Biochem. J. (1968) **106**, 135
Printed in Great Britain

Inhibition of Glycosidases by Aldonolactones of Corresponding Configuration

THE SPECIFICITY OF α-L-ARABINOSIDASE

BY J. CONCHIE, A. L. GELMAN AND G. A. LEVVY
Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, and North of Scotland College of Agriculture, Aberdeen AB9 1AS

(*Received* 30 *June* 1967)

1. The previous study (Conchie, Gelman & Levvy, 1967*b*) of the specificity of β-glucosidase, β-galactosidase and β-D-fucosidase in barley, limpet, almond emulsin and rat epididymis was extended to α-L-arabinosidase. 2. The inhibitory action of L-arabinono-(1→5)-lactone was tested against all four types of enzyme, and α-L-arabinosidase was examined for inhibition by glucono-, galactono- and D-fucono-lactone. 3. In emulsin, the enzyme that hydrolyses β-glucosides, β-galactosides and β-D-fucosides also hydrolyses α-L-arabinosides. Rat epididymis resembles emulsin except that, as already noted, it lacks β-glucosidase activity. 4. In the limpet, α-L-arabinosidase activity is associated with the enzyme that hydrolyses β-glucosides and β-D-fucosides, and not with the separate β-galactosidase. 5. The effects of the different lactones on the barley preparation suggest that α-L-arabinosidase activity is associated with the β-galactosidase rather than with the enzyme that hydrolyses β-glucosides and β-D-fucosides. Fractionation and heat-inactivation experiments indicate that there is also a separate α-L-arabinosidase in the preparation.

A recent investigation of β-glucosidase, β-D-fucosidase and β-galactosidase from four sources, namely limpet, barley, emulsin and rat epididymis, concerned the specificity of these three enzymes with regard to C-4 and C-6 of their substrates (Conchie, Gelman & Levvy, 1967*b*). There was specificity for C-4 in the limpet and barley preparations as shown by the non-identity of β-glucosidase and β-galactosidase. In emulsin, on the other hand, all three enzymes appeared to be identical, indicating a lack of specificity for both C-4 and C-6. The most surprising finding was that in the limpet and barley preparations β-glucosidase was also responsible for the hydrolysis of β-fucosides, indicating a coupled lack of specificity for C-4 and C-6. The rat preparation conformed to the emulsin pattern rather than the limpet pattern, inasmuch as that β-galactosidase and β-fucosidase activity appeared to reside in a single enzyme, but this preparation was nearly devoid of β-glucosidase activity.

β-Galactosidase from calf intestine and from *Escherichia coli* apparently acts, not only on β-fucosides, but also on α-L-arabinosides (Wallenfels & Malhotra, 1960), and with the latter source, at least, the preparation resembles rat epididymis in that it is free from β-glucosidase activity (Kuby & Lardy, 1953). It was therefore decided to investigate the relationship between α-L-arabinosidase on the one hand and β-glucosidase and β-galactosidase on the other in preparations from limpet, barley, emulsin and rat epididymis.

EXPERIMENTAL

Enzyme preparations

Limpet. A limpet (*Patella vulgata*) preparation (1·77ml./g. of tissue), made by $(NH_4)_2SO_4$ fractionation of an aqueous extract as described by Conchie & Levvy (1957), had β-glucosidase and β-galactosidase activities of 94200 and 112800 *o*-nitrophenol units/g. of tissue respectively, and β-fucosidase and α-L-arabinosidase activities of 82300 and 12400 *p*-nitrophenol units/g. of tissue respectively. The solution was diluted 1:250 for β-glucosidase and β-fucosidase assays, 1:175 for β-galactosidase assays and 1:50 for α-L-arabinosidase assays.

