both DPPA and DEPC were proved useful for both the stepwise and fragment condensation approaches on a solid support.

Based on these satisfactory, preliminary experiments, we applied the method to the synthesis of porcine motilin, Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Leu-Gln-Arg-Met-Gln-Glu-Lys-Glu-Arg-Asn-Lys-Gly-Gln, which was recently synthesized by Yajima and his co-workers, using the conventional method.[14,15] As a strategy for the synthesis of motilin, its molecule was architecturally segmented into four subunits (see dotted lines in the above formula). Synthesis was carried out by a combination of the solution and solid-phase methods. The time for the coupling reactions on a solid support varied from 2 to 48 hr depending on the reactants. Z-Phe-Val-Pro ($\frac{1}{2}C_6H_6$ solvate), mp 99–102°, $[\alpha]^{20}D$ −49° (c = 0.74, DMF), was prepared from Pro-OMe by the stepwise addition of Z-Val and Z-Phe using either DPPA or DEPC, followed by alkaline treatment. This tripeptide derivative was condensed with Ile-Phe-Thr(Bzl)-Tyr(Bzl)-Gly-resin which had been prepared by the sequential incorporation of Boc-Tyr(Bzl), Boc-Thr(Bzl), Boc-Phe, and Boc-Ile into Gly-resin. The resultant resin was treated with methanol in the presence of triethylamine to give Z-Phe-Val-Pro-Ile-Phe-Thr(Bzl)-Tyr(Bzl)-Gly-OMe (monohydrate), mp 206–210°, $[\alpha]^{20}D$ −34.8° (c = 0.5, $CHCl_3$), in 48 and 68% yields using DPPA and DEPC, respectively, based on Boc-Gly-resin. Saponification of the methyl ester afforded Z-Phe-Val-Pro-Ile-Phe-Thr(Bzl)-Tyr(Bzl)-Gly-(hemihydrate), mp 198–201°, $[\alpha]^{20}D$ −23° (c = 0.6, $CHCl_3$). Boc-Leu-Gln-Arg(NO_2)-Met(monohydrate), $[\alpha]^{20}D$ −33° (c = 0.6, MeOH), was prepared from Met-OMe analogous to the preparation of Z-Phe-Val-Pro using DPPA or DEPC. The C-terminal nonapeptide resin, Gln-Glu(Bzl)-Lys(2-Cl-Z)-Glu(Bzl)-Arg(NO_2)-Asn-Lys(2-Cl-Z)-Gly-Gln-resin, prepared stepwise from Gln-resin, was successively condensed with Boc-Leu-Gln-Arg(NO_2)-Met, Boc-Glu(Bzl), and Z-Phe-Val-Pro-Ile-Phe-Thr(Bzl)-Tyr(Bzl)-Gly using DEPC in the presence of triethylamine in dimethylformamide to give the docosapeptide resin.

The resin was treated with hydrogen fluoride in the presence of anisole at −20° for 40 min, then at 0° for 40 min, followed by the usual treatment on a Dowex 1-X4 column (acetate form).[16] The deblocked peptide obtained was successively purified by column chromatography on SP-Sephadex C-25 (gradient elution with ammonium formate buffer), Sephadex G-25 (0.1 N acetic acid), QAE-Sephadex A-25 (gradient elution with ammonium formate buffer), and Biogel P4-P6 (4:1) (0.1 N acetic acid). This purified synthetic motilin appeared homogeneous in a variety of chromatographic systems[17] and gave excellent amino acid analyses,[18] after both acid hydrolysis and enzymic digestion (AP-M), the latter procedure showing the absence of racemization during synthesis.

The activity of synthetic motilin was determined using rabbit duodenum, jejunum, and colon contractile activity in vitro.[19] Synthetic motilin showed a potency similar to the natural one and the same biological activity pattern with other smooth muscle preparations of the alimentary tracts (rat, guinea pig, and rabbit).

The synthesis of motilin, as well as preliminary experiments including the Izumiya test, indicate that both DPPA and DEPC may be very efficient reagents for solid-phase peptide synthesis as well as for conventional synthesis.

