but the analysis was less accurate than that by mass spectrometry (± <5% *vs.* ± <2%). The ^{13}C nmr spectrum of the isotopic label-rearranged *n*-propylbenzene (experiment 1) was obtained with a Varian DP60-IL (modified) spectrometer using CAT of 2163 scans. Two overlapping triplets were observed corresponding to the α and β carbon atoms, at 56.8 and 43.5 ppm, respectively, calculated using an external methyl iodide reference. Integration by peak weights indicated that the ^{13}C label was distributed 53% at the α position and 47% at the β position. The proton nmr spectrum of the same sample (experiment 1) displayed a small side band upfield from the γ-methyl absorption which was a result of ^{13}C in natural abundance at the γ carbon, and which did not increase after the isotopic rearrangement (Varian HA-100 spectrometer).

These results show that during the reaction of *n*-propylbenzene with aluminum chloride in the presence of excess benzene and a trace of water at reflux, a process takes place which scrambles the α and β positions, but not the γ position of the recovered compound. It is unlikely that this rearrangement involves diphenylhexyl cations as intermediates, since such intermediates could easily rearrange to produce dilabeled and unlabeled propylbenzene molecules by shifts and intermediates fully equivalent to those required to scramble the α and β positions.

The mechanism involving diphenylpropane intermediates[1] offers a satisfactory explanation of the data, since only monolabeled *n*-propylbenzene can result from this process. The positive effect of solvent benzene concentration on the rate of scrambling the α and β positions and the slow rate at which *n*-propylbenzene is isomerized to isopropylbenzene can also be seen as logical consequences of the diphenylpropane mechanism.

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Orientation Effects in Reactions of Acyl Chymotrypsins

Sir:

An important component of the catalytic efficiency of enzymes is their ability to juxtapose reacting groups in configurations which maximize reactivity.[1] It is qualitatively clear that the distance between reacting atoms is not the only determinant of reactivity—the orientation of the reacting groups is also important. Opinions differ as to the importance of this effect.[2] Progress in this area has been impeded by a lack of unambiguous examples of the effect of orientation. We present here data concerning the reactivity of certain acyl chymotrypsins which provide clear evidence for the operation of an orientation effect.

Acyl chymotrypsins can react with nucleophiles other than water, such as methanol[3-5] and amines.[4-7] Hydroxylamine[4-6] and methoxylamine[7] are effective nucleophiles toward acyl enzymes derived from nonspecific substrates, but they are very ineffective nucleophiles toward acyl enzymes derived from specific substrates.

Ammonia, the nitrogen nucleophile which is structurally most like water, has not been studied extensively. Ammonia and primary amines are quite reactive toward furoyl chymotrypsin,[5] but no parallel study of acyl enzymes derived from specific substrates has been reported. We have measured the reactivity of ammonia toward *N*-acetyl-L-tryptophanyl chymotrypsin by two independent methods. The lack of reactivity of ammonia in this case is in striking contrast to its reactivity toward furoyl chymotrypsin and provides evidence for very specific geometric constraints in the hydrolysis of acyl chymotrypsins.

The reactivity of ammonia toward *N*-acetyl-L-tryptophanyl chymotrypsin was measured by hydrolysis of *N*-acetyl-L-tryptophanamide at pH 9.43 in the presence of 0.003–0.1 *M* ammonia containing 8.5 atom % ^{15}N. The reaction was stopped after 10–50% hydrolysis and the remaining starting material was isolated and analyzed[8] for ^{15}N. Incorporation of the heavy isotope into the amide never exceeds 0.005 atom %. Because of this small incorporation and because of the presence of a substantial isotope effect on the reaction[8,9] it is impossible to make an accurate estimate of the exchange rate. It is clear that ammonia is no more than four times as reactive as water.[10]

Because of the lack of precision of the isotopic method we also measured the reactivity of ammonia by another method. *N*-Acetyl-L-tryptophan methyl ester was hydrolyzed with chymotrypsin at pH 10.0 in the presence of 0.10 *M* ammonia. After completion of the hydrolysis, the product mixture was analyzed by quantitative column chromatography for the presence of *N*-acetyl-L-tryptophanamide.[11] Only a very small amount of amide was formed. The reactivity of ammonia toward this acyl chymotrypsin is approximately sixfold less than that of water after correction for protonation of ammonia.[10]

From these studies and from previous studies of aminolysis of chymotrypsins a very significant fact emerges. The nucleophilicity of ammonia toward acyl

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(5) P. W. Inward and W. P. Jencks, *ibid.*, **240**, 1986 (1965).

