mediate chain-propagating steps in common, the chlorination reaction (6) was used as a model to determine the source and nature of the retardation.

$$RH + CCl_4 + CO \longrightarrow RCOCl + CHCl_3 \quad (5)$$

$$RH + CCl_4 \longrightarrow RCl + CHCl_3 \quad (6)$$

A series of peroxide-initiated chlorinations was carried out using CCl_4 and cyclohexane in glass tubes containing different added materials (Table I). Filings from the metal pressure reactor had no effect on the chlorination reaction (6), while 10% acid chloride had a modest influence; however, the combination of 10% acid chloride and a trace of metal (two sandlike grains) produced a significant decrease in conversion, while acid chloride along with 3% of the chrome–vanadium steel caused a dramatic decrease in conversion. The combination of acid chloride and steel appeared to act in concert, either producing a radical-chain inhibitor or destroying the peroxide initiator.

Table I. The Influence of Metal and Acid Chloride on the Chlorination of Cyclohexane by Carbon Tetrachloride

Conversion,[a] %	Acid chloride added,[b] %	Metal added,[b] %
95	None	None
93	None	3
85	10	None
40	10	Trace
8	10	3

[a] CCl_4 converted to chlorocyclohexane and $CHCl_3$. [b] Per cent of initial CCl_4.

A tube within a tube glass liner designed to minimize contact (by transfer of liquid through the CO weep holes in the liner by means of the rocking motion of the reactor) of the liquid reactants with the metal reactor was used in subsequent reactions. Good reproducible selectivities to acid chloride of up to 84% were obtained using this liner, while conversions, although quite acceptable, were subject to variation (Table II). We suspect that transfer of variable trace quantities of contaminant, from acid chloride–metal interaction, back into the liner caused the variation in conversions during the fixed reaction interval.

Table II. Peroxide-Initiated Chlorocarbonylation of Cyclohexane Using a Concentric Tubular Glass Reactor Insert[a]

CO pressure, psi at 25°	Conversion[b] after 16 hr, %	Selectivity to acid chloride, %
8000	34–49	89 ± 0.2[c]
6000	44–46	84 ± 0.5
4000	27–41	74 ± 1.0
2000	42–55	56 ± 1.0
1000	45–72	38 ± 2.7

[a] Cyclohexane:CCl_4 2:1; 5% *t*-butyl peroxide; 130°. [b] Based on initial CCl_4 concentration, for 16-hr runs. [c] Chlorocyclohexane 11%.

The ratio of acid chloride to alkyl chloride is plotted in Figure 1 as a function of the carbon monoxide pressure. The increasing slope indicates that this ratio is

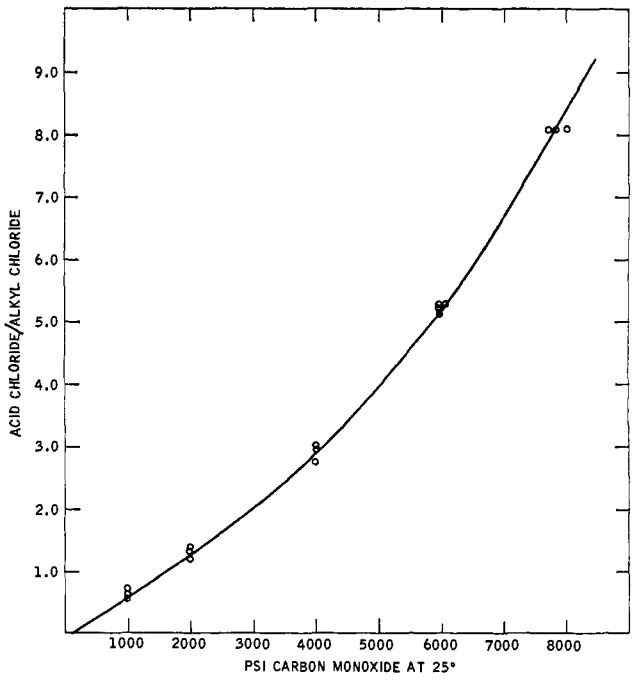

Figure 1. Effect of CO pressure on the ratio of acid chloride to alkyl chloride.

proportional to about the 1.3 power of the CO pressure. The 0.3 power may represent the deviation of CO concentration dependence on pressure from Henry's law.

