Kinetic Effects of Alkyl Quaternary Ammonium Salts on the Methanesulfonylation of the Acetylcholinesterase Catalytic Center. Significance of Substituent Volumes and Binding Enthalpies[1]

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Abstract: The problem of which thermodynamic property is measured by the effects of enzyme regulators is briefly analyzed. The working hypothesis is offered that enthalpies of binding may be informative in this respect. It was previously shown that while ΔG binding for some 35 tetramethylammonium (TMA) salts on acetylcholinesterase (AChE) is largely insensitive to structural effects, ΔH binding is markedly sensitive. The possibility offered itself that ΔH binding and degree of conformational change may be interrelated. To test this hypothesis, advantage was taken of the known fact that certain quaternary ions stimulate AChE toward irreversible methanesulfonylation. Acceleration of this reaction by quaternary salts involves a ternary complex as the kinetically significant species and is thought to reflect conformational changes in the enzyme. We have examined homologous series of *n*-alkyl- and cycloalkyl-TMA salts on this reaction and found that all accelerate the reaction of methanesulfonyl fluoride (MSF) with the enzyme. Branching in the TMA substituents dampens acceleration. Unbranched *n*-alkyl and cycloalkyl substituents gave rise to almost symmetrical bell-shaped curves when acceleration was plotted against molar volumes. The results eatablish that the salt substituents project away (exo) from the esteratic nucleophiles (endo sites). A geometrical model of the enzyme binding cleft is proposed which accounts for the steric effects on acceleration. No correlation was found between volume effects on acceleration and on ΔG binding of the salts, respectively. However, volume effects on ΔH binding provided the basis of a tentative interpretation in physical terms of the acceleration effects. The hypothesis is submitted that ΔH binding may reflect conformational changes such as would be initiated by ligand release of water from the binding cleft.

The concept that metabolic regulators influence enzyme activity by inducing changes of conformation is a well-recognized one,[2,3] but the question arises as to which thermodynamic property is measured by the observed effects of regulatory ligands. *A priori*, the macroscopic binding free energies of effector ligands may not be expected to correlate with the relative magnitudes of the induced shifts in conformational stability because (a) the ligands may bind on more than one conformational state of the enzyme, and (b) wholesale compensation in the thermodynamic parameters may obscure any special effects of the ligands on conformationally strategic bonds. In the physical sense, a change from one to another unique enzyme conformation may be defined as a stereotyped alteration in the bookkeeping of bond-breaking and bond-making reactions, which suggests that the relative heats of ligand binding may, within the framework of a single physical interaction mechanism,[4] constitute the major thermodynamic modulus underlying the structurally induced rate effects of ligands on enzymes. Owing to enthalpy–entropy compensation effects,[4,5] ΔG binding would obviously be relatively insensitive to any special physical effects of the ligands. In a preliminary study of this fundamental problem, we have recently shown[6] that for the case of quaternary ion binding on the enzyme acetylcholinesterase (AChE), ΔG binding for some 35 salts was largely insensitive to structural effects, but in marked contrast, the van't Hoff enthalpies were markedly sensitive even to relatively small structural changes. As regards ΔH–ΔS compensation, our data gave a good correlation coefficient for linear regression, the slope of the line being 288°K (T_c or "compensation temperature"[5]). Of course, this does not establish that the compensation pattern is truly linear.[7] The data only showed that a correlation exists. Since the experimental demonstration of linear ΔH–ΔS compensation remains problematic,[7] one must reply on Leffler's criterion of "chemical consistency,"[4,8] in order to decide whether compensation corresponds to reality. Application of this criterion did support the existence of compensation since the binding enthalpies also gave a good correlation with the molar volumes of the ligands.[6] Hence our conclusion that the value of 288°K for T_c would take its origin largely in the control exerted by enzyme-bound water on the ΔH–ΔS fluctuations is reasonable enough. Encouraged by these observations, we have next turned to the difficult problem of evaluating the possible quantitative relevance of binding enthalpies to the conformational response of the enzyme to quaternary salts differing only in the size and shape of their apolar substituents. Experimentally, it is not yet possible to quantitate subtle changes of conformation in enzymes by direct methods other than X-ray crystallography. However, indirect methods may be used, one of the most useful relying on the sensitivity of active-site reactivity to changes of conformation. Accordingly, we have tried

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Table I. Acceleration Effects of the Monoquaternary Salts $R-CH_2N^+(CH_3)_3$ on the Rate of MSF Reaction with AChE