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References and Notes

(1) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
(2) Reviews: Y. S. Klausner and M. Bodanszky, *Synthesis*, 453 (1972); M. Fridkin and A. Patchornik, *Annu. Rev. Biochem.*, **43**, 419 (1974).
(3) T. Shioiri, K. Ninomiya, and S. Yamada, *J. Am. Chem. Soc.*, **94**, 6203 (1972); T. Shioiri and S. Yamada, *Chem. Pharm. Bull.*, **22**, 849, 855, 859 (1974).
(4) S. Yamada, Y. Kasai, and T. Shioiri, *Tetrahedron Lett.*, 1595 (1973).
(5) J. C. Brown, M. A. Cook, and J. R. Dryburgh, *Can. J. Biochem.*, **51**, 533 (1973); H. Schubert and J. C. Brown, *ibid.*, **52**, 7 (1974).
(6) All amino acids, except glycine, were of L-configuration unless otherwise stated.
(7) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis", Freeman, San Francisco, Calif., 1969.
(8) K. Esko, S. Karlsson, and J. Porath, *Acta Chem. Scand.*, **22**, 3342 (1968).
(9) R. M. G. Nair, A. J. Kastin, and A. V. Schally, *Biochem. Biophys. Res. Commun.*, **43**, 1376 (1971).
(10) Solid-phase peptide synthesis was carried out using a reaction apparatus designed by the Tanabe research group: T. Mizoguchi, K. Shigezane, and N. Takamura, *Chem. Pharm. Bull.*, **18**, 1465 (1970).
(11) Reported mp 137–139°, $[\alpha]^{25}D$ −72.3° (c = 1.8, MeOH). See ref 10).
(12) N. Izumiya, M. Muraoka, and H. Aoyagi, *Bull. Chem. Soc. Jpn.*, **44**, 3391 (1971).
(13) M. A. Barton, R. U. Lemieux, and J. Y. Savoie, *J. Am. Chem. Soc.*, **95**, 4501 (1973).
(14) H. Yajima, Y. Kai, and H. Kawatani, *J. Chem. Soc., Chem. Commun.*, 159 (1975).
(15) The synthesis of [13-Nle, 14-Glu]-motilin was also reported: E. Wünsch, J. C. Brown, K.-H. Delmer, F. Drees, E. Jaeger, J. Muslol, R. Scharf, H. Stocker, P. Thamm, and G. Wendlberger, *Z. Naturforsch. C*, **28**, 235 (1973).
(16) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967).
(17) TLC: Silica gel GF_{254} (Merck), R_f 0.70 in *n*-BuOH:AcOH:pyridine:water (15:10:3:12); cellulose powder (Eastman), R_f 0.69 in *n*-BuOH:AcOH:pyridine:water (15:6:10:12). Paper chromatography; Toyo filter paper 51A, R_f 0.46 in *n*-BuOH:AcOH:pyridine:H_2O (15:3:10:12).
(18) Amino acid analyses after acid hydrolysis and enzymic digestion (values in parentheses): Lys 2.2 (1.8), Arg 2.1 (1.9), Asp 1.1 (0), Thr 0.9 (Asn + Thr + Gln 6.1), Glu 5.8 (3.3), Pro 1.0 (1.0), Gly 2.0 (1.9), Val 0.8 (1.2), Met 1.0 (1.0), Ile 0.9 (1.0), Leu 1.0 (1.0), Tyr 0.8 (0.8), Phe 1.8 (2.0).
(19) W. Domschke, U. Strunz, P. Mitznegg, H. Ruppin, S. Domschke, E. Schubert, E. Wünsch, E. Jaeger, and L. Demling, *Naturwissenschaften*, 61, 370 (1974).

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The Nuclear Overhauser Effect in ^{31}P Nuclear Magnetic Resonance

Sir:

The nuclear Overhauser effect (NOE) is the change in the NMR intensity of a nuclear spin upon saturation of a second spin interacting by a dipolar mechanism with the observed spin. The NOE is commonly observed in ^{13}C NMR, where carbons directly bound to hydrogens may display up to three times greater intensity due to dipolar interactions between the two nuclei. In this paper we report observation of a $^{31}P\{^1H\}$ NOE in a variety of phosphorus containing compounds. In our case saturation of all the protons in the sample can produce, under extreme narrowing conditions, a maximum increase in the intensity of the phosphorus resonance, or a nuclear Overhauser effect enhancement (NOEE), of 124%.[1] Failure to achieve the full enhancement may be due to competing relaxation mechanisms (other than the proton-^{31}P dipolar mechanism upon which the NOE depends) or to motions which are slower than required for the extreme narrowing limit. Quantitatively, the NOE enhancement in the ^{31}P, ^1H case in the extreme narrowing limit may be expressed as

$$\text{NOEE} = \rho_{\text{d-d}}(\gamma_H/2\gamma_P)/(\rho_{\text{d-d}} + \rho^*) \quad (1)$$

where γ represents the respective magnetogyric ratios, $\rho_{\text{d-d}}$ is the relaxation rate of ^{31}P due to proton–phosphorus dipole-dipole interactions, and ρ^* is the relaxation rate due to other relaxation mechanisms.

