Host–Guest Interactions. The Binding Mode of 6-Nitrobenzisoxazole-3-carboxylate to Quaternary Ammonium Macrocycles

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In order to investigate the mode of substrate binding and the factors involved in catalysis by artificial host compounds, the rate augmentation of the decarboxylation of 6-nitrobenzisoxazole-3-carboxylate (**4**) in the presence of the non-aggregating macrocyclic quaternary ammonium salts (**1**)—(**3**) was analysed. The kinetic results indicate that (**1**) and (**3**) consisting of 27-membered macrocycles form host–guest complexes with (**4**) whereas (**2**) having 21-membered rings does not. The macrotricycle (**3**) is the most effective catalyst, exhibiting a temperature-independent maximum rate enhancement of 110. The rate and binding constants and their temperature dependence for (**3**) leads to the conclusion that (**4**) penetrates with the nitroaromatic moiety first into the molecular cavity of (**1**) and (**3**). This mode of association is impossible with (**2**) for steric reasons. The cavity of (**3**), however, is large enough to accommodate two substrate molecules thus allowing the observation of the rare case of co-operative substrate binding to a low molecular weight enzyme model.

Enzyme specificity and catalysis appear to be vitally dependent on the correct arrangement of substrate binding and catalytically active functionality at the active site.[1] Artificial systems, which mimic enzyme action, should therefore allow control of the positions and orientations of the corresponding functional groups. However, some of the, in terms of rate enhancement, most powerful enzyme-like catalysts (micelles,[2] macroions,[3] functionalized polymers[4]) are poor models in this respect due to their dynamic and rapidly rearranging structure.

The expression of specificity, which is the underlying motivation for the construction of artificial enzymes,[5] requires the design and synthesis of molecular receptors with fixed topology of anchor groups for substrate binding. Whereas a great variety of anchor groups exist, which might serve as subsites for cationic and hydrophobic moieties of a substrate molecule,[6] the collection of host structures that bind anions is still very limited.[7] Our approach to the synthesis of artificial anion hosts focused on the quaternary ammonium macrocycles (**1**)—(**3**),[8] because of their chemical stability and pH-independent anion binding power, although the preparation proved rather laborious. Complexation studies[9] of (**2**) and (**3**) with inorganic and organic anions demonstrated that these compounds indeed form 1:1 inclusion complexes in aqueous solution. The rigorous conservation of the complex stoicheiometry even under extreme concentration relations, the prevalence and enhancement of the complexation power in 90% methanol, and the observation of steric discrimination on anion binding set the experimental basis for the statement that anion binding is not the result of some aggregation phenomenon of the macrocycles. As with the cyclodextrins these totally synthetic host compounds possess a number of features which suggest that the macrocyclic quaternary ammonium salts themselves might serve as enzyme models. They can provide a molecular cavity of unambiguous topology with a positive electrostatic potential inside encircled by lipophilic walls. From preliminary kinetic studies[10] and from the known factors involved in micellar and macroionic catalysis[2–4] one can conclude that this combination of features sets the stage for the stabilization of soft anionic transition states of suitable size relative to the ground state. Thus, the corresponding reactions would be accelerated. The investigation of rate effects could add another tool to the arsenal of methods available to determine the mode of binding in the host–guest complex. This knowledge is necessary for the rational design of specific molecular receptors consisting of covalently linked subsites.

The extraction of the information on the mode of host–guest association from rate data calls for a simple preferably monomolecular reaction probe.

The decarboxylation of 6-nitrobenzisoxazole-3-carboxylate (**4**) appeared to be an attractive candidate in this respect, since it has been thoroughly studied[11] and it was shown to be a fragmentation without an intermediate and with the transition state being more delocalized (softer) than the ground state. In addition this decarboxylation is insensitive to acid–base catalysis and small alternations in ionic strength and it has been explored under a variety of catalytic conditions including the rate effects in the presence of surfactants[12] and modified polymers,[13,14] so that comparison with those systems might be gratifying.

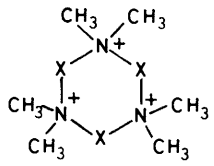
Experimental

N.m.r. measurements were performed on a Bruker WP-200 spectrometer. Complexation studies between substrates (**5**) and (**9**) and the host compounds (**3**) and CTAF (hexadecyltrimethylammonium fluoride) were conducted in D_2O solution containing 0.1M-KF and dioxane (3.750 p.p.m.) and t-butyl alcohol (1.237 p.p.m.) internal standards at ambient temperature (20 °C). Aliquot portions of a host stock solution were added to the substrate solution in an n.m.r. tube and the volume was kept constant by a jet of nitrogen. H.p.l.c. analysis used Waters instrumentation (pump 6000A; u.v. detector M 440) and a Nucleosil RP-18, 7 μm column.

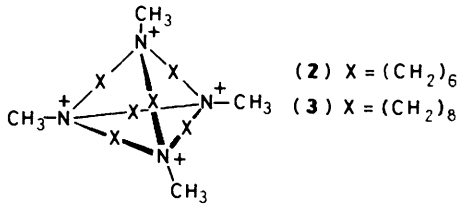
Materials.—The syntheses of the host compounds (**2**) and (**3**)* have been described.[8,9] Compound (**1**) was obtained from an intermediate in the synthesis of (**3**)[8] as follows.