R-TMA	[Salt] × 10^{-3} M/l.	$\alpha = k/k_0$	ΔH binding, kcal/mol
H	175.77[a]	6.0 ± 0.1	−6.60 ± 0.2[f]
	29.16[b]		
n-Propyl (C_3)	38.00[a]	7.3 ± 0.1	−5.22 ± 0.3[f]
	8.24[c]		
n-Butyl (C_4)	15.67[a]	8.5 ± 0.1	−5.40 ± 0.4[f]
	12.00[a]		
n-Pentyl (C_5)	17.76[c]	11.1 ± 0.2	−4.55 ± 0.3[f]
	70.71[a]		
n-Hexyl (C_6)	7.27[a]	8.3 ± 0.3	−4.49 ± 0.4[f]
	3.63[a]		
n-Heptyl (C_7)	6.40[c]	6.8 ± 0.3	−4.40 ± 0.2[f]
	19.15[a]		
n-Nonyl (C_9)	6.19[c]	6.0 ± 0.1	−4.26 ± 0.3[f]
	4.47[a]		
n-Undecyl (C_{11})	0.38[c]	4.8 ± 0.2	−2.75 ± 0.4[f]
	3.00[a]		
2-Methyl-*n*-pentyl	1.00[a]	4.0 ± 0.2	
	0.47[a]		
Cyclobutyl (cC_4)	2.64[a]	12.4 ± 0.2	−3.10 ± 0.2[g]
	1.40[a]		
Cyclopentyl (cC_5)	3.77[a]	38.4 ± 0.3	−3.00 ± 0.7[g]
	1.69[a]		
Cyclohexyl (cC_6)	5.88[a]	57.0 ± 0.5	−0.85 ± 0.5[g]
	3.38[a]		
Cycloheptyl (cC_7)	62.58[b]	23.0 ± 0.5	−4.65 ± 0.1[g]
	41.77[b]		
Cyclooctyl (cC_8)	8.84[c]	11.5 ± 0.3	−5.00 ± 0.5[g]
	4.55[b]		
Bicyclo[2.2.1]-heptyl (B_7)	55.16[c]	2.5 ± 0.2	0.00 ± 0.3[g]
	28.84[d]		
2-Bicyclo[2.2.2]-octyl (B_8)	15.14[e]	4.8 ± 0.2	−0.20 ± 0.2[g]
	9.42[a]		
1-Adamantyl (Ad)	89.55[b]	1.0 ± 0.1	−3.00 ± 0.3[g]
	49.10[c]		

[a] [MSF] = 13.92 × 10^{-6} M; [AChE] = 10 units/ml. [b] [MSF] = 20.6 × 10^{-7} M; [AChE] = 80 units/ml. [c] [MSF] = 87.64 × 10^{-6} M; [AChE] = 80 units/ml. [d] [MSF] = 6.2 × 10^{-7} M; [AChE] = 80 units/ml. [e] [MSF] = 30.9 × 10^{-6} M; [AChE] = 80 units/ml. [f] B. Belleau, H. Tani, and F. Lie, *J. Amer. Chem. Soc.*, **87**, 2283 (1965); B. Belleau and J. L. Lavoie, *Can. J. Biochem.*, **46**, 1397 (1968); B. Belleau, *Ann. N. Y. Acad. Sci.*, **144**, 705 (1967). [g] B. Belleau and J. L. Lavoie, *Can. J. Biochem.*, **46**, 1397 (1968).

to probe the chemical reactivity of the AChE esteratic nucleophiles in the presence of selected quaternary salts for which the thermodynamic parameters of binding are known.[6] The basis of an experimental approach was provided by Kitz and Wilson's discovery[9,10] of the large kinetic effects of certain ammonium and quaternary salts on the irreversible esterification of the active serine hydroxyl of AChE by methanesulfonyl fluoride (MSF). This observation was interpreted in terms of salt-induced changes of conformation in the enzyme,[11,12] thus providing a potentially useful tool in our search for the thermodynamic property which the observed salt effects may be a measure of. It is the purpose of this communication to report on (a) the kinetic effects of *n*-alkyl and cycloalkyltetramethylammonium salts (TMA salts) on the rate of methanesulfonylation of AChE and (b) on the interaction topographies and thermodynamic parameters probably underlying the conformational response of the enzyme to salt binding.

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Experimental Section[13]

Reagents. All the TMA salts used in these studies were reported previously. The structures are defined in Table I. Bovine erythrocyte AChE from Nutritional Biochemicals was used throughout. All other chemicals were reagent grade.