(6) F. J. Kezdy, G. E. Clement, and M. L. Bender, *ibid.*, **238**, 3141 (1963); R. M. Epand and I. B. Wilson, *ibid.*, **238**, 1718 (1963); S. A. Bernhard, W. C. Coles, and J. F. Nowell, *J. Amer. Chem. Soc.*, **82**, 3043 (1960).

(7) Vishnu and M. Caplow, *ibid.*, **91**, 6754 (1969).

(8) M. H. O'Leary and M. D. Kluetz, *J. Amer. Chem. Soc.*, in press.

(9) M. H. O'Leary and M. D. Kluetz, *ibid.*, **92**, 6089 (1970).

(10) This figure was calculated by incorporating 55.5 *M* for the water concentration and considering the pK_a of ammonia to be 9.25.

(11) A DEAE-cellulose column was used. The analysis was made by Ehrlich assay on concentrated effluent fractions. In some experiments a small amount of *N*-acetyl-L-tryptophanamide was added before the enzymatic hydrolysis in order to check the reliability of the procedure. The expected increase in yield of amide was observed. Under the conditions of our experiments an insignificant fraction of the amide formed would be hydrolyzed.

chymotrypsins derived from nonspecific substrates is much greater on a molar basis than that of water, as expected from the results of studies of nonenzymatic reactions of amines with esters in aqueous solution. However, the nucleophilicity of ammonia toward acyl chymotrypsins derived from specific substrates is equal to or less than that of water—a result quite different from what is observed in nonenzymatic reactions.[12] The purpose of this communication is to propose an explanation for this anomaly.

Henderson[13] has shown that in crystalline acyl chymotrypsins a water molecule is hydrogen bonded to an imidazole nitrogen[14] of histidine-57. He suggested that a similarly bound water molecule is present in solution, and that this water molecule is correctly placed for attack on the carbonyl carbon of the acyl group. Formation of the required carbon–oxygen bond involves only slight motions of the water oxygen and the carbonyl oxygen. The remainder of the acyl enzyme is quite rigid, particularly in the case of acyl groups derived from specific substrates, and little or no motion of the acyl group is required during the reaction. According to this mechanism, there are two possible explanations for the abnormal reactivity of ammonia toward *N*-acetyl-L-tryptophanyl chymotrypsin: (1) ammonia does not bind to the reactive site; (2) ammonia binds, but does not react. We will present a number of arguments against the first possibility and will present a detailed explanation of this phenomenon in terms of the second.

The occurrence of a specifically bound water molecule in crystalline acyl chymotrypsins does not necessarily indicate that in aqueous solution water is bound to that site with any appreciable strength. There is some question as to whether we should consider a binding site for water at all; alternatively, in solution this region might be filled by randomly oriented solvent molecules. It is likely, however, that the configuration with a specifically aligned water molecule is a configuration of some (albeit limited) thermodynamic stability.[15] This being so, it is reasonable to consider a water binding site, although the argument to follow could equally well be constructed without consideration of a specific binding site for water.

The similarity between water and ammonia and the weakness of the binding of water to the site in question make it unlikely that ammonia would be excluded from this site. Solvation of bound water or ammonia by bulk solvent undoubtedly occurs, and there is no reason to suspect that binding of the two species would be appreciably different. In addition, the reactivity of ammonia toward acyl chymotrypsins derived from nonspecific substrates indicates that ammonia binds satisfactorily in those cases.[16]

Thus, it seems unlikely that the enzyme excludes ammonia from the water binding site. The other possibility is that ammonia binds properly, but once bound is unreactive. We believe that this unreactivity results from improper alignment of ammonia when bound to the active site. Ammonia is slightly larger than water, and forms longer hydrogen bonds[17] (by about 0.3 Å). The longer hydrogen bond results in the ammonia nitrogen being sufficiently close to the carbonyl carbon that reaction would occur, except that the orientation of ammonia is incorrect. Although hydrogen bonds are weak and easily distortable, a very large distortion from equilibrium would be required to align the nitrogen properly for attack. In addition, this distortion would be expected to interfere with the facile proton transfer between the nucleophile and histidine-57.

The high reactivity of ammonia with furoyl chymotrypsin[5] provides further support for this hypothesis. This acyl group does not fit the substrate binding site of the enzyme very tightly, but instead has considerable freedom of motion. This motion allows the carbonyl group to adopt a conformation suitable for attack by bound ammonia.

Thus, the high hydrolysis rates of acyl chymotrypsins derived from specific substrates reflect in part an optimization of the histidine–water–carbonyl group orientation. Substitution of ammonia for water results in a sufficient distortion of the geometry that the reactivity of ammonia is low. In the case of acyl chymotrypsins derived from nonspecific substrates, the flexibility of the histidine–water–carbonyl group geometry is sufficient that the reactivity of ammonia is much higher than that of water, as is the case in model reactions. However, the reactivity of water is much reduced compared to what it was in the case of specific substrates because the carbonyl group is no longer locked in the optimum geometry for reaction. The reactivities of alcohols,[3–5] hydroxylamine,[4–6] and methoxylamine[7] with acyl chymotrypsins are also consistent with this explanation.

