubes. The pD values were determined by adding a correction factor of 0.4 (Lumry *et al.*, 1951) to the measured pH values (obtained with a Radiometer PHM63 digital pH meter).

All nmr measurements were performed on a Varian XL-100 spectrometer, with the deuterium resonance of $D_2O$ serving as the internal lock. A saturating rf field of *ca.* 0.25 mG was, except as noted, employed for the NOE measurements. The magnitude of this field was approximated from a plot of $f_2(1')$ as a function of the $H_{1'}$ irradiating frequency (see, Schirmer *et al.*, 1970).

Signal integration was performed both electronically and mechanically (use of a compensating planimeter); due to spin–spin couplings, changes in signal areas were generally not reflected in changes in peak heights. Electronic integration was carried out twice, alternatively recording five traces with the saturating field off-resonance, then five traces with the saturating field on-resonance; spectra for mechanical integration were five times alternatively recorded as above. The results of the two procedures, each performed twice on different occasions, did not differ significantly (±0.03), and were averaged. Irradiation of either a blank portion of the spectrum or the residual HDO peak caused no observable change in the signal areas of $H_2$ or $H_6$ relative to their value in the absence of a saturating field. Addition of $10^{-3}$ M EDTA had no observable effect on the high-resolution spectrum.

Nicotinamide mononucleotide presents a far from ideal case for measuring NOE enhancements, and a few words concerning experimental difficulties are in order (the high-resolution spectral parameters for $NMN^+$ above and below the pH for the second phosphate ionization are given in Sarma and Mynott, 1973b). The easiest and most accurate NOE enhancements to measure are $f_2(1')$ and $f_6(1')$ since the $H_{1'}$ resonance lies in a region of the spectrum unencumbered by other signals and can therefore be irradiated without altering the magnetizations of other resonances. Additionally, the $H_2$ and $H_6$ resonances do not overlap either with themselves or with other resonances, and are thus readily measured.

Since the observed low values for $f_2(2')$ and $f_6(2')$ play a significant role in the present analysis, it is necessary to demonstrate that $H_{2'}$ was indeed irradiated and that sufficient power was put into the signal to cause saturation. The $H_{2'}$ resonance frequency was located by observing the collapse of the $H_{1'}$ doublet ($J$ = 5.4 Hz) to a singlet; as spin 38, Hoffman and Forsén, 1966), it is clear that both conditions are fulfilled.

For the $NMN^+$ sample at pD 4.8, the chemical shift difference between $H_2$ and $H_6$ is *ca.* 17 Hz. We therefore found it advantageous to use a slightly weaker saturating rf field for the determinations of $f_{1'}(2)$ and $f_{1'}(6)$, in order to minimize the possibility of simultaneously altering the magnetizations of $H_2$ and $H_6$. The saturating field was decreased until the value for $f_{1'}(2)$ was approximately zero when the saturating field was shifted 15 Hz off the $H_2$ reference. Possibly, this results in a slight underestimation of $f_{1'}(2)$ and $f_{1'}(6)$. Such a procedure was not necessary at pD 7.0, where the chemical shift difference was 23 Hz.

Since the chemical shift difference between $H_{5'}$ and $H_{5''}$ was small, and there were large spin–spin couplings between and to these resonances, it was not possible to saturate either of them selectively. We thus attempted to satu- decoupling will require more power than saturation (see p rate them simultaneously using a single decoupling frequency; it is doubtful that sufficient power was available to accomplish this. Triple irradiation was not attempted.

The chemical shift differences between $H_{3'}$ and $H_{2'}$ or $H_{4'}$ are not overly large: as a result, it is conceivable that saturation of any one of these signals resulted in a slight disturbance of the remaining signals. We did not attempt to compensate for this.

