FUNGAL CELLULASES

XVIII.* ETHYL L-LACTATE AS AN ACCEPTOR FOR THE β-GLUCOSIDASE OF *STACHYBOTRYS ATRA*

By M. A. JERMYN†

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Summary

Ethyl L-lactate in dilute aqueous solution appears to depress the overall activity of β-glucosidase as well as acting as an acceptor, and the two effects cannot be disentangled. The effects of solute interaction on the properties of the enzyme were studied in isolation by using another water-soluble ester, methyl acetate. This solute affects the shape and height of the pH–activity curve and the relation between pH and Michaelis constant. The sum of these effects appears as an activation or a depression, according to the pH.

I. INTRODUCTION

Ethyl L-lactate was chosen for investigation as the type of an acceptor for the *S. atra* β-glucosidase that had a high affinity for the enzyme but was none the less inhibitory. Before its behaviour had been investigated very far, it became apparent that unspecific effects of activation or repression could not be ignored in this case. A related solute that was not an acceptor was therefore chosen for study. This solute had to be an ester (for meaningful comparison with ethyl lactate), water-soluble to at least 1M, reasonably stable in aqueous solution, and of sufficiently low molecular weight that aqueous solutions of appropriate molarities would not differ too much from water in physical properties. The sum of these considerations led to the choice of methyl acetate.

II. MATERIALS AND METHODS

Methyl acetate (British Drug Houses) was fractionally distilled and the fraction boiling at $57 \pm 0{\cdot}5$°C stored in the refrigerator. The material as received was in fact essentially pure, and the main function of the fractionation was to remove small traces of hydrolysis products. The hydrolysis of methyl acetate in pure water, like that of other esters, is autocatalytic; molar solutions of freshly distilled methyl acetate in freshly glass-distilled water only show a rapid fall in pH at room temperature (15–20°C) after 8–24 hr. Solutions containing methyl acetate were therefore made up immediately before use.

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† Division of Protein Chemistry, CSIRO Wool Research Laboratories, Parkville, Vic.

Ethyl lactate (British Drug Houses) as supplied contained large amounts of material both more and less volatile than ethyl lactate. The material was distilled once in bulk to get rid of most of these impurities, and the appropriate cut fractionated a number of times to yield a product, b.p. (754 mmHg) 154±1°C. Although there was no indication of the provenance of the lactic acid, the purified ester had $[\alpha]_D^{21}$ −9·2° and was thus approximately 95% ethyl L-lactate. Commercial ethylene glycol monoacetate was fractionally distilled to give a product, b.p. (760 mmHg) 188±1°C.

The methods used have been outlined in Part XV of the series (Jermyn 1966*a*).

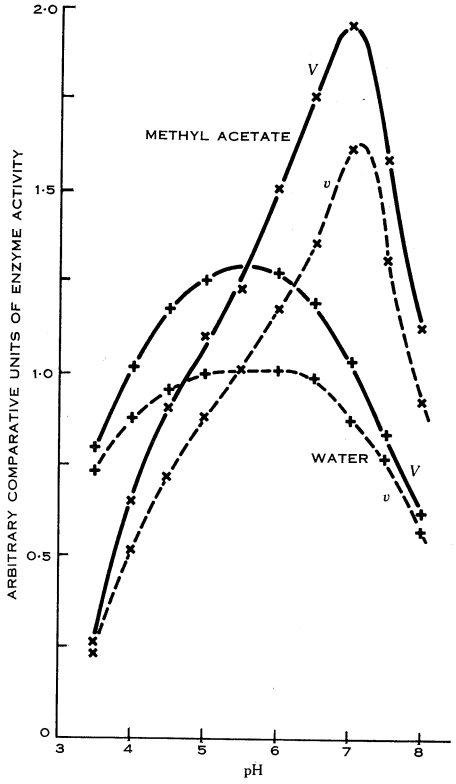

Fig. 1.—Effect of 1M methyl acetate on the pH–activity curve for the decomposition of phenyl β-D-glucopyranoside in McIlvaine buffers at 28°C by the β-glucosidase of *S. atra. v*, Substrate concentration 2×10^{-3}M; *V*, infinite substrate concentration.