The scope and mechanism of the chlorocarbonylation of a variety of paraffins with CCl_4 and CO is presently being investigated.

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Separation of Conformers. I. Axial and Equatorial Isomers of Monosubstituted Cyclohexanes

Sir:

The possibility of separating axial and equatorial isomers or boat forms of monosubstituted cyclohexanes has aroused the curiosity of chemists ever since the existence of different conformations in the molecules was postulated.[1] Claims of the isolation of conformational isomers of monosubstituted cyclohexyl compounds (stable at room temperature) have been made,[2] but the validity of these reports has been effectively challenged.[3] Now that data for the energy barriers of the chair–chair[1] interconversion of cyclohexane[4] and derivatives are

(1) H. Sachse, *Ber.*, **23**, 1368 (1890); *Z. Physik. Chem.*, **10**, 203 (1892); E. Mohr, *J. Prakt. Chem.*, **98**, 315 (1918); C. W. Becket, K. S. Pitzer, and R. Spitzer, *J. Am. Chem. Soc.*, **69**, 977, 2488 (1947).

(2) Examples are found in: (a) M. Quadrat-I-Kuda, *J. Indian Chem. Soc.*, **8**, 277 (1931); (b) D. M. Cowan, G. H. Jeffrey, and A. I. Vogel, *J. Chem. Soc.*, 1862 (1939).

(3) S. Goldschmidt and G. Grafinger, *Ber.*, **68**, 279 (1939); R. F. Miller and R. Adams, *J. Am. Chem. Soc.*, **58**, 787 (1936).

(4) F. R. Jensen, D. S. Noyce, C. H. Sederholm, and A. J. Berlin, *ibid.*, **84**, 386 (1962).

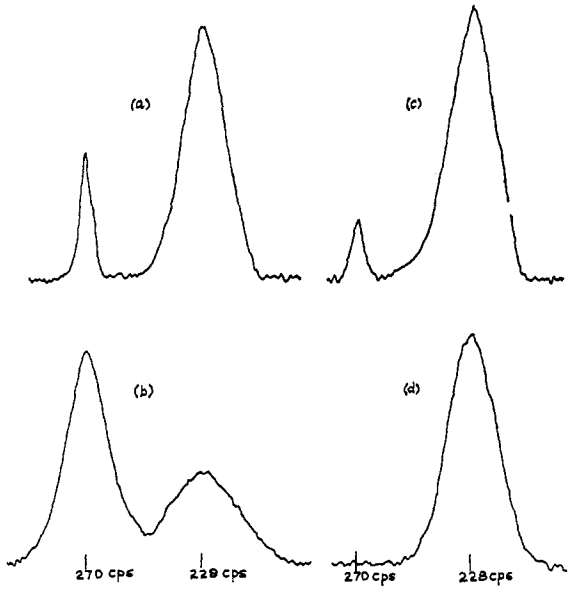
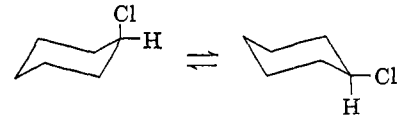


Figure 1. Methine proton resonances of equatorial and axial chlorocyclohexanes at various temperatures (CD_2CDCl solution, 60 Mc): (a) equilibrium mixture at $-115°$; (b) axial-halogen isomer-rich solution at $-151°$; (c) solid obtained at $-151°$; (d) pure equatorial cyclohexyl chloride.

available,[5,6] the temperature at which axial and equatorial isomers have substantial lifetimes can be calculated.

