ON THE INHIBITION OF β-N-ACETYL-D-GLUCOSAMINIDASE BY 2-ACETAMIDO-2-DEOXY-D-GLUCONO-(1→5)-LACTONE

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Received August 13, 1968

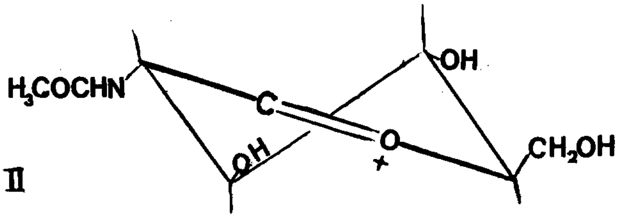
Aldono-(1→5)-lactones are powerful, highly-specific, competitive inhibitors of enzymes which catalyse the hydrolysis of glycosides of corresponding configuration (Conchie, Hay, Strachan and Levvy, 1967). No rationalisation of this phenomenon has yet been advanced; however, its generality would suggest an explanation in terms of some special property of aldono-(1→5)-lactones in relation to the active sites of glycosidases. Studies on the pig epididymal β-N-acetyl-D-glucosaminidase/2-acetamido-2-deoxy-D-glucono-(1→5)-lactone system indicate that the specificity and high affinity of the lactone for the enzyme probably arise from ease of inhibitor-binding due to the flexibility of the lactone ring and/or to the stereochemical and conformational similarities between the lactone and the transition state in the enzyme-catalysed pyranoside hydrolysis.

Materials and Methods

Ethyl and aryl 2-acetamido-2-deoxy-β-D-glucopyranosides were synthesized according to Leaback and Walker (1957), (1961). Crystalline 2-acetamido-2-deoxy-D-glucono-(1→5)-lactone was a gift from Dr. N.M. Cross.

The 60 MHz NMR spectra were performed on concentrated D_2O solutions with a Varian A60 spectrometer with DSS as internal standard and, where necessary, using C.A.T. accumulated signals. Several evaporations from fresh D_2O solutions were carried out in cases where H_1 signals fell close to that of HDO.

The β-N-acetyl-D-glucosaminidase (EC.3.2.1.30) used was fraction 6(b) of the preparation of Findlay and Levvy (1960) and, unless otherwise stated, was assayed at 30° in 0.05M sodium citrate buffer pH 4.3.



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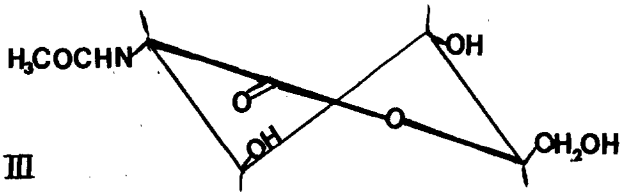

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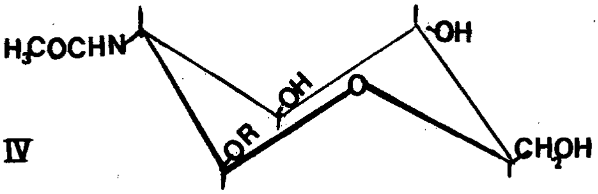

IV

Results and Discussion

The inhibition of pig epididymal β-N-acetyl-D-glucosaminidase (EC.3.2.1.30) by 2-acetamido-2-deoxy-glucono-(1→5)-lactone (I) is an especially good example of the phenomenon of inhibition of glycosidases by aldono-(1→5)-lactones of corresponding configuration. With an inhibitor constant (K_i) of about 5 x 10^{-4}mM, the lactone (I) is easily the most powerful competitive inhibitor of this enzyme yet known (Findlay and Levvy, 1960; Leaback and Walker, 1967).

Michaelis constants (K_m) of the enzyme for aryl and alkyl 2-acetamido-2-deoxy-β-D-glucopyranoside substrates are about 1-3mM and vary little with pH (Leaback, 1967) or the nature of the aglycone (Table I). The maximum velocity ($V_{max.}$) of the catalysed reaction varied with pH such

TABLE I

2-acetamido-2-deoxy-β-D-glucopyranoside (IV)	K_m (mM)	Relative $V_{max.}$	Appropriate Hammett Constant	H_1 resonance (60 MHz)		Probable conformation
				p.p.m.	$J_{1:2}$	
Methylumbelliferyl	1.2	2.08	0.95*	-**	-	-
p-nitrophenyl	1.73	2.25	1.24	4.55	7.5	C1
o-nitrophenyl	1.63	2.20	1.24	-**	-	-
p-acetylphenyl	0.89	1.45	0.38	4.65	8.5	C1
phenyl	1.53	1.00	0.00	4.85	7.5	C1
Ethyl	3.2	13	-	5.47	7.5	C1

*Calculated from the pK' (7.8) of 4-methylumbelliferone estimated at 30° and the equation given by Barlin and Perrin (1966). Other σ values were from latter source.

**No satisfactory spectra have been obtained for these materials.

as to indicate (Fig.2) the participation of an acid group or groups on the enzyme with a pK of 6.5-7.0. A plot of log $V_{max.}$ against the appropriate Hammett constant for the particular aglycone gave a line with positive slope (Fig.2). This indicates the participation in the catalysed reaction of basic or nucleophilic groups on the enzyme (cf. Lowe, Sheppard, Sinnott and Williams, 1967).

Together with the high 'acetamido' specificity and the retention of configuration of the catalysed reaction (Leaback and Walker, 1967), these properties of the enzyme are strikingly like those of egg-white lysozyme (Vernon, 1967; Lowe *et. al.*, 1967) and suggest that a similar reaction mechanism obtains; that is, concerted acid/base or acid/nucleophile catalysis with an ionic half-chair pyranose transition state of the kind (II).

