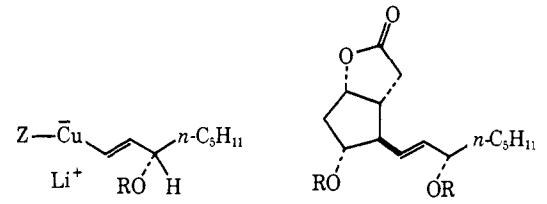
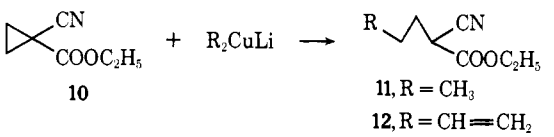
$CDCl_3$) due to $COOCH_3$ at δ 3.84 and 2 olefin H as a quartet centered at 6.08) in 95% yield. Thermolysis of **3** in the presence of copper powder (Fisher, electrolytic, 10 g/g of **3**) in xylene (20 ml/g of **3**) at reflux under nitrogen for 6 hr afforded in *ca.* 50% yield[8] the tricyclic lactone ester **4**[6] (infrared max (neat) at 5.62 and 5.77 μ, no olefinic proton peaks in the nmr spectrum). Reaction of **4** with divinylcopperlithium[9] in ether (2.0 equiv) at $-12°$ for 19 hr followed by addition of aqueous ammonium chloride solution and extraction gave the vinylcyclopentane lactone ester **5**, which was treated directly with dry lithium iodide (5 equiv) in pyridine[10] (2 ml/g of **5**) at reflux for 3 hr to produce the desired lactone **6**[6] in *ca.* 37% yield[8] as a colorless oil, ir max ($CHCl_3$) 5.65 μ (γ-lactone), 6.09, 10.10, 10.85 ($-CH{=}CH_2$). The structure and stereochemistry of this product were established by comparison with an authentic sample obtained by the reaction of the previously prepared lactone aldehyde **7**[4a] with methylenetriphenylphosphorane.[11]

Having established the feasibility of the conversion **4** → **5** by homoconjugate addition of vinyl, we are now studying the synthesis and combination of the optically active **4** with the (*S*)-vinylcopper reagent **8** which should afford an intermediate **9** that has previously been converted to prostaglandins.[4b] A report of this work will be made in due course.



8 **9**

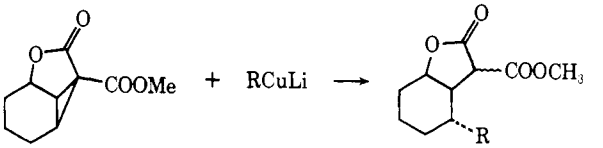
Some evidence of generality for the homoconjugate addition process involving organocopperlithium reagents has also been obtained. Reaction of ethyl α-cyanocyclopropanecarboxylate (**10**)[12] with dimethylcopperlithium (2 equiv) in ether (70 ml/g of **10**) at $-20°$ for 45 min yielded 75% of ethyl α-cyanovalerate (**11**).[6,8] Similarly, the reaction of **10** with 2 equiv



10 **11**, R = CH_3; **12**, R = $CH{=}CH_2$

of divinylcopperlithium in ether–tetrahydrofuran (10:1, 27 ml/g of **10**) at $-10°$ for 5 hr and 3° for 18 hr gave ethyl 2-cyano-5-pentenoate[6,8] (**12**) in 70% yield.

Further, the tricyclic lactone **13** with dimethylcopperlithium (1.5 equiv) in ether ($-10°$, 40 min, and 0°, 5 min) yielded **14**[6,8] (60%), and reaction of **13** with divinylcopperlithium (1.5 equiv) in ether ($-3°$, 13 hr) afforded **15**[6,8] (60% yield). The starting lactone **13**,[6] mp 93–94°, was obtained by a synthesis, starting with the methyl malonyl ester of 2-cyclohexen-1-ol, which parallels that outlined for the cyclopentyl analog **4** in Chart I.



13 **14**, R = CH_3; **15**, R = $CH{=}CH_2$

The synthetic approach to prostanoids by the scheme disclosed above has a number of potential advantages, not the least of which is brevity.[13,14]

(13) This work was assisted financially by grants from the National Institutes of Health and the Agency for International Development.
(14) We are grateful to our colleagues Drs. Ronald Ruden and David Beames for helpful suggestions.