III. Results and Discussion

(*a*) *Methyl Acetate as Added Solute*

Figure 1 shows the effect of 1M methyl acetate on pH–activity curves for the enzyme. It is at once apparent that the solute has a profound effect on the activity of the enzyme and that the direction of this effect is different at different pH values. Table 1(*a*) adds a little information to amplify the last point; the direction of the effect is independent of methyl acetate concentration, and the amount of the effect is related to concentration in the way that would be expected.

Figure 2 shows the effect of methyl acetate on the relation between pH and Michaelis constant for the decomposition of phenyl β-D-glucopyranoside. It may be seen that the effect of pH on the Michaelis constant is nearly abolished over the pH range 4–8. No convincing reason can be suggested at present for this observation.

TABLE 1

EFFECT OF INCREASING CONCENTRATIONS OF METHYL ACETATE ON THE RATE OF DECOMPOSITION OF 2×10^{-3}M PHENYL β-D-GLUCOPYRANOSIDE AT 28°C BY THE β-GLUCOSIDASE OF *S. ATRA*

McIlvaine citrate–phosphate buffers used throughout

(*a*) Experiment 1			(*b*) Experiment 2			
Methyl Acetate Concn. (M)	pH 3·5	pH 7·0	Methyl Acetate Concn. (M)	pH 5·5	pH 5·0	pH 4·5
Nil	1·00	1·00	Nil	1·00	1·00	1·00
0·01	0·93	1·06	0·25	0·99	0·97	0·92
0·03	0·77	1·30	0·5	1·03	0·94	0·87
0·1	0·47	1·59	1·0	1·04	0·91	0·82
0·3	0·37	1·77	1·5	1·02	0·88	0·79
1·0	0·32	1·82	3·0	1·03	0·88	0·77

A number of observations showed that under a variety of circumstances in the presence of methyl acetate, there were no departures from the ratio of unity between glucose and phenol liberated. The effects of methyl acetate were therefore not on the enzymic mechanism as such. Nor were they due to the gross changes in protein structure (helix–coil transitions, etc.) associated with high concentrations

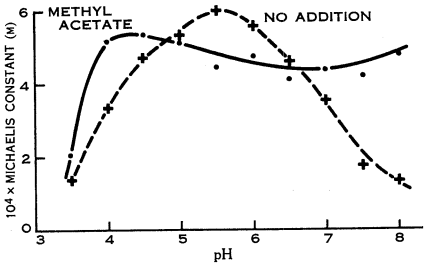

Fig. 2.—Effect of 1M methyl acetate on the pH–Michaelis constant curve for the decomposition of phenyl β-D-glucopyranoside in McIlvaine buffers at 28°C by the β-glucosidase of *S. atra*. Each point is the mean of three determinations.

of organic solvents in solution. From Figure 1 it can be seen that the pH–activity curves in the presence and absence of 1M methyl acetate cross at about pH 5·5, i.e. the measured enzyme activity is nearly indifferent to the presence of methyl acetate at this pH. In Table 1(*b*) the effect of increasing methyl acetate concentration on enzyme activity at pH 5·5, 5·0, and 4·5 is shown. Up to 3M methyl acetate (the solubility of methyl acetate in water is 3·2M) the enzyme activity remains nearly constant at pH 5·5; at pH 4·5 and 5·0 the activity falls sharply at low solute concentrations with the rate of decrease slowing with increasing solute

concentration. This is behaviour typical of a reversible protein–solute interaction, and not of the major conformational changes, usually resulting in complete "inactivation" or "denaturation", which characterize the action of high concentrations of organic solvents on proteins. Figure 3 shows data from Table 1(*a*) replotted to show a typical sigmoid interaction curve.

The best studied case of small amounts of organic solvents activating an enzyme is that of the adenosinetriphosphatase activity of myosin (Ebashi and Ebashi 1959). The order of activating ability according to type of compound is the reverse of that found in these studies, butan-1-ol being a very good activator and ethyl acetate a very poor one. The observations are usually explained by postulating a small reversible conformational change at the active centre of the enzyme; Tonomura, Sekiya, and Imamura (1962) have shown parallel activation and change in optical rotatory dispersion for the action of low concentrations of dioxan on myosin.

