Macrocyclic Enzyme Models. A Metallo[10.10]paracyclophane bearing Two Imidazolyl Groups as an Efficient, Bifunctional Catalyst for Ester Hydrolysis

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The Cu^{II} complex of [10.10]paracyclophane with one imidazolyl group on each benzene ring (Im_2[10.10]PCPCu^{II}) effectively catalyses the hydrolysis of *p*-nitrophenyl esters with a long alkyl chain by a mechanism involving acylation–deacylation cycles. Facile, Cu^{II}-assisted acyl transfer from the bound substrate to one of the imidazolyl groups with an apparent second-order rate constant of 1.3×10^3 l mol^{-1} s^{-1} in ethanol–dioxan–water (20.5 : 1 : 78.5 v/v) at 40.0 ± 0.1 °C, pH 8.12, and μ 0.10 (KCl) (Im_2[10.10]PCPCu^{II} ⟶ (Im)(ImCOR)[10.10]PCPCu^{II}) is followed by deacylation of the resulting monoacylcyclophane intermediate, which is facilitated by the imidazole-bound Cu^{II} ion, with a first-order rate constant of $\gtrsim 10^{-3}$ s^{-1}. The occurrence of catalysis was confirmed from observations for the monoimidazole analogue of [20]paracyclophane, Im[20]PCP: (a) the nucleophilic reactivity of an imidazolyl moiety in Im[20]PCP is masked upon co-ordination with Cu^{II} although the resulting complex still catalyses hydrolysis by a different mechanism, and (b) the acylated intermediate, (ImCOR)[20]PCP, does not undergo deacylation in the presence and absence of Cu^{II} ion (rate constant $<10^{-5}$ s^{-1}) under the experimental conditions. The co-ordination of Im_2[10.10]PCPCu^{II} and the role of cyclophane-bound copper in the acylation and deacylation steps are discussed.

ONE of the targets of our research on macrocyclic enzyme models is to construct a polyfunctional [20]- or [10.10]-paracyclophane which catalyses ester hydrolysis with enzyme-like turnover behaviour. Polyfunctionality is a general feature of enzyme catalysis, and a great deal of effort has been concentrated in the modification of micellar surfactants,[1,2] polymers,[3] and cyclodextrins[4,5] along these lines.

Previous papers have reported several aspects of paracyclophane catalysis which are summarized briefly as follows. (*i*) An electric charge on the cyclophane skeleton controls its aggregation behaviour in aqueous media; charged cyclophanes are monomeric over the wide concentration range which we are concerned with, while electrically neutral species tend to aggregate as their concentrations increase.[6] A neutral cyclophane co-ordinated with copper(II) ion behaves as a charged species in this respect.[7] (*ii*) The monomeric cyclophanes incorporate various hydrophobic substrates in 1 : 1 stoicheiometry. There are two geometrical modes for interaction of the paracyclophanes with substrates, penetration and face-to-face types.[8] (*iii*) Michaelis complexes derived from monofunctional cyclophanes with nucleophilic character and *p*-nitrophenyl carboxylates (substrates) lead to an obvious kinetic enhancement of intracomplex acyl-transfer from the bound substrate to the cyclophane.[6,9–12] The rate enhancement is primarily attributed to reduction in molecularity (bimolecular ⟶ pseudo-unimolecular) and should be carefully interpreted from the viewpoint of whether it is due to true enhancement of the nucleophilic reactivity. The neutral hydroxyimino-group on the electrically neutral [20]paracyclophane directly attacks an ester carbonyl of the bound substrate due to the hydrophobic effect provided by the paracyclophane skeleton.[13] (*iv*) The introduction of a second functional group with acidic character (quaternary ammonium group[14] or metal ion[7]) into a paracyclophane bearing a nucleophilic group (oxime) results in appreciable enhancement of the intracomplex acyl-transfer. An important point, however, is that the acylcyclophane intermediates thus formed do not readily undergo deacylation under the experimental conditions we have employed. Thus, the reaction is regarded as stoicheiometric rather than catalytic. In order to improve this situation and to develop an efficient catalyst which shows turnover behaviour in the hydrolysis of hydrophobic carboxylic esters, we prepared a bifunctional [10.10]paracyclophane bearing imidazolyl groups on both benzene rings and investigated its kinetic behaviour in the presence of copper(II) ion.

EXPERIMENTAL

5-Oxo[10.10]*paracyclophane-*12(13),28(29)*-dicarboxylic Acid.*[8]—5-Oxo[10.10]paracyclophane (450 mg) in carbon disulphide (20 ml) was added dropwise in 30 min at 0 °C to a mixture of aluminium chloride (7.0 g) and oxalyl chloride (3.5 g) in carbon disulphide (60 ml). After the mixture was stirred for 4 h at room temperature, ice–water (100 g) was added. The resulting mixture was further stirred for 3 h, poured into water (200 ml) containing concentrated hydrochloric acid (20 ml), and extracted with ether (100 ml × 3). The ether extract was evaporated to give a glassy material, which was subsequently stirred in 2.5% aqueous sodium hydroxide (200 ml) for 15 min and extracted with ether to remove the unchanged ketone. The aqueous layer was acidified with hydrochloric acid (pH 1) and extracted with dichloromethane (100 ml × 4). Evaporation of the dichloromethane extract gave a residue, which was purified by means of gel-filtration chromatography [Sephadex LH-20; eluant, methanol–dichloromethane (2 : 1 v/v); flow rate, 1.6 ml min^{-1}] to afford the product (103 mg), $\nu_{max.}$ (neat) 1 714 (ketone) and 1 694 cm^{-1} (carboxy); δ(CCl_4; Me_4Si) 11.39 (2 H, s, CO_2H), 7.81 (2 H, s, *o*-ArH), 7.13 (4 H, m, *m*- and *p*-ArH), 2.79—3.28 (4 H, m, *o*-benzyl), 2.49—2.79 (4 H, m, *m*-benzyl), 2.00—2.43 (4 H, m, $CH_2C{=}O$), and 0.88—1.54 (26 H, m, other CH_2).