Synthesis of (**1**). 1-[(4-Methylphenyl)sulphonyl]-1,10,19-triazacycloheptacosane[8] dihydrochloride (1.22 g, 2 mmol) was suspended in 35 w/w % HBr (8 ml) in acetic acid and phenol

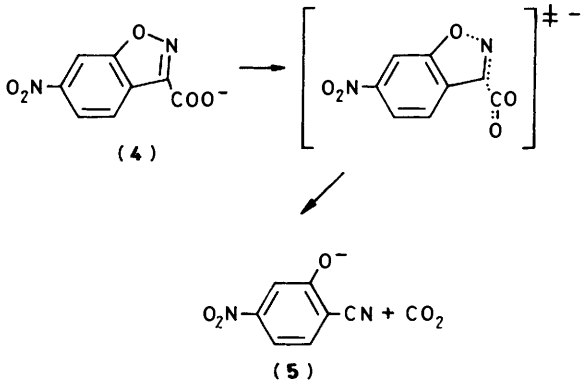
* (**1**) = 1,1,10,10,19,19-Hexamethyl-1,10,19-triazoniacycloheptacosane trifluoride; (**2**) = 1,8,15,22-tetramethyl-1,8,15,22-tetra-azoniatricyclo-[13.13.6.6^{8,22}]tetracontane tetrafluoride; (**3**) = 1,10,19,28-tetramethyl-1,10,19,28-tetra-azoniatricyclo[17.17.8.8^{10,28}]dopentacontane tetrafluoride.



(1) X = $(CH_2)_8$



(2) X = $(CH_2)_6$
(3) X = $(CH_2)_8$



(4)

(5)

(500 mg) was added. The mixture was heated at 80 °C for 20 h, then the solvent was evaporated, and the black residue was distributed between CH_2Cl_2 and water. Centrifugation gave a clear yellow aqueous phase and a black organic phase, which was extracted twice more with water. The combined aqueous layers were evaporated to dryness and the solid residue was crystallized from ethanol–ethyl acetate to give a powder (800 mg). 1,10,19-Triazaheptacosane trihydrobromide so obtained (624 mg), anhydrous sodium carbonate (1.06 g), and methyl toluene-*p*-sulphonate (1.86 g) was refluxed under N_2 overnight. The solvent was evaporated, the residue was dissolved in water, and the product was precipitated by slow addition of a saturated solution of $NaBF_4$ in water. The crystals (680 mg) were collected and recrystallized from nitromethane–methanol (10:1 v/v) to yield prisms (570 mg), δ_H (200 MHz; CD_3NO_2) 1.42 (br s, 24 H), 1.83 (br, 12 H), 3.11 (s, 18 H), and 3.33 (m, 12 H); δ_C (CD_3NO_2) 23.4, 26.9, 29.3, 52.4, and 65.3 p.p.m. (Found: C, 49.1; H, 9.0; N, 5.7. Calc. for $C_{30}H_{66}B_3F_{12}N_3$: C, 49.4; H, 9.1; N, 5.8%).

The fluoride salts of (**1**)—(**3**) were prepared by anion exchange (Dowex 1 × 8) in methanol–acetonitrile. The eluates were evaporated, the residues were redissolved in water, again evaporated, and this operation was repeated twice in order to remove all the organic solvent. The host concentrations in the final aqueous solutions were determined gravimetrically using the precipitation with sodium tetraphenylborate.

6-Nitrobenzisoxazole-3-carboxylic acid (**4**) was prepared according to the literature,[13,15] m.p. 189 °C. Stock solutions (0.1M) were prepared by quick neutralization with NaOH, adjustment of the volume, and freezing of the salt solutions in portions at −20 °C.

Synthesis of 6-nitrobenzisoxazol-3-ylacetic acid (**9**). 6-Nitrobenzisoxazol-3-ylmethanol (**6**). Carboxylic acid (**4**) (1.25 g, 6 mmol) was suspended in nitromethane (11 ml)–trimethyl borate (6 ml). Neat borane–dimethyl sulphide complex (BMS) (2 ml) was cautiously added (H_2 evolution). After 5 min at 25 °C the mixture was warmed to 65 °C, giving a clear yellow solution. Another portion of BMS (500 µl) was added after 30 min and heating was continued for 60 min. The mixture was chilled and the borane complexes destroyed by cautious addition of 5% methanolic HCl (30 ml). Evaporation gave a red residue which was distributed between ether (30 ml) and 1M-soda solution (20 ml). Washing the organic phase with soda (3 × 10 ml), drying ($MgSO_4$), and stripping off the solvent left a residue which on recrystallization from toluene gave yellowish needles (570 mg, 49%), m.p. 87—88 °C; h.p.l.c. 35% CH_3OH–30mM-HCOOH–30mM-$NaClO_4$, R_V 7.1 ml; δ_H (60 MHz; $CDCl_3$) 5.17 (s, CH_2), 8.03 (d, *J* 9 Hz, 4-H), 8.25 (dd, *J* 9 and 2 Hz, 5-H), and 8.43 (d, *J* 2 Hz, 7-H) (Found: C, 49.6; H, 3.2; N, 14.4. Calc. for $C_8H_6N_2O_4$: C, 49.5; H, 3.1; N, 14.4%).

