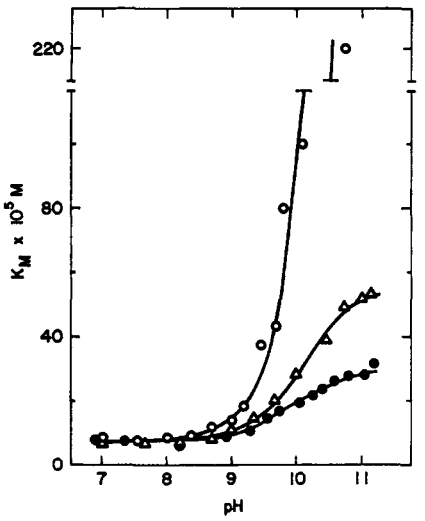

Figure 2. pH-K_m(app) profiles for the α-chymotrypsin- (○), α_1-chymotrypsin- (Δ), and δ-chymotrypsin-catalyzed (●) hydrolyses of ATME. Runs were performed at 25° in 1.6% (v/v) dimethyl sulfoxide. Each point is the average of three determinations which agreed within 10%. The solid lines are calculated from the equation $K_m(app) = K_m^{lim}(app)\{(1 + K_a^E)/[H^+]\}/\{(1 + K_a^{ES})/[H^+]\}$ using the values $pK_a^E = 9.0$, $pK_a^{ES} > 11$ for α-chymotrypsin; $pK_a^E = 9.3$, $pK_a^{ES} = 10.2$ for α_1-chymotrypsin; and $pK_a^E = 9.25$, $pK_a^{ES} = 9.75$ for δ-chymotrypsin.

buffer, pH 7.6, 0.3 M in $(NH_4)_2SO_4$. After 12 hr at 25°, the solution was made 4×10^{-4} M in diisopropyl fluorophosphate (DFP), incubated 2 hr at 25°, and dialyzed extensively against 1×10^{-4} M HCl. The protein obtained is inactive (assayed with ATME prior to the addition of DFP). It contains 1.0 mol of tyrosine/mol of protein as C-terminal[10] and 0.75 mol of threonine and 0.10 mol of alanine/mol of protein as N-termini,[11] indicating that the preparation consists mainly of II, a protein called *threo*-neochymotrypsinogen after Rovery, *et al.*[9]

Treatment of II with 5% (w/w) trypsin results in a very rapid activation, to the extent of 85–90%. The enzyme obtained after removal of trypsin and unreacted zymogen by affinity chromatography[12] contains 1.0 mol of tyrosine and 0.90 mol of leucine/mol of enzyme as C-terminal residues. N-Terminal residues were 0.80 mol of isoleucine, 0.72 mol of threonine, and 0.10 mol of alanine/mol of enzyme, indicating that the preparation consists of about 90% α_1-chymotrypsin and 10% α-chymotrypsin.

The hydrolysis of ATME was followed in a Cary-14 recording spectrophotometer as described previously.[5] K_m(app) and k_{cat} values were obtained from Eadie plots[13] of three consecutive runs at each pH. The pH dependence of K_m(app) for the α_1-chymotrypsin-catalyzed hydrolysis of ATME is presented in Figure 2 where a comparison is made with the values obtained with α- and δ-chymotrypsins. The k_{cat} values and their pH dependences were found to be the same for the three enzymes.

It can be seen that the K_m(app) values for the α_1-chymotrypsin-catalyzed reaction increase significantly less above pH 9 compared to α-chymotrypsin. The data for α_1-chymotrypsin are consistent with a dependence on a group of the enzyme with an apparent pK_a of 9.3 which shifts upon binding to 10.2. This pH dependence of K_m resembles very closely the behavior of δ-chymotrypsin.[5] Similar results have been obtained with other specific ester substrates such as *N-trans*-(2-furyl)acryloyl-L-tryptophan methyl ester and *N-trans*-(2-furyl)acryloyl-L-phenylalanine methyl ester.[14]