Bis-[N-(*imidazol*-4-*ylethyl*)]-5-*oxo*[10.10]*paracyclophane*-12(13),28(29)-*dicarboxamide* (Im_2[10.10]PCP).—A mixture of the dicarboxylic acid (195 mg) and thionyl chloride (12 ml) was stirred at room temperature for 3 h. Excess of thionyl chloride was removed *in vacuo*, and a small amount of dry tetrahydrofuran was added to the residue and evaporated. The addition and evaporation of tetrahydrofuran was repeated three times in order to remove a trace amount of thionyl chloride. A tetrahydrofuran solution (10 ml) of the bis(acid chloride) was added in one portion to an aqueous solution (20 ml) of histamine dihydrochloride (1.1 g) and sodium hydroxide (1.6 g). The mixture was stirred at room temperature for 2.5 h and extracted with ether (100 ml × 4). The usual work-up gave crude Im_2[10.10]PCP as an oil (yield *ca.* 40% by direct weighing and chromatographic analysis), which was dissolved in methanol and purified by repeated high performance liquid chromatography to give Im_2[10.10]PCP (9 mg), $\nu_{max.}$ (dichloromethane) 3 640 and 3 440 (H_2O), 1 714 (ketone), 3 240, 1 640, and 1 530 cm^{-1} (amide); δ([$^2H_4$]methanol; Me_4Si) 7.71 (2 H, s, 2-ImH), 7.18 (2 H, s, *o*-ArH), 7.13 (4 H, s, *m*- and *p*-ArH), 6.98 (2 H, s, 5-ImH), 3.60 (4 H, t, *J* 7 Hz, CH_2CH_2NHCO), 2.89 (4 H, t, *J* 7 Hz, CH_2CH_2NHCO or *o*-benzyl), 2.45—2.80 (8 H, m, *o*- and *m*-benzyl; or CH_2CH_2NHCP and *m*-benzyl), 2.39 (4 H, distorted t, *J* 8 Hz, $CH_2C{=}O$), and 1.02—2.03 (26 H, m, other CH_2) (Found: C, 69.15; H, 8.1; N, 10.2. $C_{44}H_{60}N_6O_3,3H_2O$ requires C, 68.2; H, 8.6; N, 10.85%).

Materials and conditions for chromatographic purification were Hitachi gel 3 019 (500 × 12 mm) with methanol (2.0 ml min^{-1}), Lichrosorb SI-60 (Merck; 200 × 12 mm) with hexane–methanol (2 : 3 v/v; 1.0 ml min^{-1}), and Lichrosorb SI-60 (Merck; 500 × 12 mm) with hexane–methanol (2 : 3 v/v, 1.0 ml min^{-1}) in this sequence. Some comment is required for the unsatisfactory combustion analysis data. (a) The highly hygroscopic nature of the material is partly responsible for the unsatisfactory data as confirmed by careful i.r. analysis. (b) Contamination with some lower nitrogen-containing substance, which is present in a trace amount, is suggested since the observed C : N ratio is larger than expected. By considering the difficulty we encountered in removing the monocarboxylic acid from the dicarboxylic acid (the precursor of Im_2[10.10]PCP), a contaminating species would be the monoimidazole derivative ($Im_1$10.10]PCP). Even if this is the case, the monoimidazole derivative or in general a lower nitrogen-containing derivative cannot be responsible for the observed kinetic activity by reference to the kinetic behaviour of Im[20]PCP. In spite of these comments on the combustion analysis data, the product was practically pure as confirmed by careful n.m.r. analysis.

N-(Imidazol-4-ylethyl)-10(11)-oxo[20]paracyclophane-22-carboxamide (Im[20]PCP) was prepared as reported previously.[6]

N-(*Imidazol*-4-*ylethyl*)-10(11)-*hydroxyimino*[20]*paracyclophane*-22-*carboxamide* [(Im)(NOH)[20]PCP].—A solution of Im[20]PCP (80 mg), hydroxylamine hydrochloride (70 mg), and sodium hydroxide (100 mg) in methanol (6 ml) containing a drop of water was refluxed for 3 h. The mixture was cooled to room temperature and extracted with ether (50 ml × 4). The ether extract was washed with water, dried (Na_2SO_4), and evaporated to dryness. The residue was dissolved in methanol and purified by repeated high performance liquid chromatography (Hitachi gel 3 019 with methanol) and gel filtration chromatography (Sephadex LH-20 with methanol) to afford a hygroscopic material (15 mg, 18%), δ([$^2H_4$]methanol; Me_4Si) 7.73 (1 H, s, 2-ImH), 7.18 (1 H, s, *o*-ArH), 7.11br (2 H, s, *m*- and *p*-ArH), 7.00 (1 H, s, 5-ImH), 3.62 (2 H, t, CH_2CH_2NHCO), 2.60—3.00 (6 H, m, benzyl and CH_2CH_2NHCO), 2.15—2.31 (4 H, distorted t, CH_2CNOH), and 0.90—1.78 (30 H, m, other CH_2); *m/e* 523 (M^+) (Found: C, 70.55; H, 9.4; N, 9.9. $C_{32}H_{51}N_4O_2,H_2O$ requires C, 70.95; H, 9.85; N, 10.35%).