6-Nitrobenzisoxazol-3-ylacetonitrile (**8**). Compound (**6**) (270 mg, 1.39 mmol) was dissolved in dry dichloromethane (7 ml) containing triethylamine (392 µl, 2.8 mmol). The solution was chilled to 4 °C before adding methanesulphonyl chloride (182 µl, 1.66 mmol). This mixture was stirred for 10 min at 4 °C and quenched by addition of cold water (5 ml). The organic phase was separated and extracted with ice-cold hydrochloric acid and hydrogen carbonate solution followed by drying and evaporation. The residue [methanesulphonate (**7**), pure by t.l.c. on silica with benzene–ethyl acetate 4:1 v/v, R_F 0.4] was dissolved in acetonitrile (7.5 ml)–methanol (5 ml). An aqueous potassium cyanide solution (5 ml; 2.5M) was added with stirring at 25 °C. The mixture rapidly darkened and became black within 1 h. The progress of the reaction was monitored by t.l.c. [system as above, R_F (**9**) 0.5]. After 4 h the solvent was stripped off, the residue was taken up in dichloromethane–methanol, and subjected to preparative t.l.c. on silica (eluant as above). The product was collected, eluted with methanol, and finally crystallized from toluene to yield light yellow needles (113 mg, 40%), m.p. 162—163 °C; *m/z* (e.i.) 203 (M^+, 100%), 173 (12.4), 157 (13.9), 130 (38.0), 129 (25.1), and 102 (31.1); δ_H (200 MHz; $CDCl_3$) 4.22 (s, CH_2), 8.06 (dd, *J* 8.7 and 0.6 Hz, 4-H), 8.34 (dd, *J* 8.7 and 1.9 Hz, 5-H), and 8.56 (dd, *J* 1.8 Hz and 0.6 Hz); $\nu_{max.}$(KBr) 2 240 cm^{-1} (C≡N).

6-Nitrobenzisoxazol-3-ylacetic acid (**9**). Compound (**8**) (61 mg, 300 µmol) was dissolved in acetic acid (2 ml) and 6M-hydrochloric acid (7 ml) was added. The slightly turbid mixture was heated at 100 °C for 4 h and then brought to dryness *in vacuo*. Crystallization from toluene gave light brown crystals which were sublimed (140 °C at 7 Pa) and recrystallized. Off-white needles (50 mg, 73%), m.p. 144—145 °C; *m/z* (e.i.) 222 (M^+, 32.3%), 204 (30.9), 178 (100), 132 (17.4), and 104 (20.2); δ_H (200 MHz; $CDCl_3$) 4.18 (s, CH_2), 7.88 (dd, *J* 8.8 and 0.6 Hz, 4-H), 8.25 (dd, *J* 8.8 and 1.7 Hz, 5-H), and 8.50 (dd, *J* 1.6 and 0.6 Hz), OH resonance appears as a very broad signal at δ 2.7—4.0.

Kinetic Measurements.—The reactions were followed spectrophotometrically observing the production of 4-nitrosalicylonitrile anion at 405 nm. Runs were initiated by addition of an aliquot portion of the substrate stock solution to the thermostatted optical cell containing TAPS buffer (Aldrich) (pH 8.0; 0.05M), while varying the amounts of the host stock solution and potassium fluoride to adjust the ionic strength to 0.5. The change in absorbance relative to a standard was recorded with an Eppendorf single beam photometer equipped with a programable cuvette-changer and recorder. First-order rate constants, $[host]_o \gg [substrate]_o$, were calculated from the integrated rate equation $\ln(a_\infty - a^t) = -k_{obs}t + \text{const.}$ for reactions with $t_{\frac{1}{2}} < 200$ min, taking the final absorbance a_∞