Kinetic Methods. The initial velocities of ACh hydrolysis by AChE were determined at 25 ± 0.1°, pH 7.4, under a CO_2-free nitrogen atmosphere, by the pH-stat method (Copenhagen Radiometer TTTIC, Ole Dich recorder with syringe attachment and automatic drive). The relative velocities of methanesulfonylation of AChE were estimated using a modification of the method of Kitz and Wilson.[9,10] Salt solutions prepared from double-distilled water and made 0.1 M in NaCl and 0.02 M in $MgCl_2$ were used for incubations and assays. The enzyme concentrations in the incubation experiments were 12, 14, or 80 units/ml. The temperature was 25 ± 0.1°, and the initial MSF concentrations ranged from 10^{-5} to 10^{-6} M depending on the enzyme concentration. At time intervals, 0.25-ml portions of incubation solution were assayed without delay for residual AChE activity against ACh at a concentration of 3.4 × 10^{-4} M in a final volume of salt solution of 3 ml (low enzyme concentration, 12 or 14 units/ml) or 25 ml (high enzyme concentration, 80 units/ml). Controls from which only MSF was omitted were run in parallel. In this manner, the small inhibitory effects of the quaternary salts on the velocity of ACh hydrolysis in the assay medium were automatically compensated for in the computation of acceleration or inhibition effects on the MSF reaction. The rate of disappearance of enzyme activity is proportional to the velocity of the MSF reaction with the active serine hydroxyl of the enzyme.[9,10] The methanesulfonyl enzyme is stable.[9,10] The concentrations of the effector quaternary salts in the incubation mixtures were saturating with respect to the enzyme as could be readily estimated from their known binding constants.[6,14] Nevertheless, two separate kinetic runs using two different saturating concentrations were carried out in order to ensure that the observed kinetic salt effects were the maximum obtainable. The complete data are assembled in Table I. Semilog plots (Figure 1) of the time course of the reactions gave the α values,[9,10] the ratio of the rate constants k/k_0.

Kinetics of Acceleration. For purposes of comparison with previous observations[9,10] on the kinetics of acceleration effects on AChE from electric eels, the two prototype salts TMA and *n*-butyl-TMA were evaluated as described above, except that the relation between the pseudo-first-order rate constants for methanesulfonylation and the MSF concentration was established at fixed salt concentrations. The data are summarized in Figure 2.

Binding Enthalpies and Free Energies. These were reported previously.[6,14] For convenience, they have been included in Table I.

Results

Kinetics of Acceleration. The time course of the MSF reaction in the absence and presence of two quaternary salt prototypes (TMA and *n*-butyl-TMA) is illustrated in Figure 1, where it can be seen that pseudo-first-order kinetics are obeyed. Plots of the rate constants for methanesulfonylation in the presence of the same salt prototypes *vs.* MSF concentration are also linear (Figure 2), in agreement with a mechanism where the ternary complex AChE–salt–MSF is the kinetically significant species.

Structure–Activity Relationships. (a) *n*-Alkyl-TMA Salts. In this series (Table I), peak stimulation of the MSF reaction occurs with the *n*-pentyl substituent (molar volume of 115 ml). Plots relating α (the rate constant ratios) to the TMA substituent volumes and the later to ΔG binding[14,15] are shown in Figure 3. Plots where ΔG is substituted by ΔH binding[15] are shown in Figure 4, where the effect of methyl branching

(13) Abbreviations used are: TMA = tetramethylammonium; AChE = acetylcholinesterase from bovine erythrocytes; ACh = acetylcholine; R-TMA = *n*-alkyl- or cycloalkyl-TMA; MSF = methanesulfonyl fluoride.
(14) B. Belleau, H. Tani, and F. Lie, *J. Amer. Chem. Soc.*, **87**, 2283 (1965).
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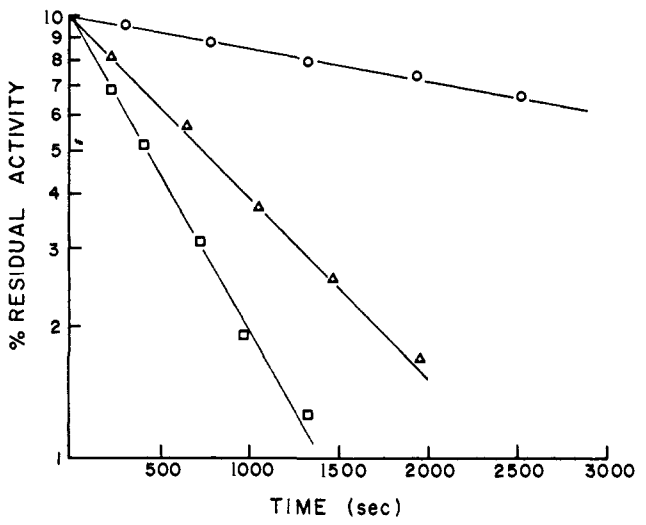

Figure 1. Time course of the methanesulfonylation of AChE: O—O = control, [MSF] 2.53 × 10^{-5} *M*; △—△, with TMA at 2 × 10^{-2} *M*; □—□, with *n*-butyl-TMA at 2 × 10^{-2} *M*; temperature 25°; pH 7.4.