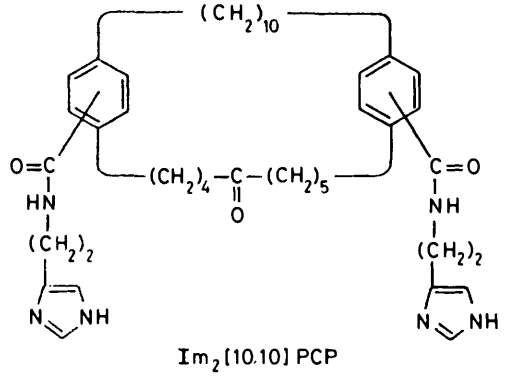
Kinetic Measurements.—The pH and kinetic measurements were the same in principle as described previously.[6,14] Each run was initiated by adding 30 μl of a stock solution of an ester substrate dissolved in dioxan to a reaction medium (3.0 ml) pre-equilibrated at 40.0 ± 0.1 °C in a thermostatted cell set in a Union Giken SM-401 high sensitive spectrophotometer. The medium was prepared by mixing a stock solution of a paracyclophane in ethanol, an aqueous buffer, a stock solution of $Cu(NO_3)_2$ in water, and an appropriate amount of ethanol. The stock solution of copper nitrate was standardized by chelatometric titration. The ionic strength of each reaction mixture was maintained at 0.10 with KCl. A 3 μl sample of a concentrated stock solution of a substrate (1.0×10^{-3} mol l^{-1}) in dioxan was injected to initiate the reaction, in cases when the second addition of a substrate was performed, to avoid any drastic change in compositions of the reaction medium. The second-order rate constant (k_2) was obtained from the initial rate of reaction (V) and the initial concentrations of catalyst and substrate by the equation $V = k_2[PCP]_0[S]_0$.

RESULTS AND DISCUSSION

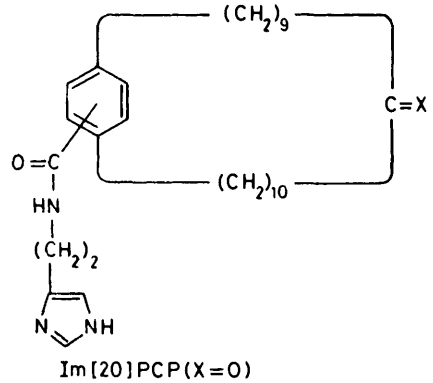
Release of *p*-nitrophenol from *p*-nitrophenyl dodecanoate was accelerated in the presence of Im_2[10.10]PCP in a manner similar to that observed for the reaction of Im[20]PCP with the same substrate.[6] The effect of Cu^{2+} ion in the Im_2[10.10]PCP-catalysed reaction was investigated under the following conditions: temperature, 40.0 ± 0.1 °C; medium, ethanol–dioxan–water (20.5 : 1 : 78.5 v/v) at pH 8.12 and μ 0.10 (KCl); initial substrate concentration, 1.0×10^{-6} mol l^{-1}; initial cyclophane concentration, 1.0×10^{-6} mol l^{-1}. The initial substrate and cyclophane concentrations and composition of the medium indicated above were so chosen that both substrate and cyclophane are in their monomeric states. The critical concentration for aggregation of *p*-nitrophenyl dodecanoate in the present medium at pH 10.26 was readily evaluated kinetically in a manner described previously [15] from the correlation between the spontaneous hydrolysis rate and the initial concentration on the logarithmic scale (Figure 1). A break point, which corresponds to the c.m.c., is observed at 2×10^{-6} mol l^{-1}. A smooth correlation of the catalytic rate constant with the concentration of Im_2[10.10]-PCP indicates that no aggregation takes place in the concentration range investigated here (Figure 2).

The apparent second-order rate constant for the reaction of Im_2[10.10]PCP with the ester substrate is plotted in Figure 3 as a function of Cu^{2+} concentration. The rate constant sharply increases with increasing Cu^{2+} concentration until it reaches a maximum at a slight excess of Cu^{2+} over Im_2[10.10]PCP. A further increase in the Cu^{2+} concentration shows practically no effect on the rate. This observation indicates that the copper

complex of Im_2[10.10]PCP (Im_2[10.10]PCPCu^{II}) has a larger reactivity than Im_2[10.10]PCP and the complex persists even with a 46-fold excess of Cu^{2+} ion; the stability constant of the copper complex must be very



Im_2[10.10] PCP



Im[20]PCP(X=O)
(Im)(NOH)[20]PCP (X=NOH)

high since the complex is almost completely formed at a 1 : 1 molar ratio of copper ion to Im_2[10.10]PCP. Figure 2 shows the correlations of the pseudo-first-order rate constant with the concentration of Im_2[10.10]PCP in the presence (4.6×10^{-5} mol l^{-1}) and absence of Cu^{2+} ion. Both systems exhibit a kinetic feature consistent