In addition to reporting observation of the $^{31}P\{^1H\}$ NOE, this paper gives examples of the kind of information the phenomenon can provide. The existence of a NOE for ^{31}P permits accumulation of information concerning relaxation mechanisms of the phosphorus nucleus, as well as conformational and motional information about molecules.

^{31}P spectra were obtained on a JEOL-PS100/EC-100 Fourier transform spectrometer at 23°C. NOE enhancement values were calculated (±10%) from the ratio of the intensity of fully decoupled spectra to the intensity of spectra in which the proton decoupler was gated to remove simultaneously the NOE and ^{31}P–^1H coupling.[2] In order to assure full relaxation of the spins, a repetition rate of 3–5 T_1 was used. Phosphate salt solutions contained 20 m*M* ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetate (EGTA) to eliminate reductions of the NOE due to paramagnetic impurities. To exclude contributions to the NOE from solvent protons, some molecules were examined in D_2O. Deuterated phosphite and hypophosphite were prepared by exchange of the phosphorus bonded hydrogens in DCl.[3]

Table I lists $^{31}P\{^1H\}$ nuclear Overhauser effect enhancements (NOEE) for several kinds of phosphorus compounds. In order to ensure that paramagnetic impurities do not contribute to ρ^* in eq 1, 20 m*M* EGTA was added to the salt solutions to complex with any trace amounts of metal ions that might be present. A representative experiment was performed with AMP to check the effectiveness of the EGTA. Both T_1 and NOE were measured for the phosphorus of AMP with the 20 m*M* EGTA and compared to T_1 and NOE for the same sample in the presence of 1.5 m*M* $CuCl_2$. For the two samples, T_1 and NOE were within experimental error of each other. Therefore, the NOE measured can be considered to be free of paramagnetic attenuation.

The NOE enhancements of Table I exhibit a range from 0% (for the fully deuterated acids) to the full NOEE possible, 124%, in the extreme narrowing limit. In the latter limit, the fraction of the total possible NOEE actually observed is a measure of the dipolar contributions to the relaxation of the observed phosphorus. Two salts, HPO_3^{2-} and $H_2PO_2^-$, with protons bonded to the phosphorus show full or nearly full NOE. These results demonstrate that the phosphorus bound proton dominates the phosphorus relaxation by dipole-dipole interactions and that other mechanisms are not contributing significantly. The acids of these salts exhibit an attenuated NOE, which indicates significant contributions from ρ^* in eq 1 due to mechanisms such as spin rotation, chemical shift anisotropy, and chemical exchange.

In general, within classes of compounds such as the acids, the salts, or the alkyl phosphites, those molecules with protons directly bonded to phosphorus give rise to greater NOEE than the species in which the protons are farther from the phosphorus. This generalization is consistent with the inverse sixth power dependence of the dipolar interaction on the interspin distance. Some support for these interpretations is found in an independent analysis, where the dipolar contribution to relaxation of phosphorus in trimethylphosphate was estimated as 66%.[4] This result corresponds to an NOEE of 82%, somewhat greater than our observed value. Both analyses suggest significant contributions to relaxation from other than the dipolar mechanism.

Table I. ^{31}P NOE Enhancements

Compound	NOEE[a]	Conditions
PO_4H_3	30%	H_2O
$PO_4H_2^-$	30	
PO_4H^{2-}	30	
PO_4^{3-}	30	
DPO_3D_2	0	D_2O
HPO_3H_2	60	H_2O
HPO_3H^-	70	D_2O
HPO_3^{2-}	100	
D_2PO_2D	0	
H_2PO_2H	40	H_2O
$H_2PO_2^-$	130	D_2O
$P(OCH_3)_3$	10	Neat
$OP(OCH_3)_3$	30	
$HP(OCH_3)_2$	60	
AMP^{2-}	70	D_2O
ATP^{4-}	10	
ATP^{4-}	50	H_2O
$P_2O_7H_4$	10	
Glycerophosphate^{2-}	60	D_2O
Glycerophosphorylcholine^0	70	
Phosphorylcholine$^-$	30	

[a] NOEE is reported as percent enhancement of signal intensity to the nearest 10%.