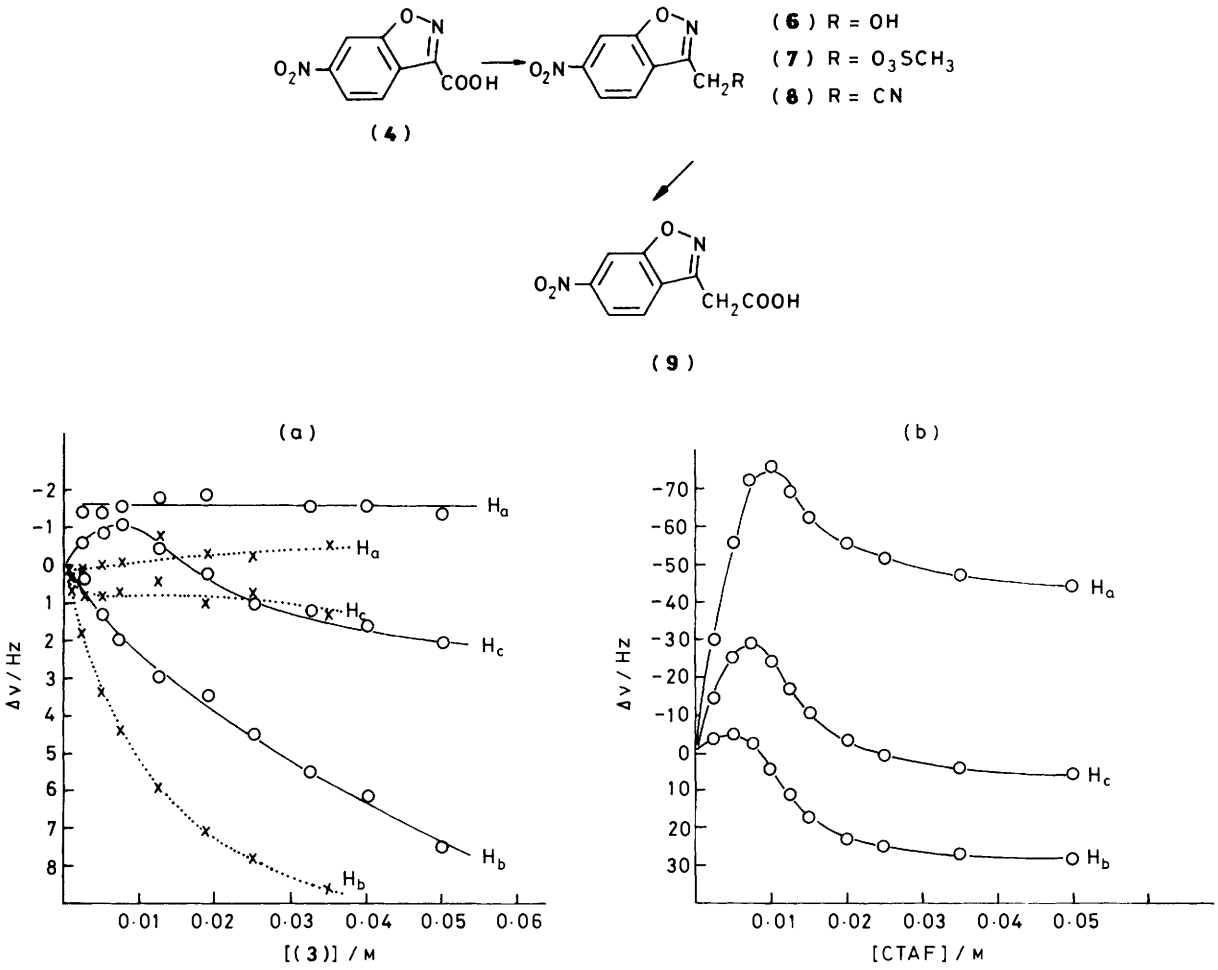


Figure 1. Dependence of chemical shift changes Δν of aromatic substrate protons on the concentration of quaternary ammonium salts in D_2O with 0.1M-KF at 20 °C: (a) (**5**) (x; 0.005M), (**9**) (o; 0.01M) *versus* (**3**) (b) (**9**) (o; *versus* CTAF)

after 10 half-lives or by the Guggenheim method for reactions with $t_{\frac{1}{2}}$ from 200 to 2 500 min. Initial rates under the conditions $[substrates]_o \gg [host]_o$ were measured in duplicate in 0.1 cm cells (250 μl volume) and processed using the extinction coefficient ε_{405} 2 720 l mol^{-1} cm^{-1}. The temperature dependence of the decarboxylation was determined with the aid of a thermostatted cell compartment, which controlled the temperature to within ±0.1 °C. Error limits (Table 2) were calculated by the worst case method.

Results

Addition of any of the quaternary ammonium macrocyles (**1**)—(**3**) to an aqueous solution of (**4**) speeded up the decarboxylation. To confirm that this effect was not due to an aggregation phenomenon of the ammonium salts the changes of aqueous solutions of (**1**)—(**3**) (as fluorides) on dilution were examined. All three macrocycles showed a linear dependence ($r > 0.99$) of the equivalent conductance *versus* $\sqrt{c}$ between 5×10^{-5} and 0.04M. No break or sudden change of slope indicative of micelle formation was found.

Though this result was expected from the symmetrical structures of the macrocycles and recalling that the critical micelle concentration of decyltrimethylammonium bromide [a compound with the same ratio of hydrophobic carbon atoms per hydrophilic centre as (**3**) but with a more favourable structure for micellation] is even higher (0.065M [2c]) than the most concentrated solutions employed in this study, we sought supplementary evidence from investigating the interaction of the substrates and macrocycle (**3**) by n.m.r. methods. However, the rather rapid decarboxylation of (**4**) in the presence of (**3**) obstructed a direct study. Therefore the surrogate substrate (**9**) was used, which resembles the genuine substrate very much according to charge, chemical nature, and molecular dimensions but is chemically stable.

In solutions of (**9**) or (**5**) (as sodium salts) and (**3**) (as fluoride salt) in D_2O the positions of the proton resonances were independent of the concentrations from 0.001 to 0.05M, confirming the absence of micelle formation in the substrate solutions. Successive additions of aliquot portions of a stock solution of (**3**) to a solution of (**9**) or (**5**), respectively, shifted the signal of the proton in the *meta*-position to the nitro substituent progressively to lower field, whereas the *ortho*-protons kept their position almost fixed (Figure 1). The resonance of the side-chain methylene protons of (**9**) remained hidden under the solvent peak. There was no apparent line broadening and no novel signals appeared. The signals of the chain methylene groups of (**3**) but not that of the *exo*-methyl groups experienced a small upfield shift (*ca.* 5 Hz) on addition of the first aliquot portion but returned to the positions determined in the absence of (**9**) in the case of a major excess of (**3**) over (**9**). The macrocycle (**3**) influences the proton shifts of educt surrogate (**9**) and product (**5**) very similarly, which suggests a similar interaction mode with either substrate. Although the changes in shift were

Table 1. Kinetic parameters at 298 K in aqueous solution of the decarboxylation of (**4**) the presence of (**1**)—(**3**) at $W_o \gg S_o$[a]