Barley. An enzyme preparation from barley of the Ymer variety (1ml./g. of dry barley) was made by $(NH_4)_2SO_4$ fractionation of an aqueous extract as described by Conchie *et al.* (1967*b*). The β-glucosidase and β-galactosidase activities of the preparation were 29800 and 7850 *o*-nitrophenol units/g. of dry barley respectively, and the β-fucosidase and α-L-arabinosidase activities were 13400 and 567 *p*-nitrophenol units/g. of dry barley respectively. The dilutions of the preparation for the various assays were as

follows: β-glucosidase and β-fucosidase, 1:150; β-galactosidase, 1:40; α-L-arabinosidase, 1:2·5.

Emulsin. An enzyme preparation from partially defatted sweet-almond meal (0·09g. in 30ml.) was prepared as described by Conchie *et al.* (1967*b*). The preparation had activities as follows: β-glucosidase and β-galactosidase 1455000 and 295000 *o*-nitrophenol units/g. of emulsin respectively; β-fucosidase and α-L-arabinosidase 1380000 and 66650 *p*-nitrophenol units/g. of emulsin respectively. The enzyme solution was diluted 1:20 for β-glucosidase and β-fucosidase assays, 1:5 for β-galactosidase assays and 1:2 for α-L-arabinosidase assays.

Rat epididymis. A rat epididymal preparation (2ml./g. of tissue), prepared as described by Levvy, Hay & Conchie (1964), had a β-galactosidase activity of 36000 *o*-nitrophenol units/g. of tissue, and β-fucosidase and α-L-arabinosidase activities of 3200 and 1416 *p*-nitrophenol units/g. of tissue respectively. The preparation was diluted 1:60 for β-galactosidase assays, 1:8 for β-fucosidase assays and 1:6 for α-L-arabinosidase assays.

Enzyme assays

β-Glucosidase, β-galactosidase and β-fucosidase. These were assayed as described by Conchie *et al.* (1967*b*).

α-L-Arabinosidase. The incubation mixture contained 1ml. of 0·2M-Na_2HPO_4–0·1M-citric acid buffer (McIlvaine, 1921), pH4·5, 2ml. of 4mM-*p*-nitrophenyl α-L-arabinoside and 0·5ml. of enzyme preparation, the volume being made up to 4ml. with water or inhibitor. After 1hr. the reaction was stopped with 0·4M-glycine–NaOH buffer, pH10·8, and the liberated nitrophenol measured as described for β-glucosidase assays by Conchie *et al.* (1967*b*).

Inhibitors

Solutions of D-galactono-(1→5)-lactone were prepared from D-galactono-(1→4)-lactone solutions as described by Levvy, McAllan & Hay (1962), except in studies of rat epididymal enzyme when the (1→5)-lactone was prepared by platinum-catalysed oxidation of D-galactose as described by Conchie, Hay, Strachan & Levvy (1967*a*).

D-Glucono-(1→5)-lactone was purified as described by Levvy *et al.* (1964).

Solutions of D-fucono-(1→5)-lactone and L-arabinono-(1→5)-lactone were obtained by platinum-catalysed oxidation of D-fucose and L-arabinose respectively, as described by Conchie *et al.* (1967*a*).

All inhibitor solutions were freshly prepared and used immediately, and concentrations are expressed in terms of starting material.

Substrate

p-Nitrophenyl α-L-arabinoside was prepared as described by Feier & Westphal (1956), except that in the preparation of tetra-*O*-acetylarabinose the chloroform solution was washed several times with an equal volume of ice-cold 0·1N-NaOH, followed by washing with ice–water and drying overnight with $CaCl_2$. Omission of this treatment gave a syrup that did not crystallize. Deacetylation of *p*-nitrophenyl tri-*O*-acetyl-α-L-arabinoside was carried out by the method of Leaback (1960).

RESULTS

Inhibition of α-L-arabinosidases by aldonolactones. Table 1 shows the concentrations of glucono-, galactono-, fucono- and L-arabinono-(1→5)-lactone required for 50% inhibition of α-L-arabinosidase activity in preparations from limpet, barley, emulsin and rat epididymis. Wherever the concentration of inhibitor permitted, inhibition could be made to approach 100%. Except with limpet arabinosidase, arabinono-(1→5)-lactone was a much less powerful inhibitor of the enzyme than were some of the other lactones tested, suggesting that in such cases there was lack of specificity with respect to the hydrolysis of arabinosides. This was further borne out by the action of arabinonolactone against the related enzymes in the preparations (Table 2).

