

## Inhibition of Glycosidases by Aldonolactones of Corresponding Configuration

### PREPARATION OF (1→5)-LACTONES BY CATALYTIC OXIDATION OF PYRANOSES AND STUDY OF THEIR INHIBITORY PROPERTIES

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1. A method was devised for the preparation of (1→5)-lactones from pyranose sugars and uronic acids by platinum-catalysed oxidation with gaseous oxygen in aqueous solution at acid pH. It was applied to mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine, glucuronic acid, galacturonic acid, galactose, L-arabinose and D-fucose. 2. Only the first three yielded products that could be obtained in the solid state without decomposition. In every case, however, the oxidation product in aqueous solution behaved as the aldono-(1→5)-lactone, and was more inhibitory towards the appropriate glycosidases than any aldono-lactone preparation hitherto examined. 3. The stabilities of the oxidation products were studied, and their interconversion with the (1→4)-lactones was demonstrated. Ring-opening does not appear to be mandatory for this isomeric change, which in some instances is very rapid. 4. To explain all the inhibitory effects observed with aldono-lactones on glycosidases of corresponding configuration, it is tentatively postulated that inhibition may be due entirely to the (1→5)-lactone, and that any inhibitory effect seen with the (1→4)-lactone is a measure of the extent and speed of its conversion into the (1→5)-lactone in aqueous solution.

The oxidation of carbohydrates by platinum catalysts in various forms has been intensively studied for a considerable time, notably by Heyns and his co-workers (for review see Heyns & Paulsen, 1962). Under neutral or mildly alkaline conditions, the primary alcohol group on C-6 of aldoses can readily be converted into a carboxyl group, provided that the even more easily-oxidized group on C-1 has been blocked. This method has proved particularly valuable in the syntheses of various glycosiduronic acids from the corresponding glycosides (Marsh, 1952; Tsou & Seligman, 1952, 1953; Marsh & Levvy, 1956, 1958; Bollenback, Long, Benjamin & Lindquist, 1955). Under controlled conditions in the presence of alkali, aldoses can readily be oxidized to aldonic acids (Heyns & Heinemann, 1947; Heyns & Stöckel, 1947). Further oxidation under these conditions leads to decarboxylation at C-1 and the formation of several degradation products.

The competitive inhibition of various glycosidases by aldono-lactones corresponding to the substrates in configuration has been described in previous papers (Levy, 1952; Conchie, 1954; Conchie & Levy, 1957; Findlay, Levy & Marsh, 1958; Levy, McAllan & Hay, 1962; Levy, Hay &

Conchie, 1964). During these investigations, evidence was obtained that the (1→4)-lactones of galactonic acid and 2-acetamido-2-deoxygalactonic (*N*-acetylgalactosaminic) acid could be transformed by heating in acid solution into even more inhibitory entities that were almost certainly the corresponding unknown (1→5)-lactones. This occurs to some extent in the enzyme assays, and may account entirely for the inhibitory effects observed with the (1→4)-lactones. Since the (1→5)-lactones were stable for at least short periods in acid solution, it appeared possible that they could be prepared by catalytic oxidation of aldoses at an acid pH. This was found to be the case, and pure crystalline mannono-(1→5)-lactone was obtained in this way (Levy *et al.* 1964).

The process has now been extended to other monosaccharides, namely glucuronic acid, galacturonic acid, *N*-acetylglucosamine, *N*-acetylgalactosamine, galactose, L-arabinose and D-fucose (6-deoxy-D-galactose), and in all cases the products obtained in solution were more inhibitory towards their respective glycosidases than were samples of the corresponding (1→4)-lactones. None of these (1→5)-lactones was known in the crystalline state, and it has not proved possible to isolate pure solids,

since evaporation of their solutions may lead to considerable conversion into the (1→4)-lactone, as well as ring opening. Nevertheless, studies of the oxidation products in solution indicate that they are in fact the (1→5)-lactones.

The present work describes the conditions for preparing solutions of the (1→5)-lactones, together with their inhibitory properties, stability and inter-conversion. A preliminary account of this work has already appeared (Conchie, Hay & Levvy, 1963).