In order to investigate this phenomenon, a 0.35-g sample of chlorocyclohexane was placed in an nmr tube and diluted (dissolved) to a total volume of 0.6 ml with CD_2CDCl at $-80°$.[7] The solution was then cooled to $-150°$ and the nmr spectrum examined. After 1 hr, the axial-methine proton (equatorial chlorocyclohexane) resonance centered at 228 cps downfield from tetramethylsilane (TMS) had decreased substantially in intensity compared to the equatorial-methine proton (axial chlorocyclohexane) resonance centered at 270 cps downfield from TMS. The axial:equatorial halogen ratio was 58:42 (Figure 1b) at this point, indicating the preferential crystallization of the equatorial isomer. The mother liquor was then removed by suction, and the crystals were washed with additional CD_2CDCl and dissolved in 0.5 ml of CD_2CDCl. The nmr spectrum of the resultant solution at $-150°$ (Figure 1c) showed 95% equatorial chlorocyclohexane. Several attempts at purification of the equatorial isomer by crystallization from CD_2CDCl always gave about 5% axial isomer as contaminant

The solution of the 95% equatorial chlorocyclohexane in CD_2CDCl at $-150°$ was warmed rapidly to $-125°$. The equatorial-methine hydrogen resonance (δ = 270 cps) gradually grew in intensity. After about 20 min. an nmr spectrum of the methine proton resonances was very similar to that shown in Figure 1a with the ratio of the peak areas constant. At $-115°$, the axial: equatorial chloride ratio (eq 1) is 13:87.



(1)

However, 100% pure equatorial chlorocyclohexane was prepared by cooling very slowly (*ca.* 5°/0.5 hr) a 0.09-g sample of chlorocyclohexane through its melting point range (−43.9°). After crystallization was complete, the sample was cooled to $-151°$ and 0.5 ml of CD_2CDCl was added. The nmr spectrum of the resultant solution gave only one methine proton resonance (Figure 1d), that being the relatively broad axial-methine proton resonance (δ = 228 cps). None of the sharper equatorial-methine proton resonance (δ = 270 cps) could be detected even in a substantially enlarged spectrum. The nmr spectrum was repeatedly scanned over a period of about 2 hr at $-151°$, and no changes were observed. These results are in agreement with our prediction that the half-life for interconversion at $-150°$ is many hours.

Similar experiments were carried out with trideuteriomethoxycyclohexane. A sample of the neat liquid in an nmr tube was cooled slowly to $-150°$ and the resulting solid was dissolved in CD_2CDCl. Examination of the methine proton resonance indicated the presence of only equatorial trideuteriomethoxycyclohexane. When the solution was warmed to $-96°$, both forms appeared. At about $-70°$, the resonances coalesced. These results are in contradiction to the report of Reeves and Strømme that the interconversion is still very fast at $-110°$.[8] This change is difficult to observe with the undeuterated compound because the methine and methyl resonances occur at approximately the same position. The *A* value of the trideuteriomethoxyl group at $-96°$ is 0.56 ± 0.02 kcal/mole.

Although a number of workers have obtained and characterized conformational isomers of compounds such as 1,2-dichloroethane,[9] 1,2-dibromoethane,[9] 1,1,-2,2-tetrabromoethane,[10] and chlorocyclohexane,[11] as solids, the present results are unique in that conformational isomers have been separated, wholly or in part, and examined in *solution*.

The availability of these isomers and nonequilibrated mixtures provides a means of measuring the rate constant for the chair–chair interconversion at temperatures very different from those used for determining the rates by conventional nmr site-exchange methods.[5] Reliable enthalpies and entropies of activation can likely be obtained from these sets of data, and this information should be useful in understanding the nature of the transition state.

An attempt is underway to improve the techniques in the hope of obtaining pure axial chlorocyclohexane. Our plans also include the separation of *meso* and *dl* rotational diastereomers of ethane derivatives having high rotational barriers and to attempt to resolve the enantiomorphic pair.

(5) L. W. Reeves and K. O. Strømme, *Can. J. Chem.*, **38**, 1241 (1960).

(6) A. J. Berlin, Ph.D. Thesis, University of California, Berkeley, 1961.

(7) All experiments reported here were carried out in standard nmr tubes utilizing the Varian HR-60 spectrometer fitted with a special variable temperature probe.

