The Effect of Leaving Group Tendency in the Chymotrypsin-catalysed Hydrolysis of Aryl Acetates

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The chymotrypsin-catalysed hydrolyses of a number of substituted aryl acetates have been studied. For all the esters with poorer leaving groups the reactions obey simple first-order kinetics. Esters with a 4-nitro-group show ' burst ' kinetics but this is not the case for esters with equally good leaving groups but no 4-nitro-group. This distinction does not occur when pyridine is the catalyst.

THE mechanism of chymotrypsin catalysed hydrolysis of aryl acetates is shown in the Scheme,[1] where E–OH is the enzyme and C is the Michaelis–Menten complex. Hartley and Kilby [2] and Gutfreund and Sturtevant [3] have shown that, in the cases of 4-nitrophenyl and 2,4-dinitrophenyl acetate, the hydrolysis step (k_3) is the slow step. The consequence is a ' burst ' in the production of phenol as the enzyme is acetylated, followed by much slower production of phenol as the acetylated enzyme is hydrolysed and free enzyme regenerated (k_3). A typical plot of phenol formation as a function of time, obtained by stopped-flow spectrophotometry in the present study, is shown in Figure 1. A similar result was obtained with

$$\text{E–OH} + \text{CH}_3\text{CO}_2\text{Ar} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{C} \xrightarrow{k_2} \text{E–OCOCH}_3 + \text{ArOH}$$
$$\text{E–OCOCH}_3 \xrightarrow[k_3]{\text{H}_2\text{O}} \text{E–OH} + \text{CH}_3\text{CO}_2\text{H}$$

SCHEME

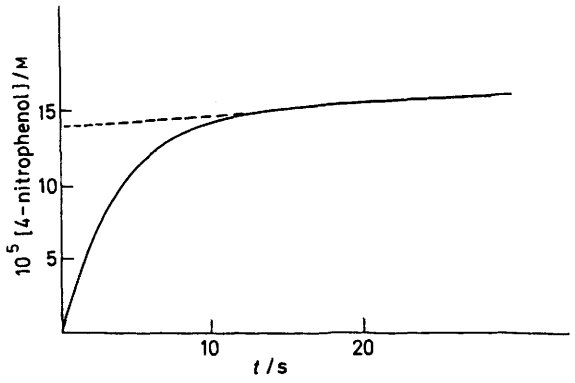

FIGURE 1 Variation of 4-nitrophenol concentration with time in the hydrolysis of 4-nitrophenyl acetate catalysed by chymotrypsin: $[CH_3CO_2Ar]_0 = 3 \times 10^{-4}$M, $[E\text{–}OH]_0 = 1.6 \times 10^{-5}$M

3,4-dinitrophenyl acetate. These three esters have good leaving groups and it is not unreasonable to assume that this is the cause of rapid acetylation and, hence, the kinetic form.

Some confirmation of this view was obtained by a study of the chymotrypsin-catalysed hydrolysis of aryl acetates with poorer leaving groups. The esters used are listed in Table 1. Here production of phenol obeyed first-order kinetics and this is consistent with change of rate-determining step to acetylation (k_2). If hydrolysis of acetylated enzyme is fast then it is kinetically important in the production of phenol. The enzyme behaves well; the reaction is of the first-order over three half-lives and the final absorbance corresponds to complete conversion of ester to phenol. By application of the steady state approximation to the concentration of C,

TABLE 1

Acetylation of chymotrypsin [a] by aryl acetates [b] at 25.3° and pH 7.60

	$k_2K_s^{-1}$/ l mol^{-1} s^{-1}	pK_a [c]	λ [d]/ nm
Phenyl acetate	2.3	9.98	275
4-Chlorophenyl acetate	3.7	9.38	293
4-Bromophenyl acetate	4.3	9.36	278
4-Ethoxycarbonylphenyl acetate	4.7	8.47	294
3-Nitrophenyl acetate	7.7	8.40	400
4-Cyanophenyl acetate		7.95	290
3,5-Dinitrophenyl acetate	656	6.73	406
2,6-Dinitrophenyl acetate	32	3.61	426
4-Nitrophenyl acetate	23 000	7.14	410
3,4-Dinitrophenyl acetate	30 000	5.53	430
2,4-Dinitrophenyl acetate	28 000	3.97	426