The present results are very similar to those obtained with thiolsubtilisin.[18] In that case, the geometry at the active site is distorted slightly by the substitution of a sulfhydryl group for the usual hydroxyl group. Nonspecific substrates show the expected reactivity toward the modified enzyme, but specific substrates are unable to achieve the proper alignment for reaction and are completely inert.

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(12) A similar conclusion could be drawn from existing data concerning the nucleophilicity of hydroxylamine toward acyl chymotrypsins.[4–7] However, the arguments to follow are less strong in that case because of the structural differences between hydroxylamine and water.

(13) R. Henderson, *J. Mol. Biol.*, **54**, 341 (1970).

(14) In the case of indoleacryloyl chymotrypsin, this water is also hydrogen bonded to the carbonyl oxygen of the acyl group, but this hydrogen bond is of questionable importance in the case of better substrates.

(15) The lack of evidence in various kinetic studies for a binding site for methanol or other nucleophiles does not argue against this proposal, but merely means that the nucleophile concentrations accessible are inadequate to saturate the site.

(16) "Binding" of water or ammonia to the active site consists of removing it from its normal solvated, hydrogen-bonded state in the bulk solvent and hydrogen bonding it to one or more groups at the active site of the enzyme. The net change is only in the location of the molecule, and probably not in the number of hydrogen bonds. Further, if the abnormal reactivity of ammonia which we have observed is due to impaired binding, then water must bind more strongly than ammonia to the active site by at least 10^3, which requires that the binding energy of water must be at least 4 kcal/mol greater than that of ammonia. However, it seems unlikely that the binding energy of water is as large as 4 kcal/mol.

(17) G. C. Pimentel and A. L. McClellan, "The Hydrogen Bond," W. H. Freeman, San Francisco, Calif., 1960, p 289.

(18) K. E. Neet, A. Nanci, and D. E. Koshland, Jr., *J. Biol. Chem.*, **243**, 6392 (1968).

07657), the University of Wisconsin Graduate School, and the Research Corporation.

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Bis(1,3,5,7-tetramethylcyclooctatetraene)uranium(IV) and Bis(1,3,5,7-tetramethylcyclooctatetraene)-neptunium(IV). Proton Magnetic Resonance Spectrum and the Question of f-Orbital Covalency[1]

Sir:

Our previously published[2] analysis of the observed large upfield shift for bis(cyclooctatetraene)uranium-(IV), $(COT)_2U$, uranocene, indicated a large positive net contact shift that suggests some form of covalency; however, in the absence of data relating to the type of ligand MO (σ or π) containing the delocalized spin, no attempt was made to evaluate the possible role of f orbitals in such covalency. Methyl substitution provides a useful technique for characterizing the spin-containing ligand. For spin in π orbitals it is well known that an attached ring proton and methyl proton exhibit contact shifts of comparable magnitude but of opposite sign; for spin in in-plane or σ orbitals, these shifts are of the same sign with the ring-proton shift three–five times larger than the methyl shifts.[3] To apply this approach to the uranocene case we have prepared and characterized bis(1,3,5,7-tetramethylcyclooctatetraene)uranium(IV) and -neptunium(IV), $(TMCOT)_2U$ and $(TMCOT)_2Np$. These compounds were prepared in a similar fashion to the parent compounds,[4,5] using the TMCOT prepared as described by de Mayo and Yip.[6] The TMCOT compounds had similar properties to the COT compounds except for greater solubility in organic solvents. An attempt was made to prepare $(TMCOT)_2Pu$ but Pu(IV) was reduced by $(TMCOT)^{2-}$ to Pu(III).

The proton nmr spectrum of a THF-d_8 solution of $(TMCOT)_2U$ was recorded as a function of temperature on a Varian HA-100 operating in the HR mode with variable-frequency modulation. Two peaks with intensities of 3:1 were observed and can be assigned to the methyl and ring protons, respectively. The observed shifts at room temperature, referenced against $TMCOT^{2-}$, are given in Table I together with that observed for $(COT)_2U$. The ring-proton shifts and their temperature dependences are virtually the same for both complexes.[7] The proton nmr spectrum of a toluene-d_8 solution of $(TMCOT)_2Np$ was recorded at 37°. The corresponding proton peaks of the methyl group (line width ~300 Hz) and ring (line width ~2000 Hz) were much broader than for the U complex. The shifts are listed in Table I.