Host	$^1k_{cat}/s^{-1}$	^1K_D/mol l^{-1}	$\frac{^1k_{cat}}{k_{un}}$	$\frac{^1k_{cat}}{K_D}$/l mol^{-1} s^{-1}	$\frac{k_{un}}{^1k_{cat}} \cdot K_D$/mol l^{-1}
(**1**)	1.15×10^{-4}	0.46	31	2.5×10^{-4}	1.48×10^{-2}
(**2**)		3.7×10^{-2}		1.35×10^{-4}	2.56×10^{-2}
(**3**)	3.97×10^{-4}		110	1.07×10^{-2}	3.3×10^{-4}

[a] k_{un} 3.58×10^{-6} s^{-1} [μ 0.5M (KF); 0.05M-TAPS buffer, pH 8.0].

insufficient for quantitative analysis, one must conclude that they result from a specific association involving only part of the substrate (presumably the nitroaromatic moiety) and the host structure. In addition the observation of sharp averaged signals indicated that complex formation and dissociation take place beyond the fast exchange limit of a 200 MHz n.m.r. spectrometer and thus are very fast processes in relation to the chemical breakdown (decarboxylation) of (**4**).

Compared with the shift changes observed with (**9**) and (**5**) in the presence of the prototypical cationic surfactant cetyltrimethylammonium fluoride (CTAF; Figure 1) the macrocycle (**3**) displayed a completely different picture. In combination with the more indirect evidence emerging from the complexation studies[9] it appears safe to state that the quaternary ammonium macrocycles form molecularly dispersed solutions in water in the concentration range used in this study.

The analysis of the rate augmentation of the decarboxylation of (**4**) in the presence of the macrocyles followed the general reaction (1). Clean first-order reactions were observed up to

$$\begin{array}{l} W + S \underset{}{\overset{^1K_D}{\rightleftharpoons}} S \subset W \xrightarrow{^1k_{cat}} W + P \\ \quad\quad \downarrow k_{un} \\ \quad\quad P \end{array} \qquad (1)$$

three half-lives, provided the concentration of the hosts W_o exceeded that of (**4**) (S_o) by at least a power of ten ($W_o \gg S_o$). For (**1**) and (**3**) the observed first-order rate constants displayed saturation behaviour as demanded by reaction (1) {[(**1**)] 0.01—0.066M; [(**3**)] 6×10^{-3}—0.046M}. The kinetics were analysed according to the general derivation of Colter *et al.*,[16] who arrive at equation (2), from which the desired host–guest dissociation

$$\frac{1}{k_{obs} - k_{un}} = \frac{^1K_D}{^1k_{cat} - k_{un}} \cdot \frac{1}{W_o} + \frac{1}{^1k_{cat} - k_{un}} \qquad (2)$$

constant ^1K_D and the turnover number in the complex $^1k_{cat}$ can be determined.

The experimental data of the catalysis by (**1**) and (**3**) fitted to (2) with correlation coefficients $r > 0.995$ and the kinetic constants calculated from these plots appear in Table 1.

Macrocycle (**2**), however, displayed a linear relationship of k_{obs} with W_o at all experimentally attainable host concentrations ($W_o \leqslant 0.045$M). To include (**2**) in reaction (1), ^1K_D must be much higher than W_o, so that (2) can be transformed to (3).

$$k_{obs} = \frac{^1k_{cat}}{^1K_D} \cdot W_o + k_{un} \qquad (3)$$

Table 1 illustrates that (**3**) is by far the most efficient catalyst for the decarboxylation of (**4**).* The kinetic results support the view that (**4**) forms 1:1 complexes at least with macrocycles (**1**) and (**3**). The association and dissociation of those complexes must be rapid compared with decarboxylation which is rate determining.

To evaluate the factors responsible for catalysis the temperature dependence of the rate effect of (**3**) was determined. The data obtained are collected in Table 2. Surprisingly, the slopes of the Arrhenius plots of the catalysed and uncatalysed reactions are the same. The catalytic effect at saturation, $^1k_{cat}/k_{un}$, is temperature independent and solely attributable to the change in activation entropy. The entropy seems to be exclusively responsible for complex formation, too, because no significant change of ^1K_D with temperature was found.

The experimental conditions needed to determine the kinetic parameters required the catalyst concentration to exceed that of the substrate by a power of ten. This situation makes it hard to compare any model with the natural enzymes, which are characteristically effective at concentrations far below those of their substrates. The catalytic power of (**3**) in the decarboxylation of (**4**) made it possible to demonstrate the rate acceleration under the usual catalysis condition: $S_o \gg W_o$. However, since severe product inhibition occurred initial rate measurements must be applied in this case. The general reaction (1) for catalysis may be used under this set of experimental conditions, too, and equation (4) may be derived by an analogous route as above.

$$\frac{1}{v_{obs} - v_{un}} = \frac{^1K_D}{^1k_{cat}\,W_o} \cdot \frac{1}{S_o} + \frac{1}{^1k_{cat}\,W_o} \qquad (4)$$

Treatment of the rate data according to equation (4), however, resulted in plots with an upward curvature, and no extrapolation to calculate the kinetic parameters was possible. This picture was reminiscent of allosteric enzymes, where several co-operatively working binding sites can process the substrate.[17] The kinetics of these enzyme reactions is characterized by a sigmoidal dependence of rate on substrate concentration and can be described by the Hill equation (5).

$$\frac{v_{cat}}{W_o} = \frac{k_{cat}\,S^n}{K' + S^n}; \; V_{max.} = k_{cat} \cdot W_o \qquad (5)$$