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Negative Nuclear Overhauser Effects as Probes of Macromolecular Structure

Sir:

The investigation of the structure of biomacromolecules is a matter of intense current interest in molecular biology and biochemistry. Of diverse methods employed, X-ray diffraction[1] and nmr[2] have proven extremely powerful in recent years. We wish to report an extension of the nmr method which appears to have great potential in such investigations.

The nuclear Overhauser effect (NOE) is defined as the change in integrated intensity of a nuclear resonance signal arising from one nucleus caused by saturation of the signal from a second nucleus. The change occurs as a result of nuclear polarization from one nucleus to another *via* relaxation mechanisms which contribute to the spin lattice relaxation time (T_1). Detailed discussion of the chemical applications of this phenomenon have been published.[3] While intramolecular effects have been extensively studied, intermolecular effects have been relatively neglected. Two reports of intermolecular NOE's have appeared. Kaiser[4] observed an increase in the intensity of the chloroform signal when the cyclohexane signal in a chloroform–cyclohexane–TMS mixture was saturated. Chan and Kreishman[5] observed selective increases in the intensities of purine protons 2 and 8, in a solution of purine and polyuridilic acid, when specific sugar resonances on the polymer were saturated. The polymer was in a random coil form and rapid segmental motion leading to long relaxation times yielded sharp lines.[6] In both cases an increase in the intensity of the signal was observed as predicted by theory.[3]

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(8) This yield has not been optimized.
(9) E. J. Corey and R. L. Carney, *J. Amer. Chem. Soc.*, **93**, 7318 (1971).
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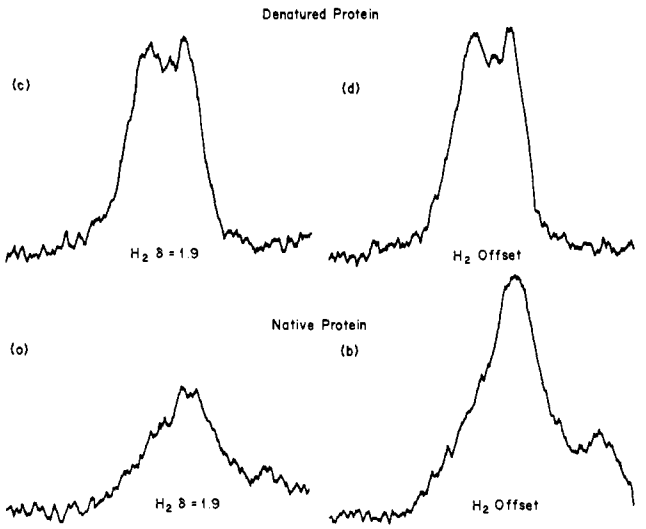

Figure 1. Phenylalanine aromatic protons of bovine neurophysin II at 250 MHz. Protein concentration 10 mg/ml in D_2O.

In a collaborative study, with Dr. E. Breslow, of the binding of the hormone lysine-vasopressin and peptide analogs to bovine neurophysin II, we have observed differential broadening of certain peptide proton signals. This selective enhancement of the transverse relaxation rates ($1/T_2$) presumably arises from the proximity of magnetic nuclei on the protein. Such a dipolar interaction could contribute to a selective enhancement of the longitudinal relaxation rate ($1/T_1$) of certain peptide protons and, in principle, an intermolecular Overhauser effect should be observable. As reported in the succeeding paper,[7] we have observed marked *decreases* in intensity of peptide proton signals, on irradiation of specific protein resonances. Intramolecular effects were also observed. Figure 1 shows the resonances at 250 MHz of residues in neurophysin II with (a) and without (b) a strong irradiation by a secondary radiofrequency at δ 1.9 ppm from DSS. A pronounced decrease in intensity is observed. When the protein is denatured, no appreciable change in intensity is detectable with (c) and without irradiation (d).

Negative NOE's[8] have been observed in the following cases: (1) nuclei having gyromagnetic ratios of opposite sign, (2) intermediacy of a third spin, and (3) occurrence of exchange modulation of scalar coupling. None of these effects are applicable in this case. A plausible explanation is as follows: the energy level diagram for a two-spin system (I, S) in which there is no scalar coupling is shown in Figure 2. The W_i's are the transition probabilities for the various dipole–dipole transitions. The fractional enhancement of the integrated intensity of I ($\langle I_z \rangle$) when S is saturated compared to the equilibrium intensity I_0 is

$$f_I(S) \equiv \frac{W_2 - W_0}{W_0 + 2W_1 + W_2} \frac{S_0}{I_0}$$

Using the equations derived by Solomon,[8] for the transition probabilities for two like spins, *i.e.*, $(\omega_I - \omega_S)^2\tau_c^2 \ll 1$, we get

$$f_I(S) = \frac{5 + \omega^2\tau_c^2 - 4\omega^4\tau_c^4}{10 + 23\omega^2\tau_c^2 + 4\omega^4\tau_c^4}$$

for the spin $^1/_2$ nuclei.