In the limpet, β-glucosidase and β-fucosidase activities have been shown to be due to a single enzyme, whereas β-galactosidase is a separate enzyme (Conchie *et al.* 1967*b*). Arabinonolactone was a powerful inhibitor of limpet β-glucosidase and β-fucosidase activity, and a considerably less powerful inhibitor of β-galactosidase activity (Table 2), whereas limpet α-L-arabinosidase activity was strongly inhibited by glucono-, fucono- and arabinono-lactone, but not by galactonolactone (Table 1). These results suggest that, in the limpet, β-glucosidase, β-fucosidase and α-L-arabinosidase activities are due to a single enzyme, which is distinct from β-galactosidase.

In barley, the relationship between β-glucosidase, β-fucosidase and β-galactosidase activities has been shown to be similar to that in limpet (Conchie *et al.* 1967*b*). Arabinonolactone was not a particularly good inhibitor of arabinosidase or any of the other glycosidases (Tables 1 and 2). Both galactono- and fucono-lactone were good inhibitors of arabinosidase, but gluconolactone was a poor inhibitor. At first sight, therefore, arabinosidase activity appears to be to some extent independent of the glucosidase and associated with galactosidase activity.

The β-glucosidase, β-fucosidase and β-galactosidase activities in emulsin are all due to a single enzyme (Heyworth & Walker, 1962; Conchie *et al.* 1967*b*). The 50%-inhibition value for each of the four lactones with emulsin arabinosidase (Table 1) proved to be of the same order as the corresponding values with the other three glycosidases (Table 2 and Conchie *et al.* 1967*b*). It is thus very probable that all four glycosidase activities are due to a single enzyme, with the fucono-, glucono-, arabinono- and galactono-lactone solutions acting in decreasing order of inhibitory power.

Previous studies on rat epididymal preparations indicated that here β-fucosidase and β-galacto-

Table 1. *Concentrations of glucono-, galactono-, D-fucono- and L-arabinono-(1→5)-lactone causing 50% inhibition of the hydrolysis of 2mM-p-nitrophenyl α-L-arabinoside by various enzyme preparations*

For description of lactones, enzyme preparations and methods of assay, see the text. Curves were drawn for percentage inhibitions at various inhibitor concentrations and the 50% values found by interpolation.

	Concn. for 50% inhibition (mM)			
Source	Gluconolactone	Galactonolactone	Fuconolactone	Arabinonolactone
Limpet	0·064	49% inhibition with 5mM	0·018	0·022
Barley	30% inhibition with 5mM	0·036	0·056	1·0
Emulsin	0·11	31% inhibition with 5mM	0·043	4·1
Rat	No inhibition with 10mM	0·030	4·6	2·4

Table 2. *Concentrations of L-arabinono-(1→5)-lactone causing 50% inhibition of the hydrolysis of substrates by enzymes from various sources*

For description of lactone, enzyme preparations and methods of assay, see the text. Curves were drawn for percentage inhibitions at various inhibitor concentrations and the 50% values found by interpolation.

	Concn. of lactone for 50% inhibition (mM)			
Substrate	Limpet	Barley	Emulsin	Rat
5mM-*o*-Nitrophenyl β-glucoside	0·068	6·5	3·6	—
2·5mM-*p*-Nitrophenyl β-D-fucoside	0·032*	4·3	2·7	0·6*
2·5mM-*o*-Nitrophenyl β-galactoside	0·32*	2·2	1·7	8·3*

* Quoted from Conchie *et al.* (1967*a*).

sidase activities are due to a single enzyme, which probably has vestigial β-glucosidase activity as a side action (Conchie *et al.* 1967*b*). Results obtained for the four lactones tested against rat arabinosidase (Table 1), when compared with results for the other glycosidases (Table 2 and Conchie *et al.* 1967*b*), suggest that the fucosidase–galactosidase is also responsible for the hydrolysis of α-L-arabinosides: this enzyme shows greatest affinity for the galactose residue and practically none for the glucose.