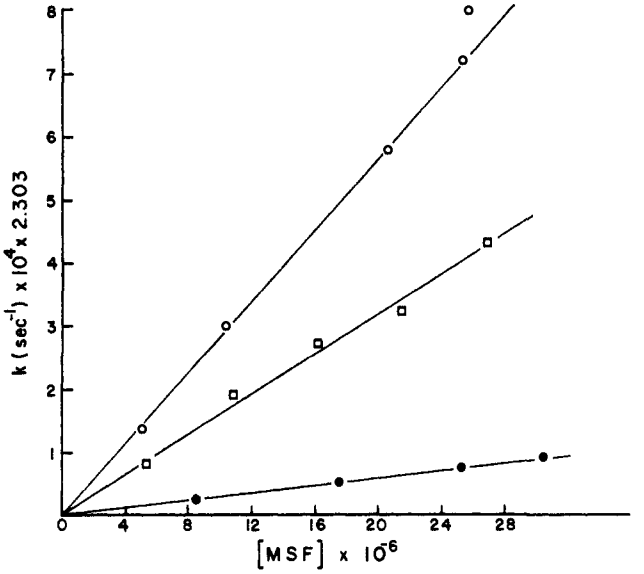

Figure 2. Variation of the rate constant for methanesulfonylation of AChE with [MSF] alone and in the presence of TMA and *n*-butyl-TMA at 2 × 10^{-2} *M*, respectively: O—O = *n*-butyl-TMA; □—□ = TMA; ●—● = control.

on α in the proximity of the cationic nitrogen as in 2-methyl-*n*-pentyl-TMA (Table I) is also illustrated. A significant decrease in stimulatory activity is observed by comparison with unbranched alkanes of equivalent molar volumes.

(b) Cycloalkyl-TMA Salts. A pattern of structure–activity relationships similar to that observed with the *n*-alkyl-TMA salts applies to the cycloalkyl analogs, except that stimulation is consistently greater. Peak stimulation ($\alpha \simeq 57$) is obtained with cyclohexyl (molar volume of 108 ml). Plots relating α to molar volumes and ΔG binding[6] are shown in Figure 5; in Figure 6 the van't Hoff enthalpies have been substituted for ΔG. Out-of-plane branching as in bicycloheptyl, bicyclooctyl, and adamantyl is detrimental to stimulation by comparison with cycloalkanes of equivalent volumes. The volume–α relation is similar to that of volume–ΔH in the unbranched series cyclobutyl to cyclooctyl.

Discussion

Topographic Parameters. It has long been recognized that the AChE active surface includes an esteratic and an anionic site.[16,17] With ACh-like substrates, the anion–cation interaction has a marked effect on the catalytic reactivity of the esteratic center as may be inferred from the decreased activity toward neutral substrates.[18] Conclusive evidence for the regulatory role of the ion–ion interaction was given by Kitz and Wilson,[9-11] who showed that methanesulfonylation of the esteratic serine hydroxyl is strongly accelerated by certain quaternary ions. Hence, the esteratic and anionic centers exist as vicinal nonoverlapping sites. An analogous situation prevails in the case of trypsin, whose activity toward the sluggish substrate *N*-acetylglycine ethyl ester is stimulated by short-chain alkylamines.[19] This is consistent with the known selectivity of trypsin for lysine derivatives since the built-in terminal ϵ-ammonium group of the latter can similarly transmit a stimulatory effect to the catalytic sites through interaction with an anionic site several bond lengths away. In a similar fashion, the rate of deacylation of acetyl- and propionyl-chymotrypsin is stimulated by indole,[20] an effector molecule which normally forms an integral part of substrates based on the trypophan structure. However, caproyl-chymotrypsin is inhibited by indole and the mechanistic aspects of these effects have been discussed by Lumry and Biltonen.[21] The case of quaternary ion stimulation of AChE toward MSF is therefore not unique in enzyme chemistry and serves to emphasize, as the other cases do, the physical significance of complementarity between natural substrates and enzymes. The idea that the esteratic and anionic centers of AChE can indeed function independently was definitely confirmed through the use of the specific anionic site directed irreversible inhibitor *N*,*N*-dimethyl-2-phenylaziridinium chloride (DPA).[22] While this substance abolishes activity toward ACh, the resulting DPA enzyme displays enhanced activity toward the neutral substrate indophenyl acetate.[23] Hence, the anionic center covalently binds the DPA molecule *independently* of the esteratic center, and it is likely that a change of conformation is induced as a consequence. It is logical to conlude that the anionic center possesses an intrinsic ability to bind charged ligands carrying complementary groups. The fact brought to light in the present work, that TMA salts carrying long alkyl chains or bulky cage-like substituents stimulate rather than protect the enzyme toward MSF, establishes that the ligand substituents bind on the anionic center and thus project away from the esteratic center (or assume an exo orientation[24] relative to the catalytic nucleo-