[a] $[E\text{–}OH]_0 = 3 \times 10^{-5}$M. [b] $[CH_3CO_2Ar]_0 = 1.2 \times 10^{-4}$M. [c] p$K_a$ of conjugate phenol of leaving group, from A. Albert and F. P. Sarjeant, ' Ionization Constants of Acids and Bases,' Methuen, London, 1962 and V. Gold, D. G. Oakenfull, and T. Riley, *J. Chem. Soc.* (*B*), 1968, 515. [d] Wavelength used in kinetic studies.

the experimentally determined rate constant is given by (1). If $[E\text{–}OH] = [E\text{–}OH]_0$, where $[E\text{–}OH]_0$ is the stoicheiometric concentration of enzyme, then values of

$$k_{obs} = k_2k_1[E\text{–}OH]/(k_{-1} + k_2) \qquad (1)$$

$k_2k_1/(k_{-1} + k_2)$ can be calculated. However, under these conditions $k_2 \ll k_{-1}$ and (1) simplifies to (2). Values of k_2/K_s are given in Table 1 and an indication of the leaving

$$k_{obs} = k_2k_1[E\text{–}OH]/k_{-1} = k_2[E\text{–}OH]/K_s \qquad (2)$$

tendency of the phenate ion is given by the pK_a of the conjugate phenol.

For those esters showing ' burst ' kinetics the situation is much more complex. Complete mathematical analyses of the kinetics of phenol production have been developed [3,4] but insufficient data were obtained in the present study to permit complete analysis. However, the initial slope of the first part of the curve shown in Figure 1 is $[E\text{–}OH]_0[CH_3CO_2Ar]_0k_2/K_s$, where $[CH_3CO_2Ar]_0$ is the initial concentration of ester, and values of k_2/K_s obtained in the present study are given in Table 1.

The values of k_2/K_s for the esters showing first-order

[1] A. Williams, ' Introduction to the Chemistry of Enzyme Action,' McGraw-Hill, London, 1969, p. 27.

[2] B. S. Hartley and B. A. Kilby, *Biochem. J.*, 1954, **56**, 288.

[3] H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, 1956, **63**, 656.

[4] F. Kezdy and M. L. Bender, *Biochemistry*, 1962, **1**, 1097.

kinetics do not give a good Hammett plot using σ^- constants. The two dinitrophenyl esters have been omitted owing to doubts about additivity. A better Hammett plot is obtained if the σ constants are used (Figure 2), and this may indicate that there is little separation of the anionic leaving group in the transition state. If K_s is constant then ρ (0.70) is a measure of changes in k_2. The value is low for attack of a nucleophile [5] on an ester linkage. It may well be that both K_s and k_2 vary with the substituent and that the linear Hammett plot is largely fortuitous. This conclusion is not inconsistent with the results of Bender and Nakamura.[5]

For the last three esters listed in Table 1, all of which show ' burst ' kinetics, values of k_2/K_s are much greater. The improved leaving tendency should increase k_2 but, judging from the values of k_2/K_s for other esters, there

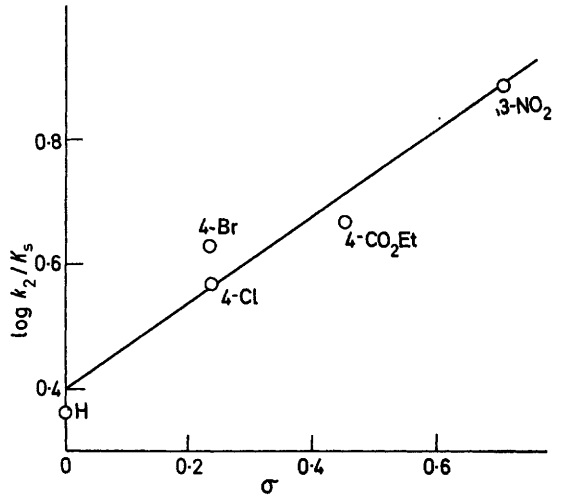

FIGURE 2 Hammett plot for the hydrolysis of aryl acetates catalysed by chymotrypsin

there must also be a decrease in the size of K_s. Although the leaving tendency of the phenate anions within this group of esters varies considerably, k_2/K_s is essentially constant and so K_s must show considerable variation.

