ON THE MECHANISM OF HYDROLYSIS OF N-ACYLAMINO ACID NITROPHENYL ESTERS

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Received May 2, 1966

Nitrophenyl esters have found increasing use as substrates for proteolytic enzymes since the classical "burst" experiments of Hartley and Kilby (1952, 1954), using p-nitrophenyl acetate and α-chymotrypsin (see, for example, Zerner and Bender, 1964). The mechanism of hydrolysis of esters involves nucleophilic attack at the carbonyl carbon (Bender, 1960). Previous studies using labile esters of N-acylamino acids have assumed that hydrolysis occurs by a similar nucleophilic attack, and it has been further assumed that hydrolytic enzymes accelerate hydrolysis by permitting more efficient nucleophilic attack at the carbonyl carbon (Bender and Kézdy, 1964).

It has been reported (Wenger, Urheim and Rottenberg, 1962) that hippuric esters exhibit unusually high reactivities toward neutral and alkaline hydrolysis. Further, N-methyl-N-acylamino acid esters are reported to be exceedingly bad substrates for α-chymotrypsin (Peterson, Hubele and Niemann, 1963). Therefore, we have investigated the hydrolysis of p-nitrophenyl hippurate (PNPH) and p-nitrophenyl benzoylsarcosine (PNPBS), to ascertain whether any special chemistry obtains which might be of importance in the enzymatic reactions. In this communication, we report on the mechanism of non-enzymatic hydrolysis of p-nitrophenyl hippurate. The

kinetics of hydrolysis of PNPH[a] was followed spectrophotometrically in the thermostatted cell compartments of a Cary 14 spectrophotometer at 30° ± 0.1 and at four wavelengths, 250 mμ, 300 mμ, 317 mμ, 400 mμ (depending on pH). Based on the release of p-nitrophenol at 317 mμ or 400 mμ (Fig. 1) the ester has the abnormally high apparent rate constant of 1.05×10^4 M^{-1} sec^{-1} for alkaline hydrolysis. During the hydrolysis of PNPH, a highly absorbing intermediate was observed (250 or 300 mμ). The decay of this intermediate, rather than the release of p-nitrophenol was found to be rate-limiting at alkaline pH. At pH's above 8.5, a first-order constant could be determined without interference from the release of p-nitrophenol (Fig. 1). At pH's below 8.5, the decay of the intermediate must be followed at the

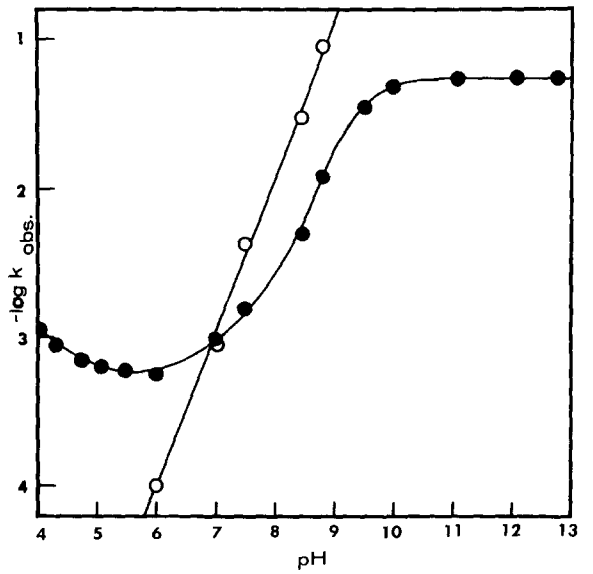

Fig. 1. pH-rate profile of PNPH (O) as determined by release of p-nitrophenol, and of 2-phenyl-oxazolin-5-one (●). Units of k_{obs}, sec^{-1}; 30° ± 0.1°; 1-3% CH_3CN in reaction mixture; measured in buffers of constant pH and increasing buffer concentrations; determinations at each pH extrapolated to zero buffer concentration.

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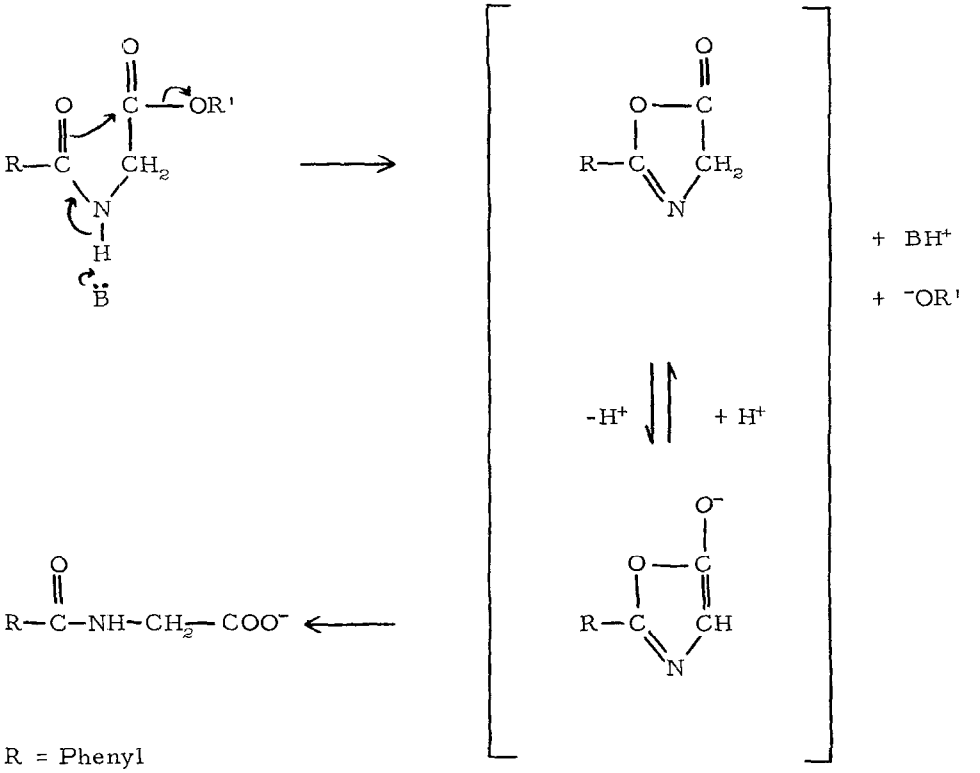
[a] M.P. 170-171°; 99.5% pure, based on release of p-nitrophenol.