The theoretical NOE enhancements, as a function of site distribution and torsion angle, were calculated directly from eq A-2 of Schirmer *et al.* (1970); a computer program was written (FORTRAN IV) for this purpose. It was assumed that all nuclei were under the extreme narrowing condition, that relaxation was exclusively dipole–dipole, that cross-correlation effects were negligible, and that a single correlation time suffices for all interaction vectors. The theoretical NOE's presented in this article involving site exchange refer to case II conditions as defined by Schirmer *et al.* (1972). NOE's for site exchange under their case I conditions were also calculated and the result of the analysis was essentially the same as that obtained using case II conditions. However, as mentioned in the text, it is extremely improbable that case I conditions prevail, *i.e.*, that $k_{\text{exchange}} \ll 1/T_1 \simeq 1\ \text{sec}^{-1}$.

The bond lengths and angles for $NMN^+$ used in our calculations were based on X-ray structural studies of nicotinamide (Wright and King, 1954) and ribose structures presented by Arnott (1970) and Lakshminarayanan and Sasisekharan (1970).

References


Altona, C., and Sundaralingam, M. (1972), *J. Amer. Chem. Soc. 94,* 8205.

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

Bachers, G. E., and Schaefer, T. (1971), *Chem. Rev. 71,* 617.

Barry, C. D., Glasel, J. A., Williams, R. J. P., and Xavier, A. V. (1974), *J. Mol. Biol. 84,* 471.

Bell, R. A., and Saunders, J. K. (1973), *Top. Stereochem. 7,* 1.

Blumenstein, M., and Raftery, M. A. (1973), *Biochemistry 12,* 3585.

Courant, R. (1937), Differential and Integral Calculus, Vol. 1, London, Blackie and Sons, Ltd., p 344.

Dubb, H. E., Saunders, M., and Wang, J. H. (1958), *J. Amer. Chem. Soc. 80,* 1767.

Egan, W., Forsén, S., and Jacobus, J. (1973), *J. Chem. Soc., Chem. Commun.,* 42.

Haschemeyer, A. E. V., and Rich, A. (1967), *J. Mol. Biol. 27,* 369.

Hoffman, R., and Forsén, S. (1966), *Progr. Nucl. Magn. Reson. Spectrosc. 1,* 38.

Jacobus, J. (1971), *Biochemistry 10,* 191.

Jordan, F., and Pullman, B. (1968), *Theor. Chim. Acta 9,* 242.

Kaplan, N. O. (1960), *Enzymes, 2nd Ed. 2,* 105.

Kaplan, N. O., and Sarma, R. H. (1970), *in* Pyridine Nucleotide-Dependent Dehydrogenases, Sund, H., Ed., Berlin, Springer Verlag, p 39.

Lakshminarayanan, A. V., and Sasisekharan, V. (1970), *Biochim. Biophys. Acta 204,* 49.

Lumry, R., Smith, E. L., and Glantz, R. R. (1951), *J. Amer. Chem. Soc. 73,* 4330.

Meadows, D. (1972), *Methods Enzymol. 26,* 638.

Noggle, J. H., and Schirmer, R. E. (1971), The Nuclear Overhauser Effect: Chemical Applications, New York, N.Y., Academic Press.
Olsen, W. K. (1973), *Biopolymers 12,* 1787.
Rhodes, L. M., and Schimmel, P. R. (1971), *Biochemistry 10,* 4426.
Saran, A., Perahia, D., and Pullman, B. (1973), *Theor. Chim. Acta 30,* 31.
Sarma, R. H., and Kaplan, N. O. (1969), *Biochem. Biophys. Res. Commun. 36,* 780.
Sarma, R. H., and Mynott, R. J. (1973a), *J. Chem. Soc., Chem. Commun.,* 975.
Sarma, R. H., and Mynott, R. J. (1973b), *J. Amer. Chem. Soc. 95,* 1641.
Schirmer, R. E., Davis, J. P., Noggle, J. H., and Hart, P. A. (1972), *J. Amer. Chem. Soc. 94,* 2561.
Schirmer, R. E., Noggle, J. H., Davis, J. P., and Hart, P. A. (1970), *J. Amer. Chem. Soc. 92,* 3266.
Son, T. D., and Chachaty, C. (1973), *Biochim. Biophys. Acta 335,* 1.
Son, T. D., Guschlbauer, W., and Gueron, M. (1972), *J. Amer. Chem. Soc. 94,* 7903.
Sundaralingam, M. (1969), *Biopolymers 7,* 821.
Sundaralingam, M. (1973), *in* The Jerusalem Symposia on Quantum Chemistry, Vol. 5, Bergman, E. S. and Pullman, B., Ed., Jerusalem, Jerusalem Academic Press, p 417.
T'so, P. O. (1970), *in* Fine Structure of Protein and Nucleic Acid, Fasman, G. D., and Timasheff, S. N., Ed., New York, N.Y., Marcel Dekker, p 49.
Wilson, H. R., and Rahman, A. (1971), *J. Mol. Biol. 56,* 129.
Wright, W. B., and King, G. S. D. (1954), *Acta Crystallogr. 7,* 283.
Yathindra, N., and Sundaralingam, M. (1973), *Biopolymers 12,* 2261.