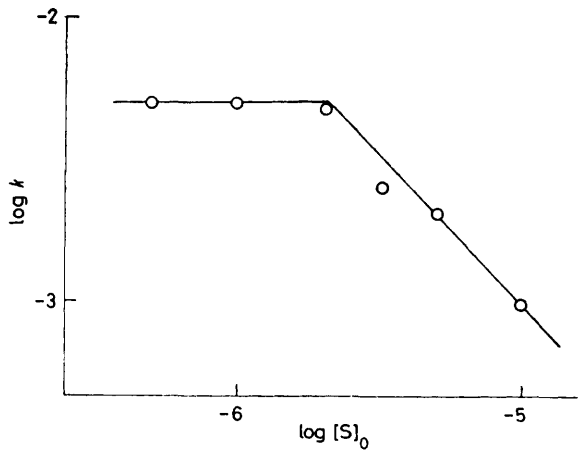


FIGURE 1 Correlation of first-order rate constant (in s^{-1}) with initial ester concentration $[S]_0$ (in mol l^{-1}) for hydrolysis of *p*-nitrophenyl dodecanoate in ethanol–dioxan–water (20.5 : 1 : 78.5 v/v) at 40.0 ± 0.1 °C, pH 10.26, and μ 0.10 (KCl)

with a mechanism which involves pre-equilibrium complexation of the cyclophane with the substrate (binding constant, K_b), followed by pseudo-intramolecular acyl transfer (rate constant, k). Both binding and rate constants were evaluated from usual double reciprocal plot of $(k_{obs} - k_{hyd})^{-1}$ *versus* $[Im_2[10.10]PCP]^{-1}$: for

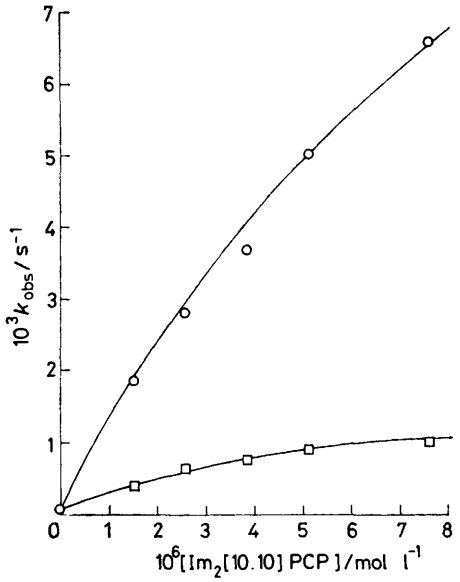


FIGURE 2 Correlations of pseudo-first-order rate constant (k_{obs}) with concentration of Im_2[10.10]PCP for *p*-nitrophenol release from *p*-nitrophenyl dodecanoate (1.0×10^{-6} mol l^{-1}) in ethanol–dioxan–water (20.5 : 1 : 78.5 v/v) at 40.0 ± 0.1 °C, pH 8.12, and μ 0.10 (KCl); in the presence (4.6×10^{-5} mol l^{-1}) (○), and absence (□) of $Cu(NO_3)_2$

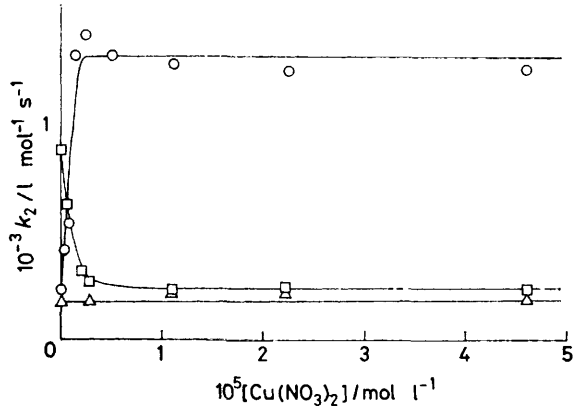


FIGURE 3 Correlations of second-order rate constant (k_2) with concentration of $Cu(NO_3)_2$ for *p*-nitrophenol release from *p*-nitrophenyl dodecanoate (1.0×10^{-6} mol l^{-1}) as catalysed by equimolar amounts of Im_2[10.10]PCP in ethanol–dioxan–water (20.5 : 1 : 78.5 v/v) (○), Im[20]PCP in ethanol–dioxan–water (20.5 : 1 : 78.5 v/v (△), and Im[20]PCP in ethanol–dioxan–water (10.9 : 1 : 88.1 v/v) (□) at 40.0 ± 0.1 °C, pH 8.12, and μ 0.10 (KCl)