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(21) R. Lumry and R. Biltonen in "Structure and Stability of Biological Macromolecules," S. Timasheff and G. Fasman Ed., Marcel Dekker, New York, N. Y., 1969, Chapter 2.
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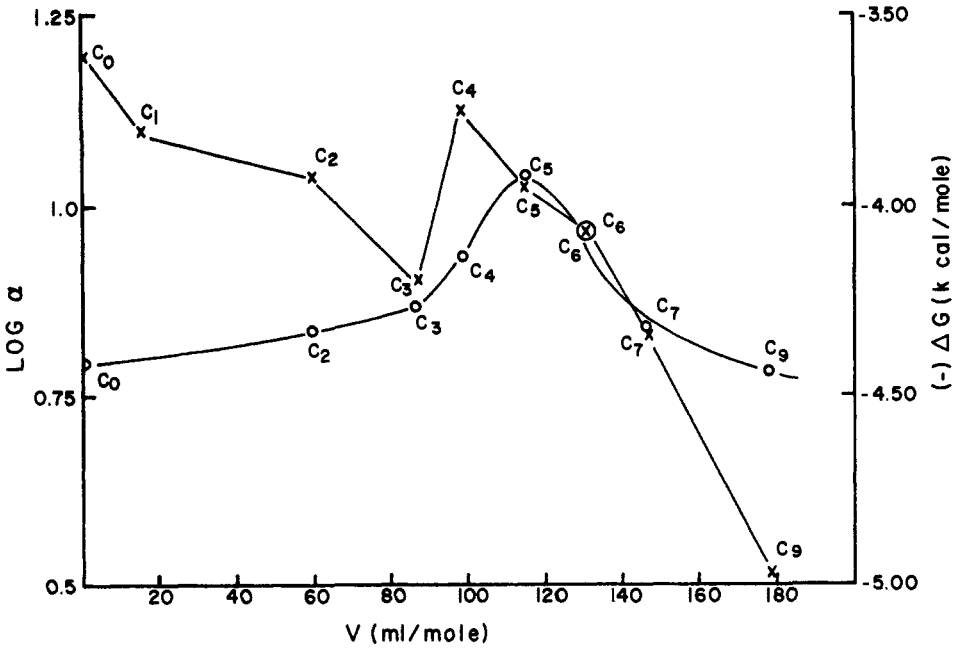

Figure 3. Plots of *n*-alkyl molar volumes[8] *vs.* α (○—○) and Δ*G* binding (×—×).[14]