Except in barley, α-L-arabinosidase activity appears to be associated with β-D-fucosidase activity in the above experiments.

Separation of limpet glycosidases. The β-fucosidase and β-galactosidase activities in a limpet preparation can be readily separated by heating at 27° and pH 8·5 for 2hr. to destroy the β-galactosidase activity (Levvy & McAllan, 1963*a*): in such a 'fucosidase-enriched' preparation the β-glucosidase activity is enhanced to a similar extent (Conchie *et al.* 1967*b*). When a limpet preparation was subjected to this treatment, the change in α-L-arabinosidase activity exactly paralleled the change in β-fucosidase and β-glucosidase, the activities of the respective enzymes being 57, 59 and 58% of the values for the untreated preparation, whereas the activity of β-galactosidase fell to 6% of the original. This parallel enrichment of α-L-arabinosidase, β-fucosidase and β-glucosidase by the removal of almost all the β-galactosidase activity supports the evidence of identity of the three former enzymes obtained by lactone inhibition experiments. When arabinono-(1→5)-lactone was tested against the α-L-arabinosidase in such an enriched preparation, the concentration required for 50% inhibition (0·016mM) was similar to that obtained with the original preparation.

Separation of barley enzymes. When a barley extract was fractionated with ammonium sulphate as shown in Table 3, the fraction precipitating between the 50 and 80% saturation limits showed the enrichment with respect to β-glucosidase activity already noted (Conchie *et al.* 1967*b*), with a concomitant, but much smaller, enrichment of α-L-arabinosidase. When the arabinosidase in such a fraction was tested with arabinonolactone, it was inhibited (50% inhibition at 1·4mM) to the same extent as the arabinosidase in the original preparation (Table 1). Since arabinosidase appeared to be distinct from β-glucosidase in the lactone inhibition experiments, the small degree of enrichment obtained in the 'glucosidase-rich' fraction may be fortuitous. The fractionation does suggest some lack of identity at least between arabinosidase and galactosidase.

When the conditions of Conchie *et al.* (1967*b*) for making a 'galactosidase-enriched' preparation (heating a 20–80%-saturated ammonium sulphate

Table 3. *Fractionation of a barley preparation with ammonium sulphate*

The enzyme in a barley extract obtained after homogenization, autolysis and centrifugation as described in the Experimental section was precipitated with $(NH_4)_2SO_4$ between the limits of saturation shown. Assays were done as described in the text and results are expressed as μg. of nitrophenol liberated/g. of dry barley in 1 hr.

Saturation with $(NH_4)_2SO_4$ (%)	β-Glucosidase	β-Galactosidase	α-Arabinosidase	$\frac{\beta\text{-Glucosidase}}{\beta\text{-Galactosidase}}$	$\frac{\alpha\text{-Arabinosidase}}{\beta\text{-Galactosidase}}$
0–25	1300	346	16	3·7	0·045
25–35	1240	650	19	2·0	0·029
35–50	17700	5800	290	3·0	0·05
50–80	13200	850	69	15·4	0·081
Whole extract	38400	8450	490	4·5	0·058

Table 4. *Changes in the glycosidase activities in a barley preparation after adjustment to* pH 3·75 *and incubation at* 37° *for various periods*

The enzyme preparation was a 20–80%-saturated $(NH_4)_2SO_4$ fraction made as described in the Experimental section. Assays were done as described in the text, the initial activities of the preparation, expressed as μg. of nitrophenol/g. of dry barley, being: β-glucosidase, 29900; β-fucosidase, 13350; β-galactosidase, 7840; α-arabinosidase 580. Results are expressed as percentages of the initial activity.