Competing relaxation mechanisms also account for other examples of reduced NOE. All three ^{31}P resonances of adenosine triphosphate (ATP) exhibit a similar lower NOEE than adenosine monophosphate (AMP), both in D_2O. The result suggests that dipolar interactions between phosphorus nuclei may be an important contributor to relaxation in ATP. Both glycerophosphate and glycerophosphorylcholine exhibit, in D_2O, a substantially greater NOE than phosphorylcholine. Since the glycerol group participates in intermolecular hydrogen bonding with the solvent, the compounds containing it may rotate more slowly leading to a lesser contribution from spin rotation to relaxation.

Other kinds of information are potentially available from the NOE. Though the molecules of Table I are all small enough to be in the extreme narrowing limit, in the case of large molecules, or small molecules bound to large molecules, the NOE may be motionally limited and thereby give a characteristic correlation time if a suitable motional model can be found.[5] An example in ^{31}P NMR is provided by the attenuated NOE observed in phospholipid bilayer vesicles.[6]

Another kind of information concerns molecular conformation. Selective irradiation of the aromatic base protons of AMP fails to produce the NOEE reported in Table I while irradiation of the ribose protons yields the full NOEE. This result suggests phosphate–ribose dipolar interactions in AMP, which is stacked under our experimental conditions.

Perhaps the most important result here for previous and future work with ^{31}P NMR is that the wide range of NOE values observed and the various effects which can change these values will result in erroneous quantitative intensity interpretations obtained from proton decoupled spectra unless procedures, such as gating the decoupler, are employed to eliminate NOE. Intensities of ^{31}P NMR resonances have been used to determine concentrations of phosphorus containing species in solution, a method of particular interest in studies of phospholipids. However, under conditions of proton decoupling, the intensity of the phosphorus resonance will be proportional to the number of spins only if differences in T_1 and nuclear Overhauser effects are taken into account. Therefore, quantitative intensity work in which a proton decoupler was used to simplify the spectra should be revaluated to determine the nuclear Overhauser effects produced.[7]

References and Notes

(1) J. H. Noggle and R. E. Schirmer, "The Nuclear Overhauser Effect", Academic Press, New York, N.Y., 1971.
(2) R. Freeman, H. Hill, and R. Kaptein, *J. Magn. Reson.*, **7**, 327 (1972).
(3) R. B. Martin, *J. Am. Chem. Soc.*, **81**, 1574 (1959).
(4) S. W. Dale and M. E. Hobbs, *J. Phys. Chem.*, **75**, 3537 (1971).
(5) D. Doddrell, V. Glushko, and A. Allerhand, *J. Chem. Phys.*, **56**, 3683 (1972).
(6) P. L. Yeagle, W. C. Hutton, C. Huang, and R. B. Martin, *Proc. Nat. Acad. Sci., USA*, **72**, 3477 (1975).

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Carbon Isotope Effects on Proton Transfers from Carbon, and the Question of Hydrogen Tunneling[1]

Sir:

We wish to report evidence that the tunnel effect makes an important contribution to hydrogen isotope effects in proton transfers but does not control the form of the dependence of the isotope effect on the strength of the attacking base.

The three-center model predicts that hydrogen isotope effects on proton transfers should be at a maximum when the proton is half transferred in the transition state.[2,3] Base and/or substrate variation have often been observed to lead to such maxima.[4-7] More recently, the significance of the observed maxima has been questioned by Bell, Sachs, and Tranter,[8] who suggest that the maxima arise from variation in the tunnel effect contribution rather than changes in stretching force constants of the carbon–hydrogen and base–hydrogen bonds. Model calculations show that the semiclassical (the isotope effect without tunneling) and the tunnel effect contributions to carbon isotope effects on proton transfers from carbon should vary in distinctly different ways with the extent of proton transfer.[9,10] Consequently, we undertook a study of β-carbon-13 isotope effects on E2 reactions of 2-phenylethyldimethylsulfonium (**1**) and -trimethylammonium (**2**) ions with hydroxide ion in mixtures of water and dimethyl sulfoxide. Both reactions show maxima in k_H/k_D as the percentage of dimethyl sulfoxide is varied.[6,7]