## MATERIALS AND METHODS

### Enzyme preparations

**Rat preputial gland.** A female rat preputial-gland extract, prepared and completely purified as described by Levvy, McAllan & Marsh (1958), was used for all  $\beta$ -glucuronidase assays. The preparation was kept at 0° and was diluted 1:5000 with bovine serum albumin before use, so that the concentration of albumin in the final assay mixture was 0.01%. The specific activity of the preparation was 450 000 phenolphthalein units/mg. of protein.

**Boar epididymis.** A boar epididymis preparation (2 ml./g. of moist tissue), purified to stage 3 of the procedure of Findlay & Levvy (1960), was used for all *N*-acetyl- $\beta$ -glucosaminidase assays. The preparation had an activity of 2 000 000 *p*-nitrophenol units/g. of tissue. It was diluted 1:4000 with albumin solution in the same way as the rat preputial-gland preparation.

**Limpet.** A limpet preparation (2.5 ml./g. of tissue), made by  $(\text{NH}_4)_2\text{SO}_4$  fractionation of an aqueous extract (Conchie & Levvy, 1957), had a  $\beta$ -galactosidase activity of 44 000 *o*-nitrophenol units/g. of tissue and a  $\beta$ -D-fucosidase activity of 52 000 *p*-nitrophenol units/g. of tissue. The preparation, which was stored at -20°, was diluted 1:100 with water for both  $\beta$ -galactosidase and  $\beta$ -fucosidase assays. For the semi-quantitative experiments with  $\beta$ -amylase and carboxymethylcellulase the preparation was diluted 1:10 and 1:400 respectively.

**Rat epididymis.** A rat epididymis preparation (2 ml./g. of tissue) was fractionated with  $(\text{NH}_4)_2\text{SO}_4$  as described by Levvy *et al.* (1964), and stored at -20°. The  $\beta$ -galactosidase activity of the preparation was 28 600 *o*-nitrophenol units/g. of tissue, and it was diluted 1:50 before use. The  $\beta$ -D-fucosidase activity was 3700 *p*-nitrophenol units/g. of tissue and the preparation was diluted 1:8 before assay.

**Sweet potato.**  $\beta$ -Amylase from sweet potato [a crystalline suspension in  $(\text{NH}_4)_2\text{SO}_4$ ] was obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). The preparation was diluted 1:200 before use.

**Trichoderma koningii.** A cell-free filtrate from *T. koningii* was kindly supplied by Dr C. A. Marsh for use as a source of carboxymethylcellulase.

### Enzyme assays

**$\beta$ -Glucuronidase.** The hydrolysis of 0.63 mm-phenolphthalein  $\beta$ -glucuronide in 0.05 *N*-acetic acid-NaOH buffer, pH 4.5, at 38° was measured by the procedure of Levvy (1952).

***N*-Acetyl- $\beta$ -glucosaminidase.** The hydrolysis of 5 mm-*p*-nitrophenyl *N*-acetyl- $\beta$ -glucosaminide in 0.05 *M*-citric acid-

NaOH-NaCl buffer, pH 4.4, was measured as described by Findlay *et al.* (1958).

**$\beta$ -Galactosidase.** The hydrolysis of 2.5 mm-*o*-nitrophenyl  $\beta$ -galactoside in 0.125 *N*-acetic acid-NaOH buffer, pH 3.0, was measured as described by Conchie & Hay (1959).

**$\beta$ -D-Fucosidase.** The hydrolysis of 2.5 mm-*p*-nitrophenyl  $\beta$ -D-fucoside in 0.05 *M*- $\text{Na}_2\text{HPO}_4$ -0.025 *M*-citric acid buffer was measured by the method of Levvy & McAllan (1963*a,b*). The pH of assay was 3.2 for the rat epididymal enzyme, and 4.7 for the limpet enzyme.