Period of incubation (min.)	Activity (%)			
	β-Glucosidase	β-Fucosidase	β-Galactosidase	α-Arabinosidase
Untreated preparation	(100)	(100)	(100)	(100)
Zero time (adjusted to pH 3·75)	90	92	86	137
30	9	15	52	77
60	1	8	37	58
90	0	5	27	35
120	0	4	20	19

fraction at 37° and pH 3·75) were applied, there was a clear differentiation between α-L-arabinosidase and the other three glycosidases examined (Table 4). On adjusting the preparation to pH 3·75, there was an immediate rise in the α-L-arabinosidase activity, and a fall in the activities of the other glycosidases. Results of the subsequent heat treatment suggested that α-L-arabinosidase was initially the most stable of the four enzymes. After 2 hr. at 37°, its percentage residual activity had fallen to the value for β-galactosidase. β-Glucosidase and β-fucosidase were almost completely destroyed before this stage had been reached.

The instantaneous increase in α-L-arabinosidase activity on adjusting the pH of the preparation was found to be associated with the formation of an insoluble deposit. When this deposit was removed by centrifugation, the activity of the α-L-arabinosidase in the supernatant fell to the value for the untreated preparation. The deposit had no β-galactosidase activity.

From these experiments it seemed that, in barley, α-L-arabinosidase activity was due to a separate enzyme. However, neither the ammonium sulphate fractionation nor the heat-inactivation results excluded the possibility of some additional hydrolysis of α-L-arabinosides by β-galactosidase. The distinction between α-L-arabinosidase and β-glucosidase–β-fucosidase would appear to be established.

Comparison of K_i values for fucono- and arabinono-lactone with limpet, barley, emulsin and rat preparations. Table 5 shows the mean values for K_m for the various nitrophenyl glycosides and the corresponding values for K_i for fucono-(1→5)- and arabinono-(1→5)-lactone with enzyme preparations from limpet, barley, emulsin and rat epididymis. With one exception the action of the lactones was purely competitive. With barley fucosidase, arabinonolactone gave a mixture of competitive and non-competitive inhibition when results were analysed by the method of Lineweaver & Burk (1934).

In the limpet, the close similarity in K_i values for fuconolactone when tested against glucoside, fucoside and arabinoside substrates, and the considerably different value obtained with galactoside, provided strong confirmation that β-glucosidase, β-fucosidase and α-L-arabinosidase activities were due to a single enzyme. This was supported by the results obtained with arabinonolactone: the higher instability of this lactone leads to rather greater

Table 5. *K_m values obtained for various substrates with enzymes from limpet, barley, emulsin and rat, and the corresponding K_i values for* D-*fucono- and* L-*arabinono-(1→5)-lactones*

Substrate concentrations were 0·5–10mM for the glucoside, fucoside and galactoside, and 0·125–4mM for the arabinoside. For description of enzyme preparations and methods of assay see the Experimental section. Values for K_m and K_i were calculated by the method of Lineweaver & Burk (1934).

Source	Substrate	K_m (mM)	K_i (mM)	
			Fuconolactone	Arabinonolactone
Limpet	*o*-Nitrophenyl β-glucoside	1·1	0·0039*	0·017
	p-Nitrophenyl β-D-fucoside	0·9	0·0027*	0·0094
	o-Nitrophenyl β-galactoside	3·0	0·34	0·2
	p-Nitrophenyl α-L-arabinoside	0·64	0·0035	0·0047
Barley	*o*-Nitrophenyl β-glucoside	2·6	0·024*	2·0
	p-Nitrophenyl β-D-fucoside	0·9	0·023*	Mixed inhibition
	o-Nitrophenyl β-galactoside	2·1	0·24	1·0
	p-Nitrophenyl α-L-arabinoside	1·3	0·06	0·31
Emulsin	*o*-Nitrophenyl β-glucoside	7·1	0·012*	1·5
	p-Nitrophenyl β-D-fucoside	1·8*	0·013*	—
	o-Nitrophenyl β-galactoside	23·0	0·013	1·7
	p-Nitrophenyl α-L-arabinoside	0·63	0·0069	1·0
Rat epididymis	*p*-Nitrophenyl β-D-fucoside	44	—	0·69
	o-Nitrophenyl β-galactoside	0·36	1·1	1·0
	p-Nitrophenyl α-L-arabinoside	3·1	1·2	1·3