Im_2[10.10]PCPCu^{II}, $K_b = 0.6 \times 10^5$ l mol^{-1} and $k = 2 \times 10^{-2}$ s^{-1}; for Im_2[10.10]PCP, $K_b = 3 \times 10^5$ l mol^{-1} and $k = 1.5 \times 10^{-3}$ s^{-1}. Consequently, Cu^{2+} ion apparently provides dual roles: enhancement of intracomplex acyl-transfer on one hand, and reduction in substrate-binding ability on the other compared with

the metal-free catalyst. In marked contrast to the Im_2[10.10]PCPCu^{II} system, Cu^{2+} ion has no rate enhancement effect on the degradation of dodecanoate (1.0×10^{-6} mol l^{-1}) as catalysed by Im[20]PCP (Figure 3). In ethanol–dioxan–water (10.9 : 1 : 88.1 v/v), in which Im[20]PCP (1.0×10^{-6} mol l^{-1}) still exists in the monomeric form, the Im[20]PCP-catalysed reaction is reduced in rate as the concentration of Cu^{2+} ion increases and the rate levels off beyond the region where the Cu^{2+} concentration exceeds twice the amount of Im[20]PCP. The reactivity of metal-free Im[20]PCP is reduced significantly by changing the composition of ethanol–dioxan–water, the reaction medium, from 10.9 : 1 : 88.1 to 20.5 : 1 : 78.5 v/v, while that of the copper complex remains nearly the same.

The kinetic effect of Cu^{2+} ion for Im_2[10.10]PCP catalysis was found to be different from that for the

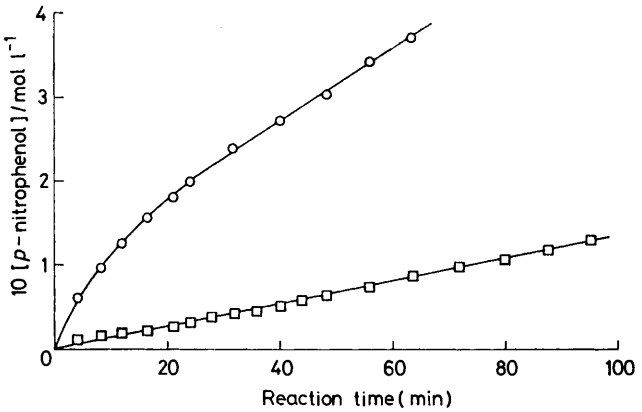


FIGURE 4 Time-courses for *p*-nitrophenol release from *p*-nitrophenyl hexadecanoate (1.0×10^{-5} mol l^{-1}) as catalysed by Im_2[10.10]PCP (1.2×10^{-6} mol l^{-1}) (○) and Im[20]PCP (0.98×10^{-6} mol l^{-1} (□) in the presence of $Cu(NO_3)_2$ (4.9×10^{-5} mol l^{-1}) in ethanol–dioxan–water (10.9 : 1 : 88.1 v/v) at 40.0 ± 0.1 °C, pH 8.12, and μ 0.10 (KCl)

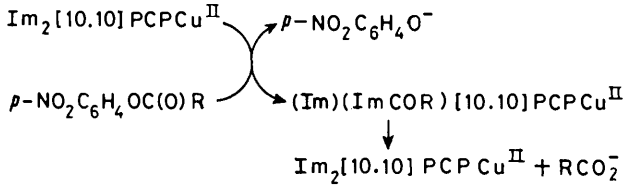
Im[20]PCP-catalysed reaction in the presence of excess of substrate. *p*-Nitrophenyl hexadecanoate (1.0×10^{-5} mol l^{-1}) was chosen as a substrate for runs in ethanol–dioxan–water (10.9 : 1 : 88.1 v/v) so that the contribution of spontaneous hydrolysis is minimized. It has been shown that the reaction of Im[20]PCP with excess hexadecanoate as well as with excess of dodecanoate in the absence of Cu^{2+} ion is stoicheiometric rather than catalytic and the amount of released *p*-nitrophenol corresponds exactly to the initial amount of Im[20]PCP.[6] Figure 4 shows the time-course for *p*-nitrophenol release from excess of hexadecanoate catalysed by Im_2[10.10]PCP (1.2×10^{-6} mol l^{-1}) and Im[20]PCP (0.98×10^{-6} mol l^{-1}) in the presence of Cu^{2+} ion (4.9×10^{-5} mol l^{-1}). Both reactions proceed beyond a stoicheiometric conversion range; the reactions are of a catalytic nature as regards the cyclophane species employed. An important difference between two systems is that Im_2[10.10]PCP-Cu^{II} displays a kinetically biphasic feature (so-called burst kinetics [16]), while Im[20]PCPCu^{II} does not.

Among the paracyclophane species, Im_2[10.10]PCP-Cu^{II}, Im[20]PCPCu^{II}, and Im[20]PCP, the mechanism of action of the last can be interpreted in the most straightforward manner. It is a nucleophilic reagent activated by an imidazolyl group and the reaction involved is a simple acyl transfer from the bound substrate to the cyclophane resulting in the accumulation of the N^{Im}-acyl intermediate; deacylation (hydrolysis) of the intermediate and hence regeneration of Im[20]PCP is too slow to be measured accurately under the present experimental conditions. The reaction effected by Im[20]PCPCu^{II} is quite different and the catalyst is regenerated during the reaction; more than a stoicheiometric amount of *p*-nitrophenol is released, and the amount released has a linear correlation with reaction time (Figure 4). Thus, the reaction is not an acyl transfer type, but most probably direct hydrolysis catalysed by Im[20]PCPCu^{II} without the intermediacy of an acylated cyclophane. Another possible mechanism involves the initial formation of an acylcyclophane intermediate, followed by rapid deacylation so as to maintain the concentration of Im[20]PCP constant during the reaction; this can be ruled out on the basis of independent measurements. The co-ordination of Cu^{2+} ion to the imidazolyl group is in conformity with a drastic change in mechanism, the nucleophilic reactivity of the imidazolyl group being masked upon complex formation, and the N^{Im}-bound Cu^{2+} ion in turn providing a catalytic site. A co-ordination interaction between Cu^{2+} ion and a carbonyl group of the ester substrate may facilitate polarization of the latter in the transition state and, consequently, the attack of external nucleophiles (H_2O and OH^-) [17] or the Cu^{2+}-bound hydroxide on the substrate [18] is presumably much enhanced.