The two esters, 3,5-dinitrophenyl and 2,6-dinitrophenyl acetate, are of special interest. The anionic leaving tendency in both is good, better than for 4-nitrophenyl acetate, but neither shows ' burst ' kinetics. The latter ester possesses special steric factors but this is not so for the former. For hydrolysis catalysed by pyridine these esters behave no differently from other nitrophenyl acetates investigated by Butler and Robertson [6] (Table 2) and so there must be a special factor in the enzyme catalysed reaction. For both, k_2/K_s is much smaller than for esters possessing equally good leaving groups, and so the abnormal factor must be the size of K_s. These esters differ from the others in not having a 4-nitro-group and we conclude that this materially affects formation of the Michaelis–Menten complex (*i.e.* the size of K_s). A

TABLE 2

Hydrolyses of phenyl acetates [a] in pyridine buffers at 25.3°

	$10^3k_{obs}/s^{-1}$		
$[pyridine]_{free}$[b]/M	3,5-Dinitro	3,4-Dinitro	2,6-Dinitro
0.08	0.65	7.4	140
0.16	1.27	15.3	300
0.24	1.83	18.2	390
0.32	2.25	24.1	510
0.40	3.13	30.7	630
10^3k/l mol^{-1} s^{-1}	7.8	76	1 480

[a] $[CH_3CO_2Ar]_0 = 10^{-5}$M. [b] Concentration of unprotonated pyridine in buffer.

possible explanation is that a site in the enzyme binds the 4-nitro-group and, in doing so, holds the substrate in a position particularly favourable for attack of the ester linkage.

4-Cyanophenyl acetate has an anion of intermediate leaving tendency. Its behaviour is consistent with the proposed mechanism in that the kinetics of 4-cyanophenol formation were not first order in 4-cyanophenol but, equally, they were not typical of an ester showing ' burst ' kinetics. The conditions for ' burst ' kinetics were set up on an analogue computer and as the value of k_2/k_3 was reduced, representing introduction of a poorer leaving group, the display approximated to that obtained in the hydrolysis of 4-cyanophenyl acetate. With very small values of k_2/k_3 the display curve became, of course, that of a first-order reaction. The result is also consistent with changes in the value of K_s and not enough data are available to distinguish between these possibilities.

EXPERIMENTAL

Materials.—Most phenols were commercial materials. 3,5-Dinitrophenol was prepared by the method of King *et al.*[7] and 4-ethoxycarbonylphenol by that of Pappo *et al.*[8] Phenols were converted to acetates by the method of Vogel [9] or Bell.[10] Chymotrypsin was supplied by Koch–Light Laboratories (crystallised three times from bovine pancreas and salt free). The concentration of enzyme was determined by weight, assuming a molecular weight of 24 800. This may overestimate the molarity [4] but the small error will be the same throughout and the conclusions will not be affected. AnalaR pyridine, KH_2PO_4, and KOH were used without further purification.

Kinetic Method.—Details of the pyridine catalysed hydrolyses of nitrophenylacetates have been given previously.[6] Chymotrypsin was dissolved in a phosphate buffer of pH 7.6 (I 0.1M) and the ester in 10^{-4}M-HCl (to suppress spontaneous hydrolysis). For the slow reactions equal volumes of the two solutions were mixed in a cuvette in the thermostatted cell holder of a Unicam SP 500 spectrophotometer. The appearance of an absorbance (see Table 1 for wavelength)

[5] M. L. Bender and K. Nakamura, *J. Amer. Chem. Soc.*, 1962, **84**, 257.
[6] A. R. Butler and I. H. Robertson, *J.C.S. Perkin II*, 1975, 660.
[7] L. C. King, M. McWhirter, and D. M. Barton, *J. Amer. Chem. Soc.*, 1945, **67**, 2089.
[8] R. Pappo, B. M. Bloom, and W. S. Johnson, *J. Amer. Chem. Soc.*, 1956, **78**, 6347.
[9] A. I. Vogel, ' Practical Organic Chemistry,' Longmans, London, 1956, p. 382.
[10] J. A. Bell, Explosives Research and Development Establishment, Technical Note No. 31, 1971.

due to formation of the phenoxide ion was monitored. First-order rate constants were calculated by the method of Swinbourne.[11]

For faster reactions the solutions were placed in the syringes of a ' Canterbury ' stopped flow spectrophotometer and the change of absorbance with time was displayed on an oscilloscope and photographed. For conversion from absorbance to phenol concentration the instrument was calibrated by the use of phenol solutions of known concentrations. In all cases the ionic strength was made 0.5M by addition of KCl.

An E.A.I. Pace TR-48 analogue computer and Beckman ' Research ' pH meter were used.

[11] E. S. Swinbourne, *J. Chem. Soc.*, 1960, 2371.

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