This result together with those reported previously on the kinetic properties of δ-chymotrypsin lead us to conclude tentatively that the ionization state of the amino group of alanine-149 is a key factor in determining the behavior of chymotrypsins at high pH. Thus, it is conceivable that the loss of the binding ability of α-chymotrypsin in the alkaline pH region is due to two, apparently unrelated, causes: (a) a major disruption or blocking of the binding site, triggered by the deprotonation of the alanine-149 amino group; (b) a minor disruptive effect caused by the deprotonation of the isoleucine-16 amino group. In the case of δ- and α_1-chymotrypsins, where the alanine-149 amino group is not free to ionize, only (b) is operative. The evidence relating the ionization state of the isoleucine-16 amino group with the decrease in k_{cat}/K_m is indirect. Furthermore much of this evidence was obtained using δ-chymotrypsin rather than α-chymotrypsin. This extrapolation is tacitly based on two questionable assumptions: (1) that the kinetic behaviors of the two enzymes at high pH are the same; (2) that the structures of both enzymes are identical, but they are not. Further work exploring the postulated involvement of alanine-149 is in progress.

Acknowledgment. This research was supported by Grant No. HEO-5726 from the National Institutes of Health, U. S. Public Health Service.

(10) Quantitative N-terminal group determinations were performed by the method of F. Sanger, *Biochem. J.*, **39**, 507 (1945). Dinitrophenyl amino acids were measured spectrophotometrically after separation by thin-layer chromatography.

(11) C-Terminal analysis was carried out using DFP-treated carboxypeptidase A by a procedure adapted from J. T. Potts, Jr., *Methods Enzymol.*, **11**, 648 (1967).

(12) P. Cuatrecasas, M. Wilchek, and C. B. Anfinsen, *Proc. Nat. Acad. Sci. U. S.*, **61**, 636 (1968).

(13) G. S. Eadie, *J. Biol. Chem.*, **146**, 85 (1942).

(14) P. Valenzuela and M. L. Bender, unpublished results.

(15) On leave from the School of Chemistry and Pharmacy, University of Chile, Casilla 233, Santiago, Chile. Recipient of a Fulbright-Hays Travel Grant.

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Substitutionally Labile Chromium(III)

Sir:

We have previously discovered that the porphyrin ligand labilizes Co(III) in its substitution reactions.[1] Recently, labilization of Co(III) has been found to occur in other types of complexes[2] and a similar mechanism proposed for substitution in a macrocyclic complex.[3] Our previous interpretation of the labilized cobalt was based either on an internal redox reaction[4]

(1) E. B. Fleischer, S. Jacobs, and L. Mestichelli, *J. Amer. Chem. Soc.*, **90**, 2527 (1968).

(2) J. Halpern, R. Palmer, and L. Blakeley, *ibid.*, **88**, 2877 (1966); D. G. DeWit, M. J. Hynes, and D. A. Sweigart, *Inorg. Chem.*, **10**, 196 (1971); H. G. Tsiang and C. H. Langford, *Can. J. Chem.*, **48**, 2776 (1970).

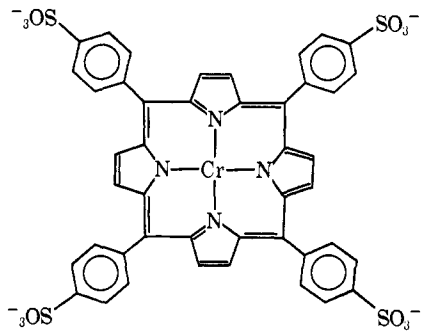
(3) J. G. Jones and M. V. Twigg, *Inorg. Chem.*, **8**, 2120 (1969).

Table I[a]

Ligand, L	Range of concentration studied	k_4, sec^{-1}	k_1k_3/k_2, $sec^{-1}\ M^{-1}$	Kinetic K_1	Spectrophotometric K_1	Spectrophotometric K_2
F^- [b]	1.0×10^{-2}–5.0×10^{-1} F	1.67×10^{-4}	5.50×10^{-3}	33.0	27	
CN^- [c]	2.0×10^{-4}–1.0×10^{-2} F	4.00×10^{-3}	1.38	345.0	150	14
Pyridine[d]	2.5×10^{-4}–4.0×10^{-2} M	9.67×10^{-2}	3.75	39.0	49	5.0

[a] All the studies were done at 25.0 ± 0.1°. [b] μ = 1.0 F, 444 nm, pH 7.0 (Tris). [c] μ = 0.1 F, 451 nm, pH 12 (OH^- = 10^{-2} M). [d] μ = 0.1 F, 444 nm, pH 7.0 (Tris).