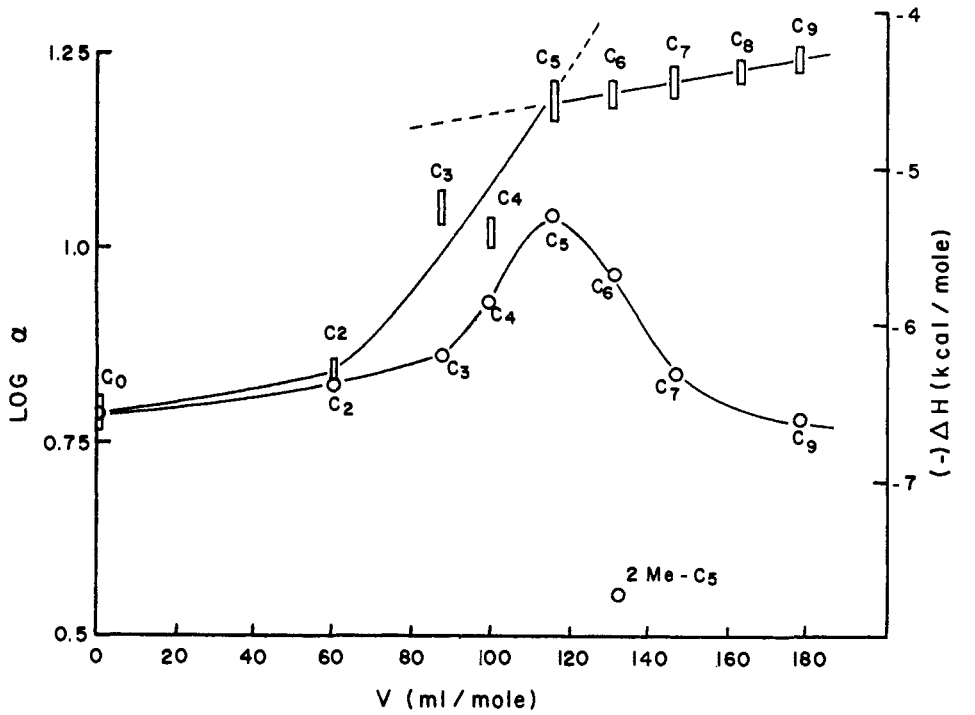

Figure 4. Plots of *n*-alkyl molar volumes[8] *vs.* α (○—○) and Δ*H* binding (□—□).[6]

philes). Nevertheless, these salts may still share with ACh the same anionic binding site since their respective interactions with the enzyme are mutually exclusive in agreement with the general laws of competitive kinetics.[6,14] While "pure" competitive behavior is not rigorously established, these observations are nevertheless consistent with the model shown in Figure 7a, where the binding center of the enzyme is represented by a conical cleft carrying an anionic site at the apex and with the esteratic nucleophiles occupying one segment (endo center). It is conceivable, although by no means essential to the hypothesis, that the cleft may be composed of two or more converging peptide chains, a possibility which is under investigation. The model allows the prediction that competitive inhibitors of ACh interacting through their substituents with the endo segment will effectively block the MSF reaction. The *m*-hydroxyphenyltrimethylammonium ion[9,10,25] would belong to this category of ligands since its phenolic group can engage in specific hydrogen

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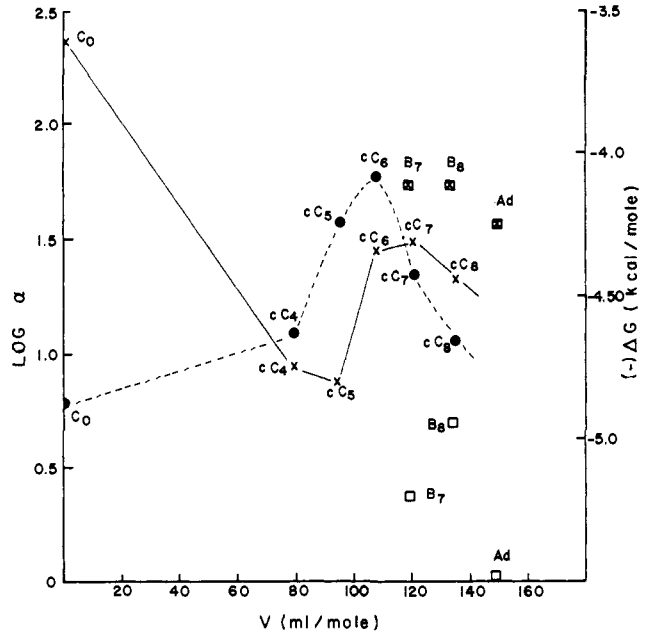

Figure 5. Plots of cycloalkyl molar volumes[6] *vs.* α (●—●) and ΔG binding (×—×).[6]

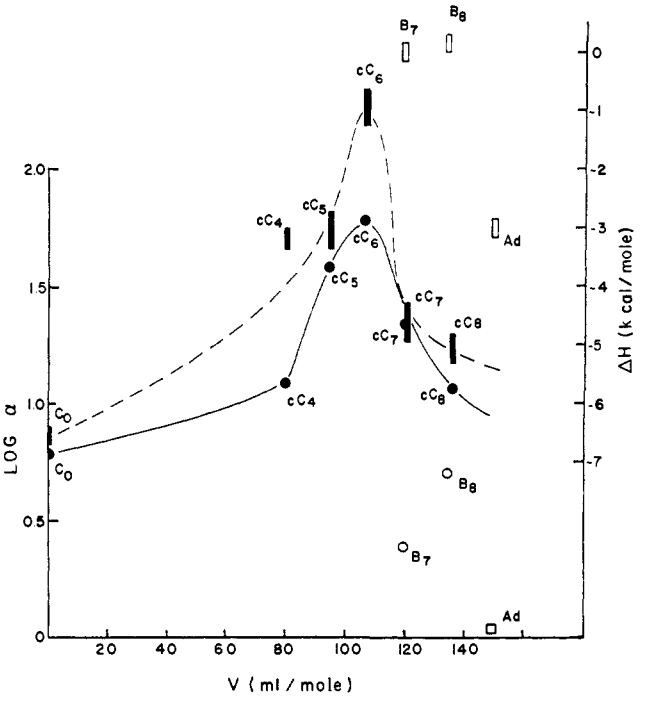

Figure 6. Plots of cycloalkyl molar volumes[6] *vs.* α (●—●) and ΔH binding (■—■).[6]