Table I. Chemical Shifts and Hyperfine Coupling Constants for $(TMCOT)_2U$, $(COT)_2U$ in THF, and $(TMCOT)Np$ in Toluene

	$(TMCOT)_2U$		$(COT)_2U$	$(TMCOT)_2Np$	
	Ring-H	Methyl-H	Ring-H	Ring-H	Methyl-H
Shifts[a]					
Observed	+41.3	+6.0	+42.6	+41.5 ± 2	−9.9
Dipolar	+7.9	+23.6	+7.9	+5.2	+13.2
Contact	+33.4	−17.6	+34.7	+36.3 ± 2	−23.1
A_F[b]	+0.98	−0.52	+1.02	+0.95	−0.61

[a] Shifts in parts per million, referenced against uncomplexed diamagnetic $TMCOT^{2-}$ and COT^{2-}. U data given at 298°K, Np data at 307°K. [b] Hyperfine coupling constants in megahertz; estimated accuracy, ±25%.

The known structure[8] of $(COT)_2U$ and an extrapolated structure based upon ionic radii for $(TMCOT)_2Np$ plus the assumption of a freely rotating methyl group were used to calculate the geometric factors.[9] The calculated dipolar shifts of the various protons were subtracted from the experimental shifts to give the net contact shifts listed in Table I. The hyperfine coupling constants A_F are also given.[10] The derived contact shifts are of opposite sign for the methyl and ring protons and their ratios [−0.5 for (TMCOT)U and −0.6 for (TMCOT)Np] are indicative of systems having considerable π spin density.[11] These results strongly suggest that the spin density in our ligands is primarily in π MO's; indeed, application of the McConnell equation, $A = Q\rho/2S$, with $Q = -63$ MHz, indicates in excess of 0.1 unpaired electron per ligand ring. Although the coupling constants are approximately the same for $U(TMCOT)_2$ and $Np(TMCOT)_2$, the U complex has only two unpaired electrons whereas the Np complex has three and suggests stronger covalency for $U(TMCOT)_2$.[12] The high magnitude of this delocalized spin density compared with that observed in more ionic lanthanide and actinide complexes implies a high degree of covalency and raises the question of f-orbital involvement in such covalency.

Direct delocalization of f electrons into a vacant ligand π MO would give proton shifts of opposite sign to those observed.[2] A more plausible mechanism is charge transfer from filled ligand π MO's to vacant f orbitals; because of exchange interaction the transfer of spin parallel to the spin on the metal ion is energetically more favorable and would leave net posi-

(1) This research was supported in part by Grant No. 13369 of the National Science Foundation. Part of this work was performed under the auspices of the U. S. Atomic Energy Commission.

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(3) (a) A. Forman, J. N. Murrell, and L. E. Orgel, *J. Chem. Phys.*, **31**, 1129 (1959); (b) J. A. Happe and R. L. Ward, *ibid.*, **39**, 1211 (1963); (c) however, *cf.* W. D. Horrocks, Jr., and D. L. Johnston, *Inorg. Chem.*, **10**, 1835 (1971); note that the symmetry of the present complexes precludes application of their arguments.

(4) A. Streitwieser, Jr., and U. Muller-Westerhoff, *J. Amer. Chem. Soc.*, **90**, 7364 (1968).

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(6) P. de Mayo and R. W. Yip, *Proc. Chem. Soc.*, 84 (1964).

(7) At $1/T = 0$ the methyl proton shift extrapolates to a nonzero intercept greater than that for the ring-proton shift. At present we have no explanation for this observation.

(8) (a) A. Zalkin and K. N. Raymond, *J. Amer. Chem. Soc.*, **91**, 5667 (1969); (b) A. Avdeef, K. N. Raymond, K. O. Hodgson, and A. Zalkin, *Inorg. Chem.*, in press; use of the refined structure of $U(COT)_2$ greatly reduces the geometric uncertainty in the calculation of the dipolar shifts. For this reason the numbers given in Table I differ from those given in ref 2.

(9) The geometric factors of the ring H and methyl H for $(TMCOT)_2U$ are -0.0020×10^{24} cm^{-3} and -0.0059×10^{24} cm^{-3}, respectively; for the ring H and methyl H of $(TMCOT)_2Np$ the factors are -0.0024×10^{24} cm^{-3} and -0.0061×10^{24} cm^{-3}, respectively. Small variations of the C–CH_3 distances have little effect on the results.

(10) Similar assumptions as discussed in ref 2 were used to calculate the $(TMCOT)_2Np$ coupling constants.

(11) D. R. Eaton and W. D. Phillips, *Advan. Magn. Resonance*, **1**, 103 (1965).

(12) G. N. La Mar, *Inorg. Chem.*, **10**, 2633 (1971).