taking place leading to $Co^{2+}P^+$ (P stands for the porphyrin ligand) or on a ligand system that interacted with the cobalt ion in such a way as to have the metal ion lose its "d^6" character. The metalloporphyrin has a very delocalized electronic structure that strongly mixes the ligand and metal-ion orbitals. This paper describes an experiment that was carried out to give us some new facts concerning this system to allow for a better understanding of the labilization. Since it is well known that "d^3" Cr(III) complexes are very inert toward substitution and also harder to reduce to Cr(II) compared to the Co(III) to Co(II) reduction, we undertook the study of the kinetics of substitution of Cr(III) porphyrins. We have employed the chromium salt of the water-soluble tetra(*p*-sulfonatophenyl)porphine (M-TPPS, I) in this study.[5,6] Equilibrium constants



I

for the chromium complex were determined by spectrophotometric methods at constant ionic strength and a temperature of 25°.[8,9]

(4) This type of redox reaction has been observed in porphyrin chemistry recently: G. Wolberg and J. Manassen, *J. Amer. Chem. Soc.*, **92**, 2982 (1970).

(5) E. B. Fleischer, J. M. Palmer, and T. S. Srivastava, *ibid.*, **93**, 3162 (1971); E. B. Fleischer and T. S. Srivastava, *Inorg. Chim. Acta*, in press.

(6) $Na_3CrTPPS$ was synthesized according to ref 5 and 7. *Anal.* Calcd for $Na_3CrC_{11}H_{18}N_4O_{21}S_4$: C, 41.74; H, 3.79; N, 4.43; S, 10.12; Cr, 4.11. Found: C, 41.64; H, 2.99; N, 4.64; S, 10.17; Cr, 4.76; magnetic susceptibility (temperature = 298°) μ_{eff} = 3.78 BM.

(7) A. Adler, F. Tango, F. Kampas, and J. Kim, *J. Inorg. Nucl. Chem.*, **32**, 2443 (1970).

(8) It should be noted that the species we have formulated as the *di*aquochromium species may be a five-coordinate *mono*aquo complex. We are carrying out further experiments to clarify this point.

(9) Other equilibria which were studied in solution are defined here.

$$\begin{array}{l} OH_2 \quad\quad L \\ |\quad\quad\ | \\ Cr\text{-TPPS} + L \rightleftharpoons Cr\text{-TPPS} + H_2O \quad\quad K_1 \\ |\quad\quad\ | \\ OH \quad\quad OH \end{array}$$

$$\begin{array}{l} L \quad\quad L \\ |\quad\quad\ | \\ Cr\text{-TPPS} + L \rightleftharpoons Cr\text{-TPPS} + OH^- \quad\quad K_2 \\ |\quad\quad\ | \\ OH \quad\quad L \end{array}$$

The 1:1 and 2:1 species are well enough separated in stability so that each species can be studied in solution.

$$\begin{array}{l} OH_2 \quad\quad OH \\ |\quad\quad\ | \\ Cr(III)\text{-TPPS} \rightleftharpoons Cr(III)\text{-TPPS} + H^+ \quad\quad pK_1 = 4.8 \\ |\quad\quad\ | \\ OH_2 \quad\quad OH_2 \end{array}$$

$$\begin{array}{l} OH \quad\quad OH \\ |\quad\quad\ | \\ Cr(III)\text{-TPPS} \rightleftharpoons Cr(III)\text{-TPPS} + H^+ \quad\quad pK_2 = 7.9 \\ |\quad\quad\ | \\ OH_2 \quad\quad OH \end{array}$$

The kinetics of the reaction of the Cr(III)-TPPS with the ligands fluoride, pyridine, and cyanide were followed spectrophotometrically in a Durrum-Gibson stopped-flow machine at 25° and ionic strength equal to 0.1 F. The pseudo-first-order rate constants, k_{obsd}, obtained from the plots of ln ([Cr(TPPS)(OH)-$(OH_2)]_t$ − [Cr(TPPS)(OH)$(OH_2]_\infty$) *vs.* time all obeyed the law

$$k_{obsd} = \alpha[L] + \beta \quad (1)$$

If a mechanism of the following type is assumed[10,11]

$$\begin{array}{l} OH_2 \\ | \\ Cr(III)\text{-TPPS} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Cr(III)\text{-TPPS} \\ | \quad\quad\quad\quad\ | \\ OH \quad\quad\quad\quad OH \end{array}$$