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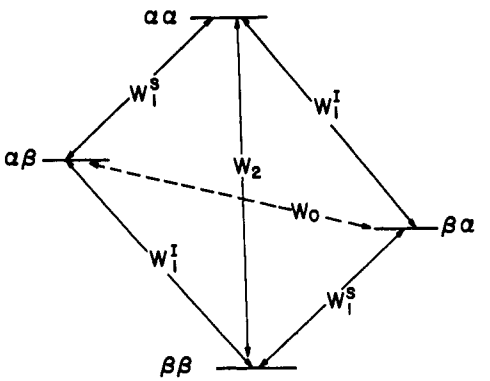

Figure 2. Energy level diagram for a two-spin system; $I = S = 1/2$.

In the extreme narrowing limit where $\omega^2\tau_c^2 \ll 1$ (*i.e.*, for small molecules having $\tau_c \approx 10^{-11}$ sec), $f_I(S) = 0.5$, which corresponds to a maximum enhancement of 50%. For large molecules, such as globular proteins, the correlation times are likely to be long and $f_I(S)$ approaches the limit -1, *i.e.*, disappearance of the signal.

Hoffman and Forsen[9] have shown that the Bloch equations modified by McConnell[10] for chemical exchange and the equations derived by Solomon[8] for a pair of mutually relaxing spins are analogous. They pointed out that for scalar interactions $W_0 > W_2$, and the coupling between the magnetizations of the two spins would have the same sign in the case of both chemical exchange and mutual relaxation. It has been shown here that $W_0 > W_2$ even in the case of a dipolar interaction provided the correlation time modulating the coupling is sufficiently long. In a chemically exchanging system saturation of the nucleus at one site results in a saturation of the signal at the second site provided[3] the exchange rate $k > 1/T_1$. In a mutually relaxing spin system, characterized by long motional correlation times, there is a transfer of spin saturation by the "flip-flop" mechanism, which can be viewed as analogous to a chemical transfer. The model presented here is qualitative and a cautionary note must be sounded regarding the use of a simple two-spin system to approximate complex multispin systems and the consequent neglect of cross correlation effects.

Additional experiments supporting this interpretation have been performed. Negative intramolecular NOE's on the aromatic resonances of insulin and lysozyme have been observed. Denaturation causes these effects to be vastly reduced or modified. In the experiments conducted so far high radiofrequency amplitudes have been used to saturate the protein alkyl proton resonances, to obtain large effects. At lower radiofrequency power levels more selective effects can be observed and structural correlations are possible in principle.

The observation of intramolecular NOE's in proteins and intermolecular NOE's on small molecules binding to biomacromolecules thus appears to offer great potential for structural investigation.

Acknowledgments. This work was supported by

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Localization of Tyrosine at the Binding Site of Neurophysin II by Negative Nuclear Overhauser Effects

Sir:

The polypeptide hormones oxytocin and vasopressin are found in neurosecretory granules, in noncovalent association with a group of closely related proteins, the neurophysins.[1] Binding studies using synthetic analogs of the hormones have demonstrated the importance of the α-amino group and the side chains at positions 1–3 in stabilizing the complex;[2–5] tripeptides containing analogs of residues 1–3 display all the principal features of the protein–hormone interaction. We wish to report the results of a 250-MHz proton nmr investigation which further elucidates the molecular details of this binding phenomenon.

Figure 1 shows the proton spectrum of the aromatic ring protons of L-Ala-L-Tyr-L-PheNH$_2$[5] (A) in the

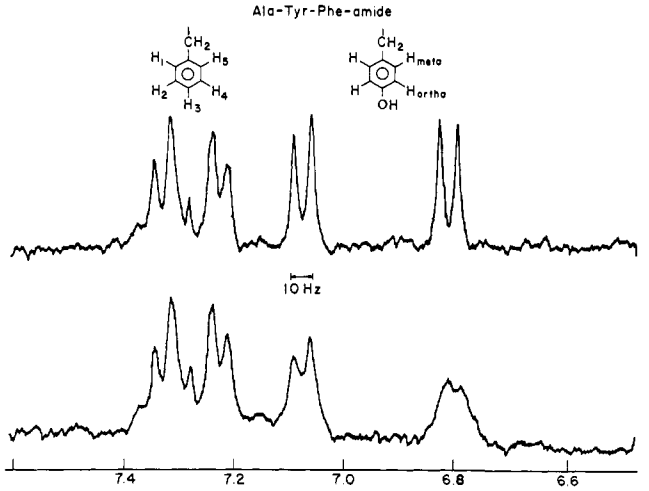

Figure 1. 250-MHz nmr spectrum of the aromatic protons of A: upper trace 2.3×10^{-3} M A in D_2O; lower trace, 0.09 equiv of NP-II added, pH 6.5.