* Quoted from Conchie *et al.* (1967*b*).

variation than usual between duplicate values. Gluconolactone when tested against arabinoside gave a K_i value (0·011mM) close to the value obtained when this lactone was tested against glucoside and fucoside substrates (see Conchie *et al.* 1967*b*).

Confirmation of the identity of all four glycosidases in emulsin is provided by the K_i values for fucono- and arabinono-lactone when tested against the various glycosides. Each lactone gave a similar K_i value, irrespective of the nature of the substrate. As with the limpet preparation, gluconolactone, when tested against emulsin arabinosidase gave a K_i value (0·029mM) close to that obtained for the lactone when tested against glucoside and fucoside substrates (see Conchie *et al.* 1967*b*).

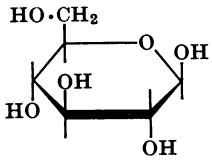
With the rat preparation, K_i values for fucono- and arabinono-lactone measured against the various substrates tended to confirm identity of the three enzymes present. Galactono-(1→5)-lactone, in this instance made from the (1→4)-lactone, gave a K_i value against the arabinoside substrate of 0·13mM, compared with values already obtained against fucoside and galactoside of 0·038mM and 0·064mM respectively (Levvy & McAllan, 1963*b*).

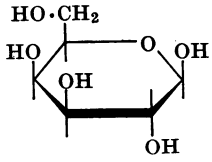
As noted in other experiments with the barley preparation, the relationship of arabinosidase to the other three enzymes did not appear to be clearly resolved. With fuconolactone the K_i value with arabinoside was intermediate between the value obtained with glucoside and fucoside on the one hand and galactoside on the other. Values for arabinonolactone did not clarify the situation since, with fucosidase, inhibition by the lactone was not purely competitive: the value with arabinosidase tended to suggest that this was, in part at least, a separate enzyme. Galactonolactone, on the other hand, gave K_i values of 0·048mM and 0·02mM when tested against galactoside and arabinoside respectively. This approximate similarity, considered together with the other results, is possibly indicative of partial identity of arabinosidase and galactosidase. The fact that fuconolactone causes small, but appreciable, inhibition (50% at 1·5mM) of barley galactosidase would then explain its rather high inhibitory power for arabinosidase (Table 5).

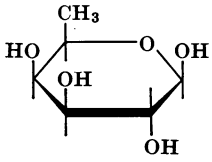
DISCUSSION

All the preparations used in this investigation have previously been shown to display a lack of specificity for the group on C-6 of the substrate, sometimes accompanied by a lack of specificity for C-4, as with emulsin. There was sometimes specificity for C-4, with a coupled lack of specificity for C-4 and C-6, as with limpet and barley (see the introduction). In all the preparations, with the exception of barley, lack of specificity for C-6 has now been shown to be associated with hydrolytic activity for α-L-arabinosides where C-5 has a hydrogen atom instead of a hydroxymethyl or methyl group. This phenomenon is rather a lack of specificity at C-5 than at C-6.

The lack of specificity for C-5 of the substrate in


β-D-Glucose


β-D-Galactose


β-D-Fucose

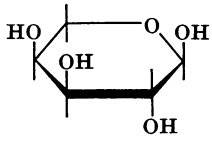

α-L-Arabinose

Table 6. *Suggested specificities of the glycosidases from different sources*

Source	Enzymes postulated	Substrates attacked
Emulsin	β-Glucosidase	β-Glucoside β-Galactoside β-D-Fucoside α-L-Arabinoside (β-Xyloside)
Limpet	β-Glucosidase	β-Glucoside β-D-Fucoside α-L-Arabinoside
	β-Galactosidase	β-Galactoside
Barley	β-Glucosidase	β-Glucoside β-D-Fucoside
	β-Galactosidase	β-Galactoside α-L-Arabinoside
	α-L-Arabinosidase	α-L-Arabinoside
Rat epididymis	β-Galactosidase	β-Galactoside β-D-Fucoside α-L-Arabinoside

emulsin has also been observed by Manners & Mitchell (1967), who found that β-glucosidase and β-xylosidase activities were due to a single enzyme in emulsin. Our emulsin preparation has no detectable β-mannosidase activity, indicating that the lack of specificity does not extend to C-2.