Care should be exercised in comparing the relative reactivities of Im[20]PCP and its copper complex. In addition to the different mechanisms involved in the degradation of carboxylic esters, further complexity arises from the aggregation status of both systems. Im[20]PCPCu^{II} behaves as a charged cyclophane and is monomeric over the wide concentration range we are concerned with,[7] whereas Im[20]PCP has a relatively low critical concentration for aggregation.[6] The reactivity of Im[20]PCP, which seems primarily governed by substrate-binding ability, shows the expected dependence on its concentration and the composition of the reaction medium; the micellar species is more reactive [6] and the monomeric form in turn has a higher reactivity in a reaction medium having a lower content of organic solvent. Thus, it is not surprising that the apparent effect of Cu^{2+} ion on the Im[20]PCP-catalysed degradation of *p*-nitrophenyl esters is dependent on the experimental conditions. At higher, micelle-forming concentrations of Im[20]PCP, the addition of Cu^{2+} ion results in a significant rate depression.[7] Such an inhibitory effect of Cu^{2+} ion is again apparent when monomeric Im[20]PCP (1.0×10^{-6} mol l^{-1}) is used in ethanol–dioxan–water (10.9 : 1 : 88.1 v/v), while in ethanol–dioxan–water (20.5 : 1 : 78.5 v/v), a medium less favourable for hydrophobic interaction, Im[20]PCP

and its copper complex have nearly the same reactivity by accident † (Figure 3).

The kinetic behaviour of the Im_2[10.10]PCPCu^{II} system differs from that of Im[20]PCPCu^{II} in two respects. First, the Im_2[10.10]PCPCu^{II} complex has a reactivity significantly higher than the metal-free species (Figure 3). Secondly, the time-course for the Im_2-[10.10]PCPCu^{II}-catalysed *p*-nitrophenol release from excess substrate displays biphasic kinetics (Figure 4). The extent of the initial burst reaction indicates that only one imidazolyl group is reactive at this stage. These observations are consistent with a two-step reaction, rapid acylation of one of the imidazolyl groups with the ester substrate corresponding to the initial burst stage, and deacylation of the resulting monoacylcyclophane intermediate, (Im)(ImCOR)[10.10]PCP-Cu^{II}, giving rise to regeneration of the original catalyst (Scheme 1). Such being the case, the acylation and deacylation processes are balanced to give a steady-state concentration of the catalyst (Im_2[10.10]PCPCu^{II}) after the burst step. With the condition that all molecules of Im_2[10.10]PCPCu^{II} are occupied by the substrate



SCHEME 1

through hydrophobic interaction in the presence of an excess amount of the latter, the initial rate (V_0) for *p*-nitrophenol release is given by equation (1), where Im_2-[10.10]PCPCu^{II} is represented as PCP, PCP·S is its association complex with the substrate, P is *p*-nitrophenol, and k_a is the first-order rate constant for acylation of one of the imidazolyl groups. The rate after

$$V_0 = dP/dt = k_a[PCP{\cdot}S]_0 = k_a[PCP]_0 \quad (1)$$

the stationary state is attained is given by equation (2), where the suffix s represents the stationary-state condition. The stationary state expression with respect to

$$V_s = dP/dt = k_a[PCP{\cdot}S]_s = k_a[PCP]_s \quad (2)$$

(Im)(ImCOR)[10.10]PCPCu^{II} (abbreviated as PCP·Ac) is given in equation (3), where $[PCP]_0$ is the total concentration of the cyclophane species and k_d is the first-order rate constant for deacylation of the acylcyclophane intermediate. The time-course of the burst kinetics

$$\begin{aligned} d[PCP{\cdot}Ac]/dt &= k_a[PCP{\cdot}S]_s - k_d[PCP{\cdot}Ac]_s \\ &= k_a[PCP]_s - k_d([PCP]_0 - [PCP]_s) \end{aligned} \quad (3)$$

(Figure 4) is analysed on the basis of equations (1)—(3): k_a, from the initial burst rate of *p*-nitrophenol release by equation (1), is 2.1×10^{-3} s^{-1}; $[PCP]_s$, from the slope of a linear portion of the curve derived from equation (2), is 3.4×10^{-7} mol l^{-1}; k_d, by equation (3), is 1.1×10^{-3} s^{-1}.