The Hill coefficient n may attain nonintegral values and expresses some measure of the interaction between the co-operative binding sites as well as their minimum number.[18] Fitting our rate data (Figure 2) to equation (6) gave the best

$$\frac{1}{v_{cat}} = \frac{K'}{V_{max.}} \cdot \frac{1}{S^n} + \frac{1}{V_{max.}} \qquad (6)$$

line at $n = 1.4$ with a correlation coefficient $r = 0.99995$ (7 points). Thus one may assume at least two binding sites with moderate 'co-operativity'.[18] To evaluate whether 'co-operativity' is due to increased binding of a second substrate molecule by host (**3**) or a higher decarboxylation rate constant

* $^1k_{cat}/^1K_D$ determines the effectiveness of the catalysts in competition with each other, whereas $k_{un} \cdot {}^1K_D/{}^1k_{cat}$ gives the host concentration at which the rate of the uncatalysed process is doubled.

Table 2. Temperature dependence and activation paramaters of the decarboxylation of (**4**) in the absence and presence of (**3**) ($W_o \gg S_o$)[a]

T/K	k_{un}/s^{-1}	$\Delta H^{\ddagger}_{un}$/kJ mol^{-1}	$\Delta S^{\ddagger}_{un}$/J K^{-1} mol^{-1}	$^1k_{cat}$/s^{-1}	$\Delta H^{\ddagger}_{cat}$/kJ mol^{-1}	$\Delta S^{\ddagger}_{cat}$/J K^{-1} mol^{-1}	^1K_D/mol l^{-1}
288.0	6.58×10^{-7}			6.25×10^{-5}			0.032
298.0	3.55×10^{-6}	122 ± 3[b,c]	$+62.8 \pm 15$[b,c]	3.90×10^{-4}	122 ± 3	$+102.6 \pm 15$	0.037
308.0	1.95×10^{-5}			1.81×10^{-3}			0.035

[a] μ = 0.5M (KF); 0.05M-TAPS buffer; pH 8.0. [b] Ref 8: $\Delta H^{\ddagger}_{un}$ 123 kJ mol^{-1}; $\Delta S^{\ddagger}_{un}$ +62.8 J K^{-1} mol^{-1}. [c] Ref. 7*a*: $\Delta H^{\ddagger}_{un}$ 134 kJ mol^{-1}; $\Delta S^{\ddagger}$ +95.9 J K^{-1} mol^{-1}

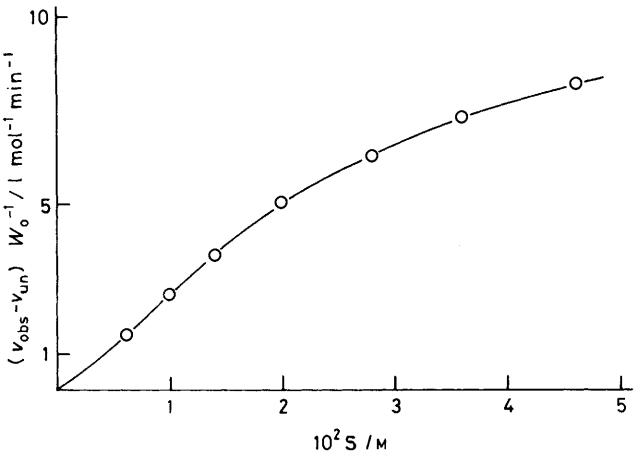


Figure 2. Catalysis of the decarboxylation of (**4**) by (**3**) at 298 K. Dependence of the observed initial rate on substrate concentration ($S_o \gg W_o$)

of the 2:1 complex or an alteration of both parameters, reaction (7) was set up. The rate of product production is then given by equation (8). Considering the simplification introduced by the

$$\begin{array}{ccccc} S + W & \overset{^1K_D}{\rightleftharpoons} & S \subset W & \overset{^1K_D}{\rightleftharpoons} & S_2 \subset W \\ \downarrow k_{un} & & \downarrow {}^1k_{cat} & & \downarrow {}^2k_{cat} \\ P & & P & & 2P \end{array} \qquad (7)$$

$$v_{obs} = k_{un}[S] + {}^1k_{cat}[S \subset W] + 2\ {}^2k_{cat}[S_2 \subset W] \qquad (8)$$

experimental conditions (initial rates: $S = S_o$; $S_o \gg W_o$) and expressing the complex concentration by equations (9) and (10) one obtains (11).

$$[S \subset W] = \frac{{}^2K_D\, S_o\, W_o}{{}^1K_D\, {}^2K_D + {}^2K_D S_o + S_o^2} \qquad (9)$$

$$[S_2 \subset W] = \frac{S_o}{{}^2K_D}[S \subset W] \qquad (10)$$

$$\frac{v_{obs}}{S_o} = \frac{{}^1k_{cat}\, {}^2K_D + 2\, {}^2k_{cat}\, S_o}{{}^1K_D\, {}^2K_D + {}^2K_D\, S_o + S_o^2} \cdot W_o + k_{un} = L_i \cdot W_o + k_{un} \qquad (11)$$

Equation (11) shows that the observed rate constant in this case is proportional to the host concentration W_o. The slopes L_i of corresponding plots at different S_{oi} depend nonlinearly on S_{oi}, but (11) is easily rearranged to (12), which allows the

$$\frac{L_i({}^1K_D + S_{oi}) - {}^1k_{cat}}{S_{oi}} = -\frac{1}{{}^2K_D} L_i S_{oi} + \frac{2\, {}^2k_{cat}}{{}^2K_D} \qquad (12)$$