$$\begin{array}{l} \quad\quad\quad\quad\quad\quad L \\ \quad\quad\quad\quad\quad\quad | \\ Cr(III)\text{-TPPS} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} Cr(III)\text{-TPPS} \\ | \quad\quad\quad\quad\ | \\ OH \quad\quad\quad\quad OH \end{array}$$

then the rate law for the approach to equilibrium is

$$-\frac{d \ln [Cr(TPPS)(OH)(OH_2)]}{dt} = \frac{k_1k_3}{k_2}[L] + k_1$$

where

$$k_{obsd} = \frac{k_1k_3}{k_2}[L] + k_4$$

in analogy with (1). A plot of k_{obsd} *vs.* [L] would give k_4 as the intercept (β), k_1k_3/k_2 as the slope (α), and the equilibrium constant for the overall reaction, $K_1 = k_1k_3/k_2k_4 = \alpha/\beta$. The values derived graphically from the study for the substitution reactions of the chromium metalloporphyrin are given in Table I. The values of the constants in Table I clearly illustrate the labilizing effect that the porphyrin ligand has upon the Cr(III) substitution reactions. A close comparison is not available for the chromium as it was for the cobalt case, but comparison of rates of anation reactions shows that the chromium–porphyrin complexes are about 10^3–10^4 times faster than "normal" chromium complexes in their substitution reactions.[12] In

(10) A. Haim and W. K. Wilmarth, *Inorg. Chem.*, 1, 573 (1962).

(11) As pK_1 is 4.8 and pK_2 is 7.9, it was assumed that the reacting species is the monohydroxy species.

(12) N. Duffy and J. E. Earley, *J. Amer. Chem. Soc.*, **89**, 272 (1967); E. Campi, J. Ferguson, and M. T. Tobe, *Inorg. Chem.*, **9**, 1781 (1970).

the cobalt case the rates were about 10^6 times faster.[1] As was expected, the Cr(III)–TPPS is not easily reduced and thus the likelihood of an internal redox mechanism explaining the lability can be ruled out. Thus it appears that the porphyrin ligand labilizes both Cr(III) and Co(III) species by some type of electronic effect which has as its origins the delocalized electronic structure of the complex in which there is very strong mixing of metal-ion orbitals and ligand orbitals causing the metal ion to lose its d^6 or d^3 character. It has recently been found that ruthenium is also labilized in its substitution reactions when complexed to the porphyrin.[13] Other molecular systems in which this type of delocalization may take place, such as in the dithiolene system, can be expected to produce labile Co(III) and Cr(III) complexes.[14]

(13) M. Tsutsui, D. Osteld, and L. Hoffman, *J. Amer. Chem. Soc.*, 93, 1821 (1971).

(14) This work was supported by the National Institutes of Health.

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Specific Alkylation of Polycyclic Hydrocarbons *via* Reductive Alkylation and Oxidative Rearrangement

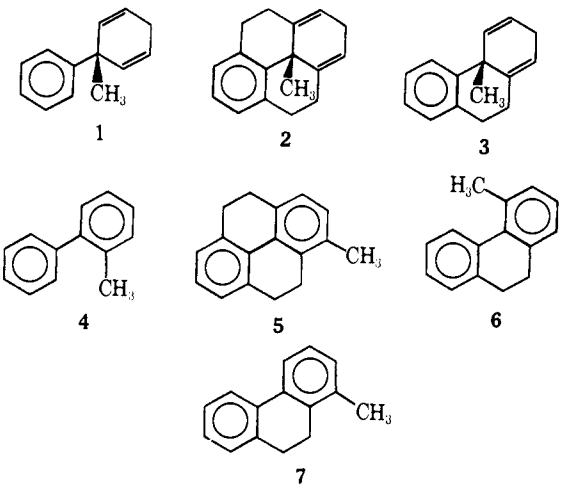
Sir:

We wish to report a novel and convenient two-step procedure for the introduction of alkyl groups into specific sites of polycyclic aromatic ring systems.