(8) L. W. Reeves and K. O. Strømme, *Trans. Faraday Soc.*, 57, 390 (1961).

(9) S. Mizushima, "Structure of Molecules and Internal Rotation," Academic Press Inc., New York, N. Y., 1954.

(10) R. E. Kagarise, *J. Chem. Phys.*, **24**, 300 (1956).

(11) P. Klaeboe, J. Lothe, and K. Lunde, *Acta Chem. Scand.*, 10, 1465 (1956); K. Kozima and K. Sakashita, *J. Chem. Soc. Japan*, 31, 796 (1958).

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Substrate Activation of Trypsin. The Effect of Enzyme Acetylation[1]

Sir:

Extensive acetylation increases the specific activity of chromatographically purified trypsin using TAME as substrate.[2,3] Various acetylation procedures using acetic anhydride and N-acetylimidazole lead consistently to enhancement of the molecular activity of trypsin (278 ± 45 μmoles of TAME hydrolyzed sec^{-1} $\mu mole^{-1}$ of functional enzyme [or sec^{-1}]). The molecular activity of $AT_{6.7}$,[2] which was employed in this study, is 1118 ± 170 sec^{-1}. Both the TAME assays ([S] = 1.6×10^{-2} *M*) and active site titrations[4] were performed at the pH optimum common to trypsin and $AT_{6.7}$ of 8.7 in 5.0×10^{-3} *M* borate, 5.0×10^{-3} *M* KCl, 1.0×10^{-2} *M* $CaCl_2$, 25°. *p*-Nitrophenyl acetate ($0.6–2.4 \times 10^{-3}$ *M*, 5 vol. % acetonitrile) was employed for the active site titrations. *p*-Nitrophenyl-α-benzyloxycarbonyl-L-lysinate could not be used because of pH-dependent departures from stoichiometry below *ca.* pH 7.5 in the case of $AT_{6.7}$. Both substrates were used for trypsin with no significant difference between the results. No detectable activity upon N-benzoyl-L-tyrosine ethyl ester was observed.

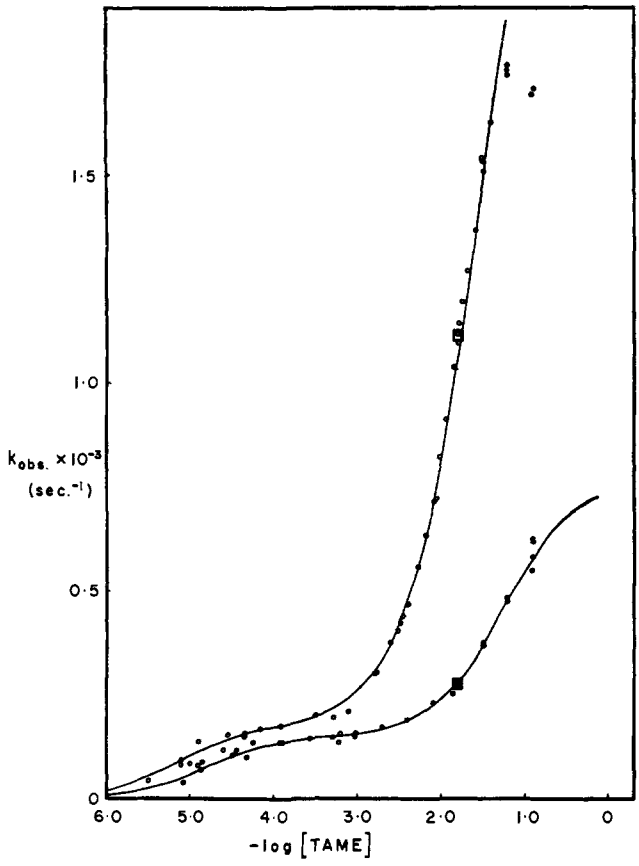

Figure 1. A comparison of the substrate concentration dependence of k_{obsd} for the trypsin- (closed circles) and $AT_{6.7}$-catalyzed (open circles) hydrolysis of TAME. Conditions: 0.20 *M* NaCl, 5.0×10^{-3} *M* KCl, 1.0×10^{-2} *M* $CaCl_2$, 25.0°, pH 8.70. Mole ratios: TAME/trypsin = $1.6 \pm 0.1 \times 10^6$; TAME/$AT_{6.7}$ = $3.8 \pm 0.0 \times 10^6$. Concentrations were established by adjustment of total solution volumes. Squares represent reference assay concentration, 1.6×10^{-2} *M*.