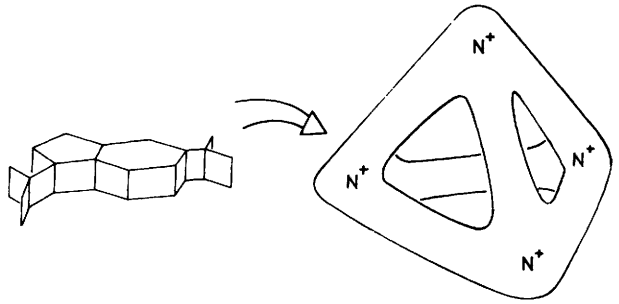


Figure 3. Illustration of the size relation of (**4**) and (**3**) in its most expanded conformation

determination of ^2K_D and $^2k_{cat}$ from the slope and intercept of a straight line, provided $^1k_{cat}$ and ^1K_D are known from a separate experiment as in the case here. When initial rates at 298 K of the decarboxylation of (**4**) in the presence of (**3**) at $S_o \gg W_o$ were plotted against W_o the strict linear dependence required from equation (11) was confirmed at seven different S_{oi} ($r > 0.995$). The slopes of these lines $L_i \cdot S_{oi}$ fitted to equation (12) with a correlation coefficient $r = 0.9993$ using the values of $^1k_{cat}$ and ^1K_D as given in Table 1. The slope and intercept of this plot were recalculated to yield ^2K_D 1.48×10^{-2} l mol^{-1} and $^2k_{cat}$ 3.27×10^{-4} s^{-1}; these data are *not* calculated from the plot, but are only given here for comparison purposes. Compared with ^1K_D and $^1k_{cat}$ (Table 1) one easily recognizes that co-operativity is due to the better binding (lower dissociation constant) of a second substrate molecule by host (**3**).

This positive co-operativity is counteracted to a small extent by a concomitant decrease in the turnover number $^2k_{cat}$. A strong net co-operative effect remains, however.

Discussion

The decarboxylation of (**4**) is well known to depend strongly on the medium. Changing the solvent may enhance the rate up to a factor of 10^7 relative to water.[11] Even in aqueous solution the rate can be accelerated 1000-fold or more by addition of aggregate forming cationic detergents[12] or functionalized polymers.[13,14] Quaternary ammonium salts, which do not form aggregates, *e.g.* tetramethylammonium salts, display no rate effect.[12] Thus it seemed necessary to confirm the aggregation status of the macrocycles (**1**)—(**3**). The conductivity measurements together with the n.m.r. studies evidenced conclusively that the macrocycles form insulated entities in aqueous solution surrounded by bulk water. The catalytic effects at $W_o \gg S_o$ can be quantitatively analysed on the basis of a rapid 1:1 complex formation prior to the rate-limiting fragmentation. Since the chemical nature of all the host compounds is identical, the differences in the affinity for the substrate as well as the magnitude of catalysis must be related to the structure of the host–guest complexes. An illustration of the relative size of (**4**) and (**3**) is given in Figure 3.

The symmetry and simplicity of the host structures which

possess a fixed topology by virtue of a high connectivity, however, offers only two principal binding modes of the guest. It may either be associated to a face of the host from the outside or it may penetrate the macrocycles to form true inclusion complexes. These alternatives are independent of the existence of a *permanent* molecular cavity in the host compounds and a decision between them should be possible from the interpretation of their substrate binding constants. Complex stability constants derived from kinetic experiments may not be accurate but the differences observed in the present case are drastic so that the arguments can be based on their relative magnitude rather than their absolute value.

If it is tentatively assumed that the mode of association of the host with substrate (**4**) is the same in every case and happened *via* a face-to-face attachment of (**4**) to the triangular sides of the macrocycles one would expect to find similar stability constants, because from a consideration of the molecular dimensions one must infer that the face of any host is larger than the substrate, so hydrophobic interactions between the binding partners are roughly identical. Within the series, (**2**) would probably form the most stable complex due to having the highest electrostatic attraction. Analogous arguments would apply if inclusion complexes were formed with every host molecule. Again, (**2**) should form a complex the stability of which is expected to exceed that of the corresponding complex with (**3**) by a power of ten as estimated from the factors governing inclusion complex stability.[9] Experimentally, however, we observe no substrate binding with (**2**) whereas the larger macrocyles (**1**) and (**3**) indeed form 1:1 complexes (Table 1). Thus one must conclude that (**1**) and (**3**) can offer a substrate binding mode that is not available for (**2**). With respect to the molecular dimensions it is reasonable to assume true inclusion complex formation of (**4**) with (**1**) and (**3**), respectively, which is not possible with the smaller tetrahedral host (**2**) due to steric repulsion.