Molecular models of the lactone (I) indicate a more flexible structure than those of the alkyl and aryl 2-acetamido-2-deoxy-β-D-glucopyranoside substrates, and a preference for a half-chair conformation such as III. This is in accord with the established planarity of the

-C-CO-O-C- grouping (Mathieson and Taylor, 1961; McConnell, Mathieson and Schoenborn, 1962) and the half-chair conformation of lactone-rings in steroids (Cheung, Overton and Sim, 1965; Sheppard and Turner, 1968).

Whereas 60 MHz NMR spectra showed (Table I) that the 2-acetamido-2-deoxy-β-D-glucopyranoside substrates adopt a C1 chair conformation in D_2O solution, signals (τ values 5.7 - 6.3) from fresh solutions of the lactone (I) were very complex and, as might be expected from a very flexible structure, were incompletely resolved even at 220 MHz.

A series of structural analogues of the lactone (I) were found to be competitive inhibitors of the hydrolysis of methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside by the enzyme; thus, inhibitor constants of 15, 8, 43, 50 and 200 mM were obtained for acetic acid, acetamide, N-methyl-acetamide, 2-acetamido-acetic acid and ethyl 2-acetamido-acetate respectively. That neither 0.2M ethyl 2-amino-acetate nor 0.2M ethyl 2-propionamido-acetate produced detectable inhibition under similar conditions, suggested that the series of lactone-analogues were competing for the enzyme's highly-specific 'acetamido' receptor site (cf. Pugh, Leaback and Walker, 1957; Leaback and Walker, 1967). Since the carbonyl

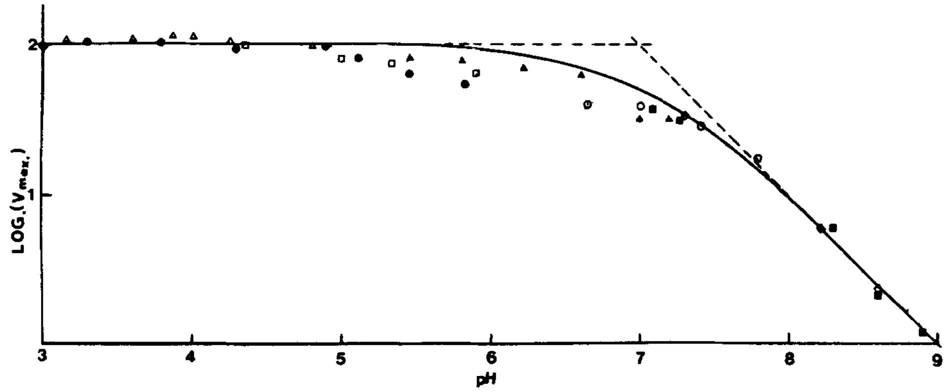

Fig.1. Results illustrated here using formate (△), phthalate (●), pivalate (□), cacodylate (▲), collidine (○), glycylglycine (■) and barbiturate (◇) buffers, approximate to the acid ionisation curve indicated above with a pK about 7.0; a somewhat lower pK (about 6.5) was indicated using certain other buffers at the basic end of this pH range.

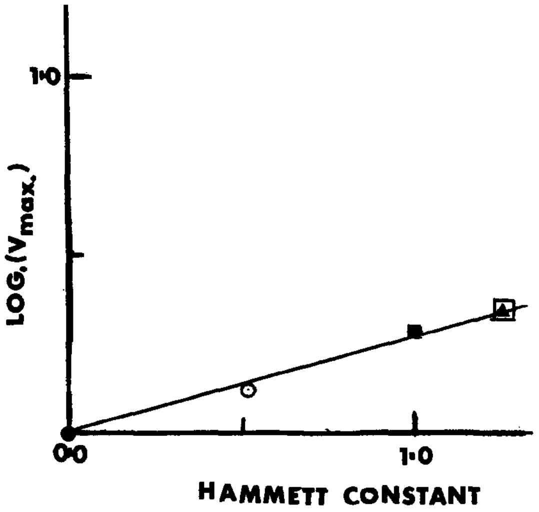

Fig.2. Plot of log $V_{max.}$ for the enzymatic hydrolysis of phenyl (●), p-acetylphenyl (O), methylumbelliferyl (■), o-nitrophenyl (▲) and p-nitrophenyl (□) 2-acetamido-2-deoxy-β-D-glucopyranosides plotted against the Hammett Constant (σ) for the appropriate aglycone.

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group did not increase affinity for the enzyme in this series, it was concluded that the high affinity for the lactone is unlikely to be a consequence of the lactone carbonyl itself but rather of some property which the group confers upon the ring.

It is suggested, therefore, that the high specificity and affinity follow from the flexibility of the lactone ring and/or the stereochemical and conformational similarities between the lactone and the transition state of the enzyme-catalysed hydrolysis of pyranoside substrates. A corollary of this would be that glycosidases which show high affinity for lactones of appropriate configuration (cf. Conchie, et. al., 1967) probably catalyse pyranoside-hydrolysis by a similar mechanism entailing intermediates with half-chair conformations.

Acknowledgments

I am indebted to Professor J.A. Elvidge of the University of Surrey and to Mr. J.K. Beaconsall of Imperial Chemical Industries Ltd. for the 60 and 220 MHz spectra respectively. Helpful discussions with

Dr. P.G. Walker and financial assistance from the National Fund for Poliomyelitis Research are gratefully acknowledged.

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