isosbestic point of PNPH and p-nitrophenol, which is of course pH-dependent. The mechanism of Chart I was postulated and is supported by the following data

CHART I



(1) The mechanism involves the intermediate 2-phenyl-oxazolin-5-one. This oxazolinone was synthesized (Crawford and Little, 1959), and Table I shows representative data in a comparison of the rates of hydrolysis of the intermediate and 2-phenyl-oxazolin-5-one.

(2) The pK_a' of the intermediate determined kinetically is 9.3_2 (Fig. 1); the pK_a' of the enol of the oxazolinone, measured independently by a spectrophotometric titration technique is 9.4.

(3) The hydrolysis of PNPH is general base-catalyzed (Table II).

TABLE I

Rates of Hydrolysis of Intermediate and 2-Phenyl-Oxazolin-5-one[a]

pH	Buffer	$10^3 k_{obs}$ Intermed.[b]	$10^3 k_{obs}$ Oxazolinone[b]
7.50	0.05 M phosphate	5.2_7[c]	5.3_3
8.47	0.01 M tris	7.8[d]	7.8
9.52	0.02 M borate	$47._0$[e]	$46._9$
9.98	0.01 M borate	$54._8$[e]	$54._9$
	0.00118 N NaOH	$54._9$[e]	$55._4$
	0.0118 N NaOH	$56._1$[e]	$56._3$
	0.0590 N NaOH	$55._2$[e]	$56._2$

[a] 30° ±0.1°. [b] in sec.$^{-1}$. [c] at 360 mμ, the isosbestic point of PNPH and p-nitrophenol. [d] at 250 mμ. [e] at 300 mμ.

(4) At pH 11, the release of p-nitrophenol is virtually instantaneous while the intermediate has a half-life of ca. 14 sec.. At pH 5, the half-life of 2-phenyl-oxazolin-5-one is ca. 17 min.. These facts allow the identification of the intermediate, whose U.V. spectrum (in chloroform) is consistent with that of 2-phenyl-oxazolin-5-one.

(5) Detailed examination of the spectral data obtained in 0.1 N sodium hydroxide shows that the reaction proceeds quantitatively through the oxazolinone.

The mechanism would lead to racemization during the hydrolysis of optically active esters, depending on the relative rates of enolization and ring opening reactions (Goodman and McGahren, 1965), and is available in principle for labile esters of all N-acylamino acids. There was no build up of the oxazolinone intermediate during the hydrolysis of methyl hippurate,

TABLE II

Effect of 2,6-Lutidine on the Hydrolysis of the p-Nitrophenyl Esters of Acetic Acid, N-Benzoylsarcosine and Hippuric Acid[a,b]

[Free Lutidine] (M)	$10^6 k_{obs}$ PNPA[c] ($sec.^{-1}$)	$10^5 k_{obs}$ PNPBS[c,d] ($sec.^{-1}$)	$10^4 k_{obs}$ PNPH ($sec.^{-1}$)
0.0245	1.8_0	3.9_3	9.3
0.0123	1.7_8	3.9_0	8.5
0.0098	1.7_8	3.9_5	8.3
0.0074	1.7_3	3.8_7	7.8

[a] pH 6.78 ± 0.00_4 (1:1 buffers). [b] μ adjusted to 0.049 by addition of KCl. [c] calculated from initial rates. [d] PNPBS has m.p. 113° and satisfactory analysis; 100.$_0$% pure based on p-nitrophenol release.

indicating that a labile ester moiety is necessary. Goodman and Stueben (1962) had previously postulated an oxazolinone intermediate to account for the racemization observed during the hydrolysis of dipeptide nitrophenyl esters in 64% (v/v) dioxan-water mixtures. Further, Williams and Young (1964) have demonstrated that 4-isobutyl-2-phenyl-oxazolin-5-one exists in equilibrium with N-benzoyl-L-leucine p-nitrophenyl ester in the presence of N-methyl piperidine in chloroform, and that racemization of the ester occurred *via* the oxazolinone.

The present work, however, constitutes the first direct demonstration of an oxazolinone intermediate in the hydrolysis of an N-acylamino acid nitrophenyl ester and shows moreover that the reaction proceeds quantitatively through the oxazolinone.

Since all specific substrates for proteolytic enzymes are N-acylamino acid derivatives, studies (enzymatic and non-enzymatic with

various nucleophiles) of labile esters and their N-methyl analogues are being actively pursued with a variety of enzymes in this laboratory to ascertain what role, if any, the hydrogen bonded to nitrogen has in enzymatic systems.

Acknowledgements

We acknowledge with thanks the indefinite loan of a Cary 14 spectrophotometer from the Wellcome Trust, London. This work was supported in part by a grant from the N.H.M.R.C. (Australia).

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