With limpet, the fact that the coupled lack of specificity for C-4 and C-6 extends to α-L-arabinosides is in agreement with the explanation already proffered for the identity of β-fucosidase and β-glucosidase (Conchie *et al.* 1967*b*), namely the elimination of steric hindrance in the combination of enzyme with substrate by the removal of an interfering group either on C-4 or on C-5 in the pyranose ring.

In barley, previously found to resemble limpet in possessing a β-glucosidase–β-fucosidase and a separate β-galactosidase, anomalous results were obtained for the hydrolysis of the arabinoside, and there appeared to be at least two enzymes with arabinosidase activity, the β-galactosidase and a third distinct enzyme. We cannot explain the specificity requirements of barley β-glucosidase–β-fucosidase; obviously the explanation mentioned above for limpet can no longer apply in this instance.

β-Galactosidase from other sources has been shown to act on α-L-arabinosides as well as β-fucosides (Wallenfels & Malhotra, 1960), and this pattern would appear to apply to our rat preparation. Rat epididymis differs from emulsin only in lacking β-glucosidase activity. It may be found that the lack of specificity of β-galactosidase in intestine is as extensive as in emulsin, so that a single enzyme hydrolyses β-glucosides, β-galactosides, β-fucosides and α-L-arabinosides (cf. Heyworth & Dahlqvist, 1962).

Our conclusions are summarized in Table 6, and the structural relationships between the different sugar residues are shown in the formulae. It is evident that testing the specificity of a glycosidase by varying the configuration at only one carbon atom at a time can no longer be accepted as conclusive for purposes of discrimination.

The authors acknowledge the skilled technical assistance of Mr I. Strachan. A.L.G. thanks Long John Distilleries Ltd. for providing chemicals and equipment.

REFERENCES

Conchie, J., Gelman, A. L. & Levvy, G. A. (1967*b*). *Biochem. J.* **103**, 609.
Conchie, J., Hay, A. J., Strachan, I. & Levvy, G. A. (1967*a*). *Biochem. J.* **102**, 929.
Conchie, J. & Levvy, G. A. (1957). *Biochem. J.* **65**, 389.
Feier, H. & Westphal, O. (1956). *Chem. Ber.* **89**, 589.
Heyworth, R. & Dahlqvist, A. (1962). *Biochim. biophys. Acta*, **64**, 182.
Heyworth, R. & Walker, P. G. (1962). *Biochem. J.* **83**, 331.
Kuby, S. A. & Lardy, H. A. (1953). *J. Amer. chem. Soc.* **75**, 890.
Leaback, D. H. (1960). *J. chem. Soc.* p. 3166.
Levvy, G. A., Hay, A. J. & Conchie, J. (1964). *Biochem. J.* **91**, 378.
Levvy, G. A. & McAllan, A. (1963*a*). *Biochem. J.* **87**, 206.
Levvy, G. A. & McAllan, A. (1963*b*). *Biochem. J.* **87**, 361.
Levvy, G. A., McAllan, A. & Hay, A. J. (1962). *Biochem. J.* **82**, 225.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
McIlvaine, T. C. (1921). *J. biol. Chem.* **49**, 183.
Manners, D. J. & Mitchell, J. P. (1967). *Biochem. J.* **103**, 43P.
Wallenfels, K. & Malhotra, O. P. (1960). In *The Enzymes*, 2nd ed., vol. 4, p. 409. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.