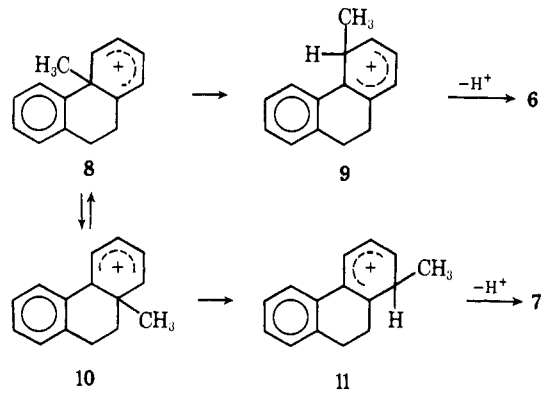
Interaction of methyl bromide with the anionic intermediates from the reaction of lithium metal with biphenyl, 4,5,9,10-tetrahydropyrene and 9,10-dihydrophenanthrene in liquid ammonia affords in excellent yield (>90%) the corresponding monomethyl 1,4-cyclohexadienes **1**, **2**, and **3**,[1] respectively. The latter, upon treatment with trityl fluoroborate[2] in 1,2-dichloroethane, undergo facile simultaneous rearrangement and aromatization to the related monomethyl derivatives (**4**, **5**, and **6** and **7**, respectively) of their hydrocarbon precursors. Compounds **6** and **7** are formed in the ratio 1.6:1, indicative of somewhat greater preference for 1,2 over 1,3 migration of the methyl group. Isomers, other than **4**–**7**, occur to the extent of <1%, if at all. Conversion of **5**, **6**, and **7** to fully aromatic methylpyrene and methylphenanthrenes is smoothly effected upon treatment with excess trityl cation reagent.[3]

Reactions of the two bridged compounds, **2** and **3**, with trityl fluoroborate are complete within 1 hr at 3–5°, whereas **1** remains unchanged under these conditions. Conversion of **1** is complete, however, in 10 min at reflux temperature.

The intermediate benzenonium ions (*e.g.*, **8**) formed on hydride abstraction from the methyl-1,4-cyclohexadienes are essentially σ complexes. They are unusual, however, in possession of both alkyl and aryl substituents on the tetrahedral carbon atom. The ethylene bridges in the benzenonium ions derived from **2** and **3** may be



expected to contribute substantially to the stabilization of these intermediates,[4] an effect probably sufficient to account for their greater reactivity relative to **1**. Also, the steady-state concentration of the initial benzenonium ions may be expected to be low relative to that of the trityl cation and to vary in the series **2** > **3** > **1**.[4] In agreement, addition of water to the product of reaction of **1** and the trityl fluoroborate reagent at 3–5° leads to recovery of **1** and trityl alcohol and no detectable quantity of a hydroxy derivative of **1**.



The benzenonium ions (*e.g.*, **9** and **11**) arising on methyl migration to a secondary position must be formed essentially irreversibly. This follows, since other isomers of **4**–**7** are not found, and the rate of proton loss from intermediates **9** and **11** (*i.e.*, aromatization) may reasonably be assumed to greatly exceed the rate of further methyl shift.[5] Product distribution, therefore, is presumably a consequence of the position of the equilibrium between **8** and **10** and the relative rates for the latter to undergo transformation to **9** and **11**, respectively. Predominance of the 4-substituted isomer of 9, 10-dihydrophenanthrene (**6**) is in agreement with the larger partial rate factor for electrophilic substitution in the 4 position compared with the 1 position of this hydrocarbon.[6]

In a typical reductive methylation, a solution of biphenyl (2.5 mmol) in anhydrous ether (75 ml) is added

(1) Direct internal alkylation of the pyrene ring system, to our knowledge, has not been previously reported. Synthesis of **3** was reported earlier: P. W. Rabideau and R. G. Harvey, *J. Org. Chem.*, 35, 25 (1970).

(2) H. Y. Dauben, Jr., L. R. Honnen, and K. M. Harmon, *ibid.*, 25, 1442 (1960).

(3) W. Bonthrone and D. H. Reid, *J. Chem. Soc.*, 2773 (1959).

(4) For a discussion of σ complexes see D. A. McCaulay in "Friedel-Crafts and Related Reactions," Vol. II, G. A. Olah, Ed., Interscience, New York, N. Y., 1964.

(5) Methyl migration is considerably more probable than is phenyl migration; see M. J. S. Dewar in "Molecular Rearrangements," Vol. I, P. de Mayo, Ed., Interscience, New York, N. Y., p 322.

(6) P. B. D. de la Mare, E. A. Johnson, and J. S. Lomas, *J. Chem. Soc.*, 6893 (1965); 5317 (1964).