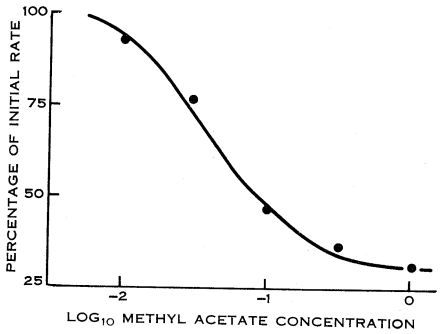

Fig. 3.—Effect of methyl acetate concentration on the rate of decomposition of 2×10^{-3}M phenyl β-D-glucopyranoside at 28°C and pH 3·5 by the β-glucosidase of *S. atra*.

It is suggested therefore that methyl acetate brings about a conformational change in the β-glucosidase molecule that leads to a variety of effects—changes in the affinity of enzyme for substrate, changes in the shape and relative height of pH–activity curve. Figure 1 shows that these changes cannot be neatly summed up as an "activation" or "inactivation". It is uncertain whether these changes are different in nature from effects of the "allosteric" type or whether they are in fact similar but devoid of the narrow specificity that has been taken as one of the criteria of the allosteric activation or inactivation.

(*b*) *Ethyl Lactate as Acceptor*

The overall characteristics of the enzymic reaction in the presence of 1M ethyl L-lactate, for comparison with those in the presence of 1M methyl acetate, are presented in Figures 4 and 5. When it is noted further that more than 98% of transfer is to ethyl lactate at this molarity of the acceptor over the entire pH range, it might be expected that Figures 4 and 5 merely repeat the comparable data for t-butyl alcohol with a much more efficient inactivating acceptor, i.e. the data given are very nearly the "true" data for the decomposition of the enzyme–glucoside–ethyl lactate complex relative to that of the enzyme–glucoside–water complex. That this is not the case follows from the data of Figure 6; the nature of these results led to the investigations with methyl acetate.

It would be unreasonable to attempt to draw a straight line through the points of Figure 6; the implication from them is clear. The addition of small amounts of ethyl lactate leads to a general fall in the rate of the total enzymic reaction without being accompanied by a corresponding transfer reaction to ethyl lactate. Similar effects involving activation rather than depression would give a convex rather than

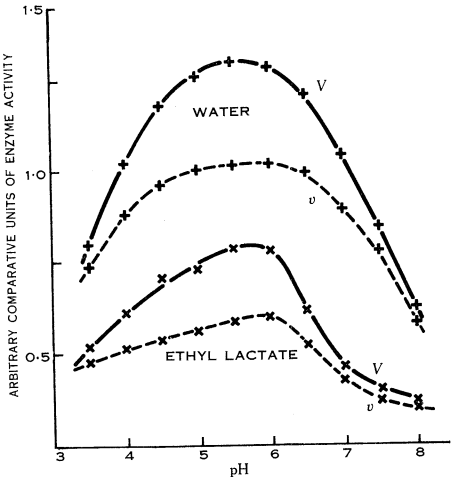

Fig. 4.—Effect of 1M ethyl L-lactate on the pH–activity curve for the decomposition of phenyl β-D-glucopyranoside in McIlvaine buffers at 28°C by the β-glucosidase of *S. atra*. *v*, Substrate concentration 2×10^{-3}M; *V*, infinite substrate concentration.

a concave curve. Since both the "activating" acceptors that have been studied in detail, hexane-1,6-diol and methanol, gave linear plots it may be presumed that there is no detectable degree of activation as a whole in these cases. However, even a perfunctory search demonstrated the existence of an "activating" acceptor that did show such an effect.

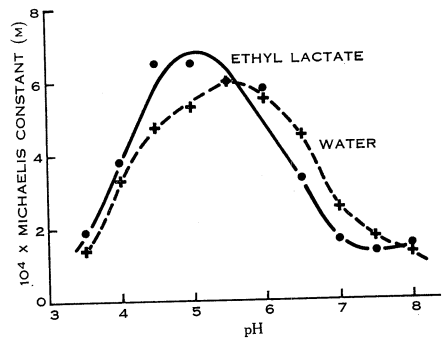

Fig. 5.—Effect of 1M ethyl L-lactate on the pH–Michaelis constant curve for the decomposition of phenyl β-D-glucopyranoside in McIlvaine buffers at 28°C by the β-glucosidase of *S. atra*. Water points, mean of three determinations; ethyl lactate points, single determination.