The consideration of the drastic decrease in K_D on going from the two-dimensional cavity of (**1**) to the three-dimensional cavity of (**3**) adds another argument in favour of the true inclusion of parts of the substrate into the host structure. If there were side-by-side attachment of host and guest, no change in K_D would be expected, because the chemical nature as well as size and conformation in (**1**) and (**3**) are identical. In contrast, substrate penetration would disclose the difference between these host compounds, because covering one face of (**1**) by a hydrophobic cap as in (**3**) (see Figure 2) should remove the water molecules still lined up around the hydrophobic portion of (**4**) that sticks out of the macrocycle (**1**). The hydrophobic effect should increase and should show up in a lowered K_D. The situation is very similar to that found with cyclodextrins. If one opening of the cyclodextrin torus is closed with a cap, the K_D values for hydrophobic substrates drop typically by a factor of 10—100.[19] An inspection of space-filling CPK-models reveals that host (**3**) can form a molecular cavity of *ca.* 7.5 Å in diameter, which is, to the first approximation, spherical. In contrast, substrate (**4**) is a flat molecule 11 Å long and *ca.* 3 Å thick. There is no way to cover the entire rigid substrate by the host structure. Inclusion complexation will always leave some part of the guest structure exposed to bulk solvent. The question arises whether the kinetic data could serve to evaluate which portion of the substrate molecule is actually surrounded by the host structure. It is reasonable to assume that this will be the hydrophobic nitroaromatic moiety and its vicinity.

This view is supported by the invariance of the (**4**) ⊂ (**3**) dissociation constant with temperature (Table 2). The enthalpy of complexation must be very small. Ion pairing in aqueous solution may show little of an enthalpic change if the water molecules in the hydration layer are reoriented and thereby exert a compensatory effect on the electrostatic attraction energy.[20] In the present cases, however, we consider the introduction of a charged group into a preformed array of opposite charges, which is shielded from the bulk solvent. This process should be accompanied by an enthalpy change.[21] The observation that substrate binding by (**3**) exclusively appears to be an entropy phenomenon renders the penetration of the carboxylate moiety into the molecular cavity of (**3**) unlikely and rather points to a hydrophobic interaction *via* inclusion of the nitroaromatic moiety of (**4**) into the host structure.[22]

The temperature dependence of $^1k_{cat}$ in the reaction using (**3**) as a catalyst presents another piece of evidence to generate a clear picture of the catalytic process. Apparently there is no difference in activation enthalpy between the uncatalysed and catalysed reactions (Table 2), but a dramatically favourable change in activation entropy. It is not plausible to accommodate these results by the assumption of a change in decarboxylation mechanism or compensatory enthalpy effects in view of the simplicity of the host structure. The molecular environments of the reacting substructure in both processes seem to be very much alike. This means that in the host–guest complex, too, the carboxylate group and its vicinity must be surrounded by water and not by the host molecule. The solvation of the hydrophilic moiety of (**4**) remains unaltered on association.

Catalysis by (**3**) of decarboxylation of (**4**) in consequence is the result of a specific interaction of host and guest and originates from the replacement of the solvation shell at a non-reacting site of the substrate by the host.

The application of experimental conditions comparable with the enzymatic situation $\{S_o \gg [(3)]_o\}$ yielded rate data which could not be interpreted in terms of reaction (1). Host–guest complexes of higher order, which were not observable under the conditions $[(3)]_o \gg S_o$, must be taken into account. If two or more substrate molecules bind to (**3**) the sigmoidal dependence of rate *versus* substrate concentration, well known from allosteric enzymes, would indicate co-operation between the binding sites. Relative to proteins the host (**3**) is very small and highly symmetrical and fairly rigid, so that it is hard to visualize two different binding sites in co-operation with each other. Co-operative effects can only be expected if two substrate molecules penetrate the central cavity.

The kinetic behaviour of this system can be described with high fidelity by assuming a 1:1 and a 2:1 complex as outlined in reaction (7). The co-operative effect deduced from the precise fit of the kinetic data to the Hill equation taking $n = 1.4$ as the apparent Hill coefficient may be split into the contributions to ground state substrate binding (K_D) and the influence on the transition state (k_{cat}). Thus the analysis according to reaction (7) yields additional confirmation on the mode of substrate binding. As it turns out, co-operation is the result of an increased affinity of the 1:1 complex towards the second substrate molecule whereas there is a small decrease in k_{cat} counteracting this co-operation. This result is readily understandable on the basis of the binding model discussed above. Since there is only a very modest effect on k_{cat} but a strong influence on K_D the mode of association of the second substrate molecule is likely to be the same as for the first, *i.e.* the hydrophobic nitroaromatic moieties will occupy the central cavity. This type of binding requires the hydrophilic carboxylate groups to extend through different sides of the host tetrahedron out into solution. So the change in the molecular environments of the moieties undergoing chemical transformation in the 1:1 *versus* the 2:1 complex will be minimal as is manifested in k_{cat}.

With the aid of space-filling molecular models one can speculate about the origin of the co-operativity. The higher affinity of the 1:1 complex towards a second substrate molecule appears to be a consequence of the mismatch of host and guest structure. On the introduction of the flat plate-like substrate

into the spherical cavity of (**3**) a void is opened which eases the uptake of one more guest molecule.

In conclusion the macrocyclic quaternary ammonium salt (**3**) emerges from the kinetic study of the decarboxylation of (**4**) to be a low molecular weight enzyme model. (i) It possesses a fixed and stoicheiometric number of active sites per molecule. (ii) Substrate binding occurs in a specific manner with the nitroaromatic moiety invading the hydrophobic interior of the host. (iii) Catalysis is brought about by increasing the activation entropy of the rate-determining step through the removal of an unfavourable component in the prior association step. (iv) The occurrence of co-operative effects demonstrates that even this feature is not coupled to the macromolecular protein structure of natural enzymes but may be observed and mimicked with simple artificial host compounds, too.

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