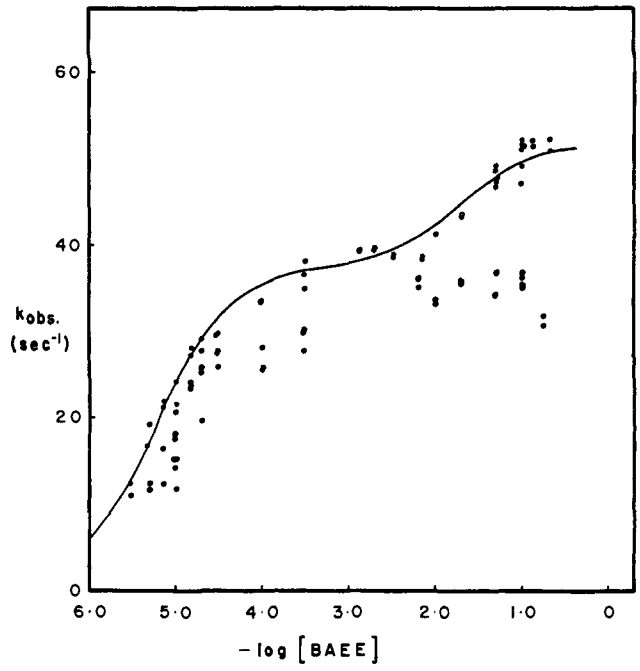

Figure 2. A comparison of the substrate concentration dependence of k_{obsd} for the trypsin- and $AT_{6.7}$-catalyzed hydrolysis of BAEE. The symbols and conditions are the same as in Figure 1. Mole ratios: BAEE/trypsin = $1.2–2.0 \times 10^5$; BAEE/$AT_{6.7}$ = $1.8–3.9 \times 10^5$.

Kinetic studies were initiated in an effort to explain this apparent selective enhancement of the catalytic capability of trypsin upon acetylation of the enzyme. The effect of TAME concentration upon k_{obsd} (μmoles of TAME hydrolyzed sec^{-1} $\mu mole^{-1}$ of functional enzyme) for trypsin and $AT_{6.7}$ are compared in Figure 1. The experimental points represent data acquired using chromatographically purified[3] enzyme preparations. All data have been corrected for substrate blanks. Enzyme blanks were negligible. Very similar results were obtained when the purification steps were omitted. In all cases the data at high TAME concentrations deviate in a systematic fashion from predictions based upon the generally accepted characteristics of the trypsin-catalysis of esters of this type involving rapid, reversible binding of a single substrate molecule followed by the first-order formation of an enzyme–substrate inter-

(1) This investigation was supported in whole by Public Health Service Grant GM11189 from the Institute of General Medical Sciences. The authors are grateful to J. T. Madison for the use of a Cary Model 15 spectrophotometer and to M. W. Anders, J. M. Hanson, and R. C. Rychert for their advice and assistance.

(2) The following abbreviations are employed: TAME, N-α-*p*-toluenesulfonyl-L-arginine methyl ester; BAEE, N-α-benzoyl-L-arginine ethyl ester; $AT_{6.7}$, Worthington two-times-crystallized, salt-free bovine trypsin (10 mg ml^{-1}) acetylated with 1 μl of redistilled acetic anhydride/mg of enzyme, 4°, pH 6.7 maintained by addition of NaOH employing a Radiometer autotitrator-titrigraph.

(3) H. L. Trenholm and J. F. Wootton, Abstracts, 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1965, p 81C.

(4) M. L. Bender, J. V. Killheffer, Jr., and R. W. Roeske, *Biochem. Biophys. Res. Commun.*, 19, 161 (1965).