bonding with the esteratic nucleophiles. Moreover, exo-complex formers possessing unusual bulk may also interfere directly with the MSF approach to the endo sites. For instance, the bulk tolerance of the cleft for ternary complex formation is exceeded in the case of the tetrabutylammonium ion[11] but not with the tetraethyl analog,[9,10] which explains the protection exerted by the former and the stimulation by the other. The specific prediction is also allowed that the steric tolerance of the cleft for ternary complex formation should increase as the bulky parts of a

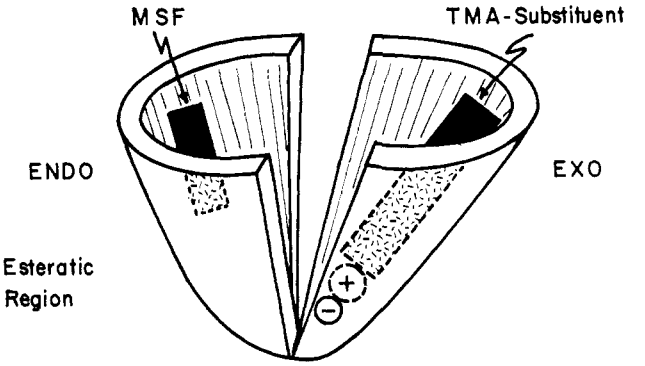

Figure 7. Schematic representation of the acetylcholine binding cleft of AChE. The apolar TMA substituents assume an exo orientation with respect to the esteratic nucleophiles where MSF reacts. The anionic sites at the apex acts as a common anchoring site for the TMA ligands and the substrate ACh whose ester function assumes an endo orientation.

quaternary ligand are moved away from the cationic nitrogen. This is neatly confirmed by the failure of bicyclooctyl- and adamantyl-TMA to inhibit the MSF reaction. This does not mean, however, that these bulky substituents have no dampening effect on acceleration. Strong evidence to the contrary was obtained by comparing the *n*-hexyl- with the 2-methyl-*n*-pentyl substituents whose molar volumes are nearly identical. The presence of methyl branching in proximity of the nitrogen in the latter caused an almost twofold decrease in α (Table I, Figure 4), thus clearly suggesting that out-of-plane groups in the bound TMA substituents probably cause the MSF probe to experience repulsions, albeit on the nonprohibitive variety. On that basis, we feel justified in concluding that the decreased stimulation observed with the bicycloalkyl substituents by comparison with unbranched cycloalkyls of equivalent volumes (Table I, Figure 6) is probably the result of nonprohibitive repulsions in the ternary complex. Since all the substituents are of the alkane type it is unnecessary to invoke specific interaction mechanisms other than repulsive forces to explain these substituent effects. It appears then that the proposed stereochemical model (Figure 7) is qualitatively consistent with all the available facts, and it remains to rationalize the acceleration effects in physical terms, keeping in mind that the kinetic response of the enzyme to the ligands must be unobscured by nonbonded repulsions in the ternary complex if any progress is to be made. In this regard, it would seem that only unbranched alkyl and cycloalkyl substituents may be used to advantage.

Thermodynamic Parameters. The bell-shaped curves relation α to substituent volumes in both the *n*-alkyl and cycloalkyl series (Figures 4 and 6) may have common physical origins since α increases and declines at nearly equivalent molar volumes. However, this does not explain the much greater activity of the ring compounds at molar volumes equal to those of their open-chain counterparts. Alkyl and cycloalkyl substituents differ in some important respects from the physico-chemical standpoint: (a) the latter are more compact (thus much shorter at equimolar volumes) and much less flexible; (b) the former, when sufficiently long, confer surface activity to ionic carriers. An octyl chain can penetrate much deeper into a protein