presence and absence of bovine neurophysin II (NP-II). The signals from the tyrosine ring are broadened, the protons ortho to the hydroxyl more so than the meta. The phenylalanine ring protons are comparatively unaffected. The tyrosine in position 2 of binding peptides can be substituted by phenylalanine with no significant effect on binding. If (*S*-Me)Cys-Phe-IleNH$_2$ (B) is used as the binding peptide the phenylalanine ring protons show differential broadening, analogous to the tyrosine protons in A. The observations are in agreement with the postulate of rapid reversible binding with specific interactions between the aromatic residue at position 2 of the peptide and unspecified residues on the protein.[3] The differential broadening of adjacent protons on the same aromatic ring suggests a dipolar contribution to the transverse relaxation rate enhancement arising from the proximity of magnetic nuclei on the protein. Such an intermolecular interaction has been considered earlier by Lanir and Navon in their study of the binding of sulfonamide inhibitors to carbonic anhydrase.[6] Strong irradiation of the correct NP-II protons should therefore produce an intensity change, due to an Overhauser effect, in the aromatic resonances from position 2 of the peptide.

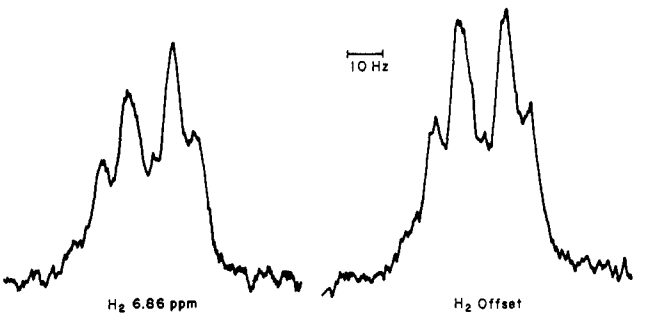

Figure 2. Phenyl protons of B in 20:1 mixture of B and NP-II with and without strong radiofrequency irradiation at δ 6.86 ppm.

Figure 2 shows the effect on the phenylalanine ring protons of irradiating a 1:20 mixture of NP-II and B at δ 6.86 ppm. A striking decrease in intensity is observed and similar effects were also observed at δ 1.9 and 3.1 ppm. The results on a variety of peptides are summarized in Table I.

Table I. Per Cent Decrease[a] in Intensity of Peptide Signals on Saturating Protein Resonances

Peptide	Resonance obsd[b]	Resonance saturated[g] δ 1.9	δ 3.1	δ 6.86
Ala-Tyr-PheNH$_2$[c]	Tyr ortho	38	36	*h*
	Tyr meta	14	24	*h*
(*S*-Me)Cys-Tyr-PheNH$_2$[d]	Tyr ortho[i]	21	19	*h*
	Tyr meta	3	9	*h*
Met-Tyr-PheNH$_2$[e]	Tyr ortho	33	35	*h*
	Tyr meta	20	29	*h*
(*S*-Me)Cys-Phe-IleNH$_2$[f]	Phe 2,3,4	20	18	22
	Phe 1,5	17	15	10

[a] Intensity decreases are crude estimates based on peak heights. There is no change in line width. [b] Refer to Figure 1 for labeling. [c] [Peptide] = 4.5×10^{-3} M, [NP-II] = 3×10^{-4} M, pH 6.5. [d] [Peptide] = 5.4×10^{-3} M, [NP-II] = 3×10^{-4} M, pH 3.5. [e] [Peptide] = 2×10^{-3} M, [NP-II] = 3×10^{-4} M, pH 6.6. [f] [Peptide] = 4.9×10^{-3} M, [NP-II] = 2.3×10^{-4} M, pH 6.5. [g] δ = ppm from DSS. [h] Observing and irradiating frequencies overlap. [i] The NOE's are concentration dependent, a factor disregarded here.

NOE's did not occur in the absence of protein or in the presence of denatured protein. Peptides containing phenylalanine only in position 3 showed no

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