A potential candidate as an acceptor producing such effects was found in ethylene glycol monoacetate (EGMA). Besides being an ester, it was known to be an activating acceptor of medium efficiency. An experiment was run at pH 7·0, where methyl acetate was known to give the highest activating effects, using various concentrations of EGMA. Both the transfer ratio and *V* relative to the reaction in the presence of water only were determined for each concentration of EGMA.

The raw data are set out in Table 2, which is of the same form as the tabulated data for ethyl lactate on which Figure 6 is based. The partition of relative V between the reactions with water and EGMA, derived from these data, are set out in Figure 7. As predicted the curve is a convex one; the lower concentrations of

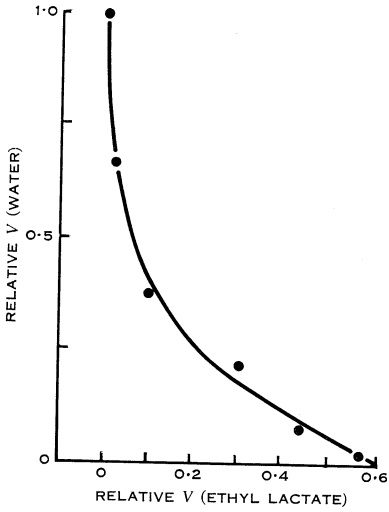

Fig. 6.—Partition of V between the reactions involving water and ethyl lactate for the decomposition of phenyl β-D-glucopyranoside in McIlvaine buffer, pH 5·0, and 28°C by the β-glucosidase of *S. atra*.

EGMA actually lead to an increased rate of reaction by the route involving water as acceptor. It can thus hardly be doubted that EGMA is a general activator of the enzyme. Since it is a less efficient acceptor than ethyl lactate, it is not even possible, as Figure 7 shows, to make a rational extrapolation to $V_W = 0$.

TABLE 2

DATA ON THE BREAKDOWN OF PHENYL β-D-GLUCOPYRANOSIDE AT pH 7·0 (McILVAINE BUFFER) AND 28°C BY *S. ATRA* β-GLUCOSIDASE IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF ETHYLENE GLYCOL MONOACETATE (EGMA)

EGMA Concn. (M)	Percentage Transfer to EGMA	Relative V	EGMA Concn. (M)	Percentage Transfer to EGMA	Relative V
0	0	1·00	0·10	43	1·66
0·01	16	1·26	0·30	58	2·00
0·03	25	1·47	1·00	73	1·16

Results similar to those set out in Figure 6 were obtained for ethyl lactate at pH 3·5, 6·5, and 8·0. The extrapolated value of V_{EL} ($\simeq 0{\cdot}6$) at pH 5·0 is thus an estimate of the relative rate of decomposition of the ethyl lactate complex of the ethyl lactate–inactivated enzyme as against that of the water complex in water alone. No true comparison on a common basis can be made. The degree to which

the sort of effects exhibited by methyl acetate and those exhibited by t-butyl alcohol have been combined in the data of Figures 4 and 5 cannot even be estimated. It is even possible that the "true" rate of decomposition of the ethyl lactate complex of the undepressed enzyme is higher than that of the water complex.

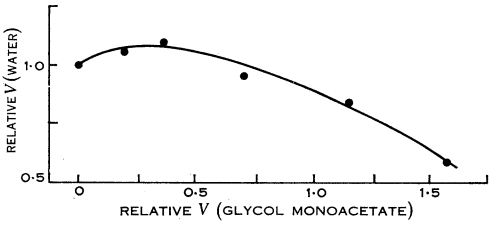

Fig. 7.—Partition of V between the reactions involving water and ethylene glycol monoacetate for the decomposition of phenyl β-D-glucopyranoside in McIlvaine buffer, pH 7·0, and 28°C by the β-glucosidase of *S. atra*.

Figure 6 and the related plots for the other pH values must be held to show that ethyl lactate is consistently a depressant between pH 3·5 and 8·0. The shape of the pH–activity plot in the presence of methyl acetate shows this substance to be depressing between pH 3·5 and 5·0 and activating between pH 5·0 and 8·0. The two esters thus both show a general interaction with the enzyme, but are not structurally near enough for these effects to be parallel.

IV. Acknowledgment

The experimental work in this paper was carried out by Miss Carol May.

V. References

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