It must be pointed out here that the biphasic kinetic behaviour is not sufficient evidence to prove that deacylation of the acylcyclophane intermediate takes place. Another possibility, which needs to be ruled out before the suggested acylation–deacylation mechanism is approved, is that the linear portion of the curve simply corresponds to the catalytic action of the copper site of the intermediate, (Im)(ImCOR)[10.10]PCPCu^{II}, by a mechanism similar to that operating in the Im[20]-PCPCu^{II} catalysis described above. To deal with this problem, it is necessary to advance an alternative kinetic criterion. The extent of deacylation of the acyl intermediate in a given period can be evaluated by kinetic titration of the regenerated catalyst using the same substrate. *p*-Nitrophenyl dodecanoate (1.0×10^{-6} mol l^{-1}) was reacted with an equimolar amount of Im_2[10.10]PCPCu^{II} (in the presence of 4.6×10^{-5} mol l^{-1} Cu^{2+}) in ethanol–dioxan–water (20.5 : 1 : 78.5 v/v) with an initial second-order rate constant (k') of 1.3×10^3 l mol^{-1} s^{-1}. The reaction (*p*-nitrophenol release) was completed in 20 min. Then, the same amount of the substrate was added again; *p*-nitrophenol release took place at the identical initial rate (k''), 1.2×10^3 l mol^{-1} s^{-1}. This observation indicates that the acyl intermediate, (Im)(ImCOR)[10.10]PCPCu^{II}, was completely deacylated before the second addition of the substrate was performed, the lower limit for the deacylation rate constant (k_d) being *ca.* 1×10^{-3} s^{-1} if 10 min (half the time required for completion of the first reaction) is taken as an upper limit for the half-life of deacylation. Although Im_2[10.10]PCPCu^{II} must be completely saturated with the substrate under biphasic kinetic conditions (Figure 4) and the deacylation of the acylcyclophane intermediate becomes the rate-determining step, only part of the cyclophane is occupied by the substrate under the conditions employed for the kinetic titration and the whole reaction turns out to be controlled by the acylation step.

On the other hand, the acyloxime of a [20]paracyclophane with both hydroxyimino and imidazolyl groups, which is co-ordinated with Cu^{2+} ion, (Im)(NOCOR)-[20]PCPCu^{II}, is far less prone to deacylation.[7] The copper ion co-ordinated to the imidazolyl group may facilitate acylation of the hydroxyimino group by a mechanism similar to that predicted for the Im_2[10.10]-PCPCu^{II} catalysis; the rate constant for acylation (k') is 2.4×10^3 l mol^{-1} s^{-1} in ethanol–dioxan–water (20.5 : 1 : 78.5 v/v). The resulting acyloxime intermediate does not undergo deacylation under the conditions employed, and re-addition of the substrate at 24 and 92 min after initiation of the initial reaction resulted in reactions with second-order rate constants (k'') of 3.9×10^2 and 4.1×10^2 l mol^{-1} s^{-1}, respectively. These values are very close to that for the reaction with Im[20]PCPCu^{II} (k 2×10^2 l mol^{-1} s^{-1}). Thus, the overall

† If the nucleophile in the Im[20]PCPCu^{II}-catalysed reaction is the Cu^{2+}-bound hydroxide,[18] its reactivity may be enhanced in a less polar medium,[18d] *i.e.*, in ethanol–dioxan–water (20.5 : 1 : 78.5 v/v) compared with ethanol–dioxan–water (10.9 : 1 : 88.1 v/v). This effect would compensate a reduction in substrate-binding ability of Im[20]PCPCu^{II} in a medium having a higher content of organic solvents.

reaction in this case does not proceed by the acylation–deacylation mechanism, but involves a stoicheiometric acylation of the hydroxyimino-group catalysed by the imidazole-bound copper ion, followed by direct catalytic hydrolysis of the substrate promoted by the imidazole-bound Cu^{2+} of (Im)(NOCOR)[20]PCPCu^{II} in the manner observed for catalysis by Im[20]PCPCu^{II}. A similar procedure was applied to the evaluation of the deacylation kinetics of (ImCOR)[20]PCP. Equimolar amounts (1.0×10^{-6} mol l^{-1}) of *p*-nitrophenyl dodecanoate and Im[20]PCP underwent reaction to afford (ImCOR)-[20]PCP; k' 0.88×10^3 l mol^{-1} s^{-1} in ethanol–dioxan–water (10.9 : 1 : 88.1 v/v). After six half-lives (108 min), a second portion of substrate (the same amount as the initial one) was added; *p*-nitrophenol release gave k'' 0.69×10^2 l mol^{-1} s^{-1}. In a separate run, Cu^{2+} ion (4.6×10^{-5} mol l^{-1}) was added after 9.5 half-lives (170 min) of the initial reaction and the mixture was left for 3.5 half-lives (60 min). Re-addition of the substrate at this stage resulted in a reaction with k'' 0.74×10^2 l mol^{-1} s^{-1}. The result indicates that only (ImCOR)-[20]PCP is accumulated and its deacylation, if any, would proceed with a rate constant $k_d < 10^{-5}$ s^{-1} without any effect by Cu^{2+} ion.