# Neural Regulation of Mammalian Fast and Slow Muscle Myosins: An Electrophoretic Analysis†

J. F. Y. Hoh*

ABSTRACT: Mammalian nerves to fast and slow muscles have the remarkable property of changing the speed of contraction of muscles following cross-reinnervation. The biochemical basis of speed transformation is the change in myosin in ATPase activity. This paper provides electrophoretic evidence for structural changes in myosin from cross-reinnervated muscles. A method is described for the separation of intact fast and slow muscle myosins by polyacrylamide gel electrophoresis. This method utilizes the fact that ATP and its analogs prevent the formation of myosin polymers in low ionic strength buffers. In this system, normal fast muscle myosin has a higher electrophoretic mobility than slow muscle myosin. Normal rat soleus myosin has a major slow and a minor fast component due to two populations of muscle fibers. The same muscle cross-reinnervated by a fast muscle nerve shows only the fast component. The normal, homogeneous fast extensor digitorum longus muscle has only the electrophoretically fast myosin, but following cross-reinnervation it shows both fast and slow components. These results suggest that mammalian motor nerves can induce or suppress the expression of genes that code for fast and slow skeletal muscle myosins.

Motor nerves exert a trophic effect upon skeletal muscles. A remarkable example of this trophic effect was the discovery by Buller *et al.* (1960) that when nerves to fast- and slow-contracting muscles in a mammalian limb were cut and sutured cross-wise, so that the fast muscle became reinnervated by the nerve to slow muscle and *vice versa* (an operation known as nerve cross-union), the time course of the isometric twitch of these muscles became altered: the fast muscle became slow, while the slow muscle became fast. Detailed physiological analysis of normal fast and slow mammalian muscles by Close (1964) revealed that they differ in the intrinsic speed of shortening of their sarcomeres. This implies that the rate of sliding of thick and thin filaments relative to each other under unloaded conditions is different in fast and slow muscles. Furthermore, Close (1969) showed that the intrinsic speeds of sarcomere shortening in cross-reinnervated muscles were reciprocally altered. These physiological studies suggest that the site of action of the neural influence on speed is the contractile material, *i.e.*, actin or myosin. The work of Bárány (1967) narrowed this to myosin since he showed that the actin-activated ATPase activity of myosins from muscles of different speed was correlated with the speed of muscle shortening, whereas myosin ATPase activity was independent of the source of actin used in the ATPase reaction. This concept of the biochemical basis of speed transformation has been corroborated in studies which showed correlated changes in myosin ATPase activity and muscle speed in cross-reinnervated muscles (Buller *et al.*, 1969; Bárány and Close, 1971).

It has been postulated that mammalian nerves bring about speed changes by regulating the expression of genes that code for myosin (Hoh, 1969; Buller *et al.*, 1969; Guth *et al.*, 1970). A major difficulty in testing this hypothesis is

† From the Department of Pharmacology, University of Illinois at the Medical Center, Chicago, Illinois, 60612, and the Department of Physiology, University of Sydney, N.S.W. 2006, Australia. *Received July 17, 1974.* This work was supported by U. S. Public Health Service N.I.N.D.S. Grant N.B. 05679 to Dr. B. Salafsky and the National Health and Medical Research Council of Australia.

* Present address: Department of Physiology, University of Sydney, N.S.W. 2006, Australia.