The substrates **1** and **2** were oxidized quantitatively with potassium permanganate to benzoic acid, which in turn was treated with hydrazoic and sulfuric acids to give carbon dioxide. This carbon dioxide was compared in an isotope-ratio mass spectrometer[11] with carbon dioxide from substrate recovered after partial (usually 40–75%) reaction. The *m/e* 44/45 ratio for the original sample (R^0) and the recovered sample (R) were substituted into eq 1, along with the fraction of reactant remaining (F).[12] The resulting isotope effects, expressed as the percentage by which k_{12}/k_{13} exceeds unity, are compared with k_H/k_D values[6,7] in Figure 1 for **1**, and Figure 2 for **2**. Each point is the result of three–ten determinations, and standard deviations of the mean run 0.03–0.18%.

$$k_{12}/k_{13} = \log F/\log (RF/R^0) \qquad (1)$$

The scatter at the higher water concentrations of the results on **1** (Figure 1) exceeds the combined standard deviations, and may result from error occasioned by the slowness of the reactions in these media. No such unusual scatter is observed in the results on **2** (Figure 2). In both cases, however, the most important point is clearly evident: the $^{12}C/^{13}C$ and H/D isotope effects depend on solvent composition in distinctively different ways.

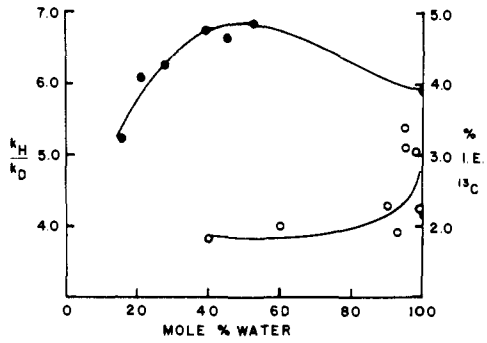

Figure 1. Observed k_H/k_D values (left-hand ordinate and solid circle) and β-^{13}C isotope effects, expressed as $(k_{12}/k_{13} - 1) \times 100$ (right-hand ordinate and open circles), for the elimination reaction of 2-phenylethyldimethylsulfonium ion with hydroxide ion in mixtures of water and dimethyl sulfoxide at 30°C.

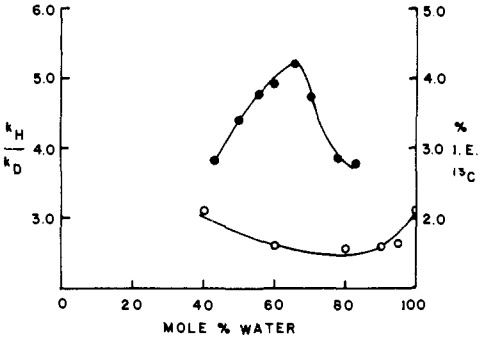

Figure 2. Observed k_H/k_D values (left-hand ordinate and solid circles) and β-^{13}C isotope effects, expressed as $(k_{12}/k_{13} - 1) \times 100$ (right-hand ordinate and open circles) for the elimination reaction of 2-phenylethyltrimethylammonium ion with hydroxide ion in mixtures of water and dimethyl sulfoxide at 60°C.

This point is important because the Bell, Sachs, and Tranter[8] model achieves variation in the tunnel effect primarily by changes in the activation energy (strictly speaking, the barrier height in whichever direction the proton transfer is exothermic, but our E2 reactions are all exothermic in the forward direction). It follows that variations in the carbon and deuterium isotope effects cannot both be controlled by tunnel-effect changes, for the activation energies, and their changes with solvent composition, should be nearly the same for all of the isotopic species. Actually, the activation energies for the reactions of **1** decrease monotonically as the water content of the medium decreases,[6] a pattern which is qualitatively consistent with the carbon isotope effects but quite inconsistent with the maximum in k_H/k_D. There is, however, no reason to believe that the variation in the carbon isotope effect would be controlled by the tunnel effect when the variation in k_H/k_D is not,[13] so we conclude that variations with solvent composition in both the $^{12}C/^{13}C$ and H/D isotope effects reflect primarily force-constant changes.

Comparison of calculated[9,10] and observed isotope effects will be considered in more detail in a full paper. Here we will simply present briefly the evidence bearing on the role of the tunnel effect. Calculations based on purely semiclassical models of the E2 transition state predict slightly *inverse* $^{12}C/^{13}C$ isotope effects in the vicinity where k_H/k_D is at a maximum.[9,14] This is clearly not the case in Figures 1 and 2, so one is forced to the conclusion that there must be a substantial tunnel effect superimposed on the semiclassical effect. A $^{12}C/^{13}C$ tunnel effect in the vicinity of 1.5–2.5% would fit the observed results. Such a tunnel effect for