than the more compact cyclooctyl ring which closely approximates *n*-butyl in length. Now the conformational versatility or flexibility of the alkyl substituents increases with chain length, but in the constrained cycloalkyl series it increases much less from cyclobutyl to cyclohexyl than from the latter to larger rings which can assume many more conformations and exhibit pseudorotation.[26] Hence, the reversal of the respective trends of α values in the two series may well have different origins: in the alkyl series it may be related to protein penetration by long-chain substituents whereas in the cycloalkyl series it may coincide with a sudden departure from the conformational constraints of small rings. The free energies of binding *vs.* volumes are uninformative in this regard since they do not correlate with the volume effects on α (Figure 3). However, the van't Hoff enthalpies *vs.* volume yield some valuable information (Figure 4): first, ΔH becomes increasingly positive up to pentyl-TMA, but then virtually levels off at the peak of acceleration. The average $\delta\Delta H/\delta\Delta S$ ratio for the binding of the short-chain alkyls up to hexyl or heptyl is 250–288°K,[6,15] but for the longer chain members it falls to about 50°K. The transfer of alkanes from an apolar to an aqueous phase is characterized by a ratio of 50–150°K.[27] The nearly athermal binding of the longer chain alkyl substituents is therefore suggestive of chain penetration of an apolar region,[28] presumably at the periphery of the cleft. One consequence may be that a distorted conformation of the enzyme is induced. This could explain the decline in α values as the chain is elongated.

In the case of the cycloalkyl series, again no correlation is found between volume effects on α and ΔG binding, respectively (Figure 5). However, a fairly good correlation between volume effects on α and ΔH binding, respectively, is found (Figure 6). Since the $\delta\Delta H/\delta\Delta S$ ratio for the binding of *all members* of the series averages 288°K, it seems to follow that the decline in the α values may not be caused by a shift of interaction mechanism such as penetration of the larger rings into the interior of apolar regions. It appears, then, that the volume effects on α would be primarily a measure of an important property of the protein which is detectable in enthalpies and entropies of binding, but as a consequence of enthalpy–entropy compensation, not clearly indicated by the free-energy changes in binding.

As already pointed out in the introductory statement, a ligand-induced change of conformation may be defined as a stereotyped alteration in the balance of bond-breaking and bond-making reactions in the protein, and it is conceivable that relative binding enthalpies may reflect the degree of alteration. In the case at hand, the degree of stimulation of AChE toward MSF correlates better with enthalpies than free energies of ligand binding (provided nonbonded repulsions between the bound ligands are absent in the ternary complex). Since we have already shown[6] that a correlation of the form $\delta\Delta H = T_c\delta\Delta S$, where T_c = 288°K, holds for the binding of some 35 TMA salts on AChE, an endothermic release of cleft-bound water molecules accounts best for the structurally induced fluctuations in the binding enthalpies.[6] Hence, the rough correlation between acceleration and enthalpies of binding may be explained by the loss of water from the cleft, as this would increase the chemical potential of the esteratic nucleophiles and relax their conformation. This latter effect would merely constitute a case of free-energy transmission from one region of the cleft to another in the form of mechanical free energy.[5] The effects of enzyme modifiers may perhaps be accounted for in similar physical terms, although much work remains to be done before generalizations can be attempted.

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(26) J. D. Roberts, 19th National Organic Chemistry Symposium of the American Chemical Society, Tempe, Ariz., June 1965, p 77.
(27) W. Kauzmann, *Advan. Protein Chem.*, **14**, 1 (1969).
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Communications to the Editor

On the Existence of Polar Conformations of Cycloheptane, Cyclooctane, and Cyclodecane

Sir:

Cyclooctane is a *polar molecule.* The application of the molecular beam electric deflection method,[1,2] *i.e.*, deflection (refocussing) of beams of these molecules in an inhomogenous electric field (quadrupole focusser), to cycloheptane, cyclooctane, and cyclodecane has revealed the existence of polar conformations[3] of these molecules.

Cyclopentane[4] appears to be nonpolar using this method. Apparently the barrier to pseudorotation is

(1) (a) L. Wharton, R. A. Berg, and W. Klemperer, *J. Chem. Phys.*, **39**, 2023 (1963); (b) W. E. Falconer, A. Büchler, J. L. Stauffer, and W. Klemperer, *ibid.*, **48**, 312 (1968).
(2) E. W. Kaiser, J. S. Muenter, and W. Klemperer, *ibid.*, **48**, 3339 (1968).

(3) (a) The X-ray analyses of Dunitz on cyclododecane and on derivatives of cyclodecane, cyclononane, and cyclooctane have been summarized in J. D. Dunitz and V. Prelog, *Angew. Chem.*, 72, 896 (1960). (b) For a recent, excellent, and comprehensive review see J. D. Dunitz, "Perspectives in Structural Chemistry," Vol. II, J. D. Dunitz and J. A. Ibers, Ed., Wiley, New York, N. Y.,1968, p 1. (c) Theoretical discussion of this area may be found in J. B. Hendrickson, *J. Amer. Chem. Soc.*, **89**, 7306, 7043, 7047 (1967).
(4) This molecule was studied with a nozzle source rather than a conventional effusion source. We have observed in electric resonance experiments that the population of internal states in molecules leaving an