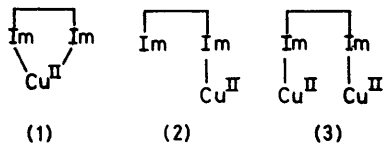
The rate constants for hydrolysis of *p*-nitrophenyl dodecanoate obtained in the present study are summarized in the Table. The striking differences in catalytic action between Im$_2$[10.10]PCP and Im[20]PCP systems

Rate constants for hydrolysis of *p*-nitrophenyl dodecanoate [a]

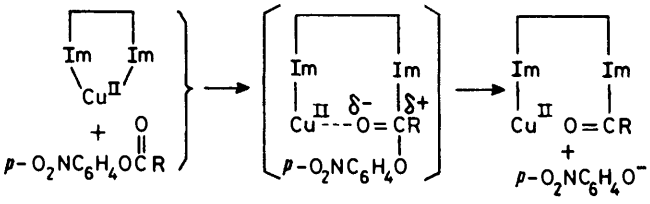
Catalyst species	Rate constant [b]	Medium
Im$_2$[10.10]PCP	k_a 0.23×10^3 l mol^{-1} s^{-1}	A
Im$_2$[10.10]PCPCu^{II}	k_a 1.3×10^3 l mol^{-1} s^{-1}	A
	$k_d \gtrsim 1 \times 10^{-3}$ s^{-1}	A
Im[20]PCP	k_a 0.18×10^3 l mol^{-1} s^{-1}	A
	k_a 0.88×10^3 l mol^{-1} s^{-1}	B
	$k_d \lesssim 1 \times 10^{-5}$ s^{-1}	B
Im[20]PCPCu^{II}	k_h 0.18×10^3 l mol^{-1} s^{-1}	A
	k_h 0.24×10^3 l mol^{-1} s^{-1}	B
	$k_d \lesssim 1 \times 10^{-5}$ s^{-1}	B

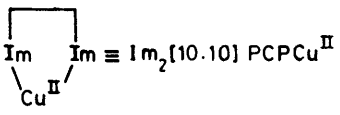
[a] At 40.0 ± 0.1 °C, pH 8.12, and μ 0.10 (KCl). [b] k_a, rate constant for acylation of the imidazolyl group; k_d, rate constant for deacylation of the acylimidazole intermediate; k_h, rate constant for the direct hydrolysis of substrate. [c] A, Ethanol–dioxan–water (20.5 : 1 : 78.5 v/v); B, ethanol–dioxan–water (10.9 : 1 : 88.1 v/v).

are as follows: (a) in contrast to Im[20]PCPCu^{II} which is lacking in nucleophilic reactivity, one imidazolyl group of Im$_2$[10.10]PCPCu^{II} acts as a nucleophile (the latter catalyst is even more reactive than Im$_2$[10.10]PCP by a factor of 5—6 without correction for statistical factors); (b) (Im)(ImCOR)[10.10]PCPCu^{II} is deacylated readily with a rate constant greater at least by a factor of 10^2 than that for (ImCOR)[20]PCP observed both in the presence and absence of Cu^{2+} ion. Thus, the catalytic efficiency of Im$_2$[10.10]PCPCu^{II}, which can be assumed to be a good model for carboxypeptidase A,[19] can without doubt be ascribed to a novel co-operation of the imidazole function and the imidazole-bound copper ion. Among the possible structures (1)—(3) for co-ordination of Cu^{2+} ion to Im$_2$[10.10]PCP, (3) is the least plausible since the co-ordination mode in this structure is the same as that expected for Im[20]PCP-Cu^{II} and there seems to be no reason to observe enhanced nucleophilic reactivity only for the bifunctional cyclophane. Structure (2) is consistent with the fact that



only one imidazolyl group acts as a nucleophile. If this were the case, the rate–[Cu^{2+}] profile in Figure 3 would indicate that the co-ordination of the second Cu^{2+} to the free imidazolyl group is inhibited to such an extent that (2) persists even in the presence of a 46-fold excess of Cu^{2+}; this does not seem likely. The chelating structure (1) is the most plausible and is consistent with the fact that an amount of Cu^{2+} ion equimolar to Im$_2$-[10.10]PCP is sufficient for complete formation of the kinetically active species. A somewhat larger amount of Cu^{2+} ion is required to reach the levelling-off stage of the reaction rate for the Im[20]PCP system, since Im[20]PCP acts as a monodentate ligand; the stability constant of the copper complex must be significantly lower than that of Im$_2$[10.10]PCPCu^{II}. If (1) is the actual co-ordination structure in the ground state for the reaction of Im$_2$[10.10]PCPCu^{II} with an ester substrate, the nucleophilicity of the imidazolyl group would be reduced due to the co-ordination effect. Such an inhibitory effect by Cu^{2+}, however, is probably more than compensated by a favourable interaction of the ester carbonyl with the copper ion in the transition state (Scheme 2). The deacylation of (Im)(ImCOR)[10.10]-

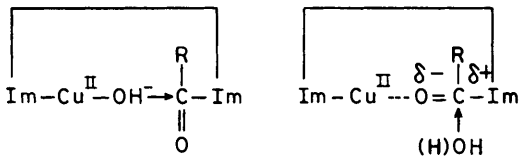




SCHEME 2

PCPCu^{II} is similar in a mechanistic sense to the Im[20]-PCPCu^{II}-catalysed hydrolysis of an ester substrate if the difference in substrate nature is not emphasized, *i.e.*, covalently bound acylimidazole for the former and noncovalently incorporated *p*-nitrophenyl ester for the latter. The most plausible reaction schemes effective in the transition state of deacylation are shown in (4) and (5).

In conclusion, Im_2[10.10]PCPCu^{II} acts as a true catalyst in the hydrolysis of carboxylic esters of hydrophobic character showing the so-called turnover behaviour. The bifunctional cyclophane co-ordinated with



(4) (5)

Cu^{2+} ion has three functions, a hydrophobic binding site with the macrocyclic skeleton, nucleophilic reactivity by the imidazolyl group, and Lewis acidity or a co-ordination reactivity by the imidazole-bound Cu^{2+} ion.

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