**$\beta$ -Amylase.** The procedure adopted was a modification of that of Whelan (1964). The incubation mixture contained 'soluble starch' (0.6%, w/v; 5 ml.), 0.2 *N*-acetic acid-NaOH buffer, pH 4.8 (1 ml.), and  $\beta$ -amylase (0.5 ml.), the volume being made up to 8 ml. with water or inhibitor solution. After incubation for 30 min. at 38°, the reaction was stopped by heating the tubes in a boiling-water bath for 5 min. Samples were removed after cooling, and reducing sugars were estimated, after suitable dilution, as described below.

**Carboxymethylcellulase.** A modification of the procedure described by Halliwell (1961) was used. The incubation mixture contained 0.2 *N*-acetic acid-NaOH buffer, pH 5.5 (0.15 ml.), CM-cellulose (Cellofas B; Imperial Chemical Industries Ltd., Stevenston, Ayrshire) solution (1%, w/v; 0.3 ml.), enzyme (0.15 ml.) and water or inhibitor solution to bring the volume to 0.75 ml. After incubation for 1 hr. at 38°, the reaction was stopped by the addition of  $\text{Na}_2\text{CO}_3$  solution (0.13%, w/v; 0.25 ml.) and the cyanide-carbonate reagent (1 ml.) of Park & Johnson (1949). Water (2 ml.) was added and the remainder of the procedure for the estimation of reducing sugars was pursued.

### General methods

**Reducing-sugar estimation.** This was done by the method of Park & Johnson (1949), except that the  $\text{FeNH}_4(\text{SO}_4)_2$  solution was made up in 0.15 *N*- $\text{H}_2\text{SO}_4$  instead of 0.05 *N*- $\text{H}_2\text{SO}_4$ .

***N*-Acetylhexosamine estimation.** Determinations of *N*-acetylglucosamine and *N*-acetylgalactosamine were done by the method of Levvy & McAllan (1959).

**Uronic acid estimation.** This was done as described by Bitter & Muir (1962).

**'Most inhibitory solutions'.** Such solutions, displaying maximum conversion into the inhibitory entity, were prepared from potassium hydrogen saccharate and from mucic acid (galactaric acid) as described by Levvy (1952) for testing against  $\beta$ -glucuronidase and from galactono-(1→4)-lactone as described by Levvy *et al.* (1962) for testing against  $\beta$ -galactosidase.

**Titration.** All titrations of lactones and acid solutions were carried out using a Radiometer Titrigraph pH-stat (Radiometer, Copenhagen, Denmark).

**Infrared spectra.** Spectra of solid products (KBr disks) were obtained by using a Unicam SP.200 Infrared Spectrophotometer.

## EXPERIMENTAL AND RESULTS

### Preparation of lactones

**Oxidation procedure.** Platinum catalyst was prepared by hydrogenating Adams' platinum oxide catalyst (Johnson, Matthey and Co. Ltd., Hatton

Garden, London) in aqueous suspension in quantities of 1–2 g. The freshly reduced catalyst was stored under water at 4° and retained its efficiency for about 2 weeks. In the experiments described below, the moist weight of catalyst was obtained after spreading it on hardened filter paper to remove the excess of moisture.

An aqueous solution of the sugar (0.05–1.0 g. in 25–50 ml.), adjusted to the required pH with hydrochloric acid and containing a suspension of platinum catalyst prepared as described above, was placed in a narrow cylindrical glass vessel fitted with a sintered-glass base through which oxygen was passed into the solution. For small quantities of sugar (up to 150 mg.) a threefold weight of moist catalyst was used, but this ratio was often decreased without loss of efficiency when larger amounts of sugar were oxidized (illustrated below for mannonolactone). The catalyst was maintained in suspension by mechanical stirring, and the oxidation vessel was immersed in a water bath kept at 40°. At the end of the oxidation period, the solution was freed from catalyst by filtration, and either diluted for immediate inhibition tests, or evaporated *in vacuo* as described below for mannonolactone. Conditions giving solutions with maximum inhibitory properties against the appropriate enzyme were determined for each sugar, and are detailed in Table 1, along with some data for the products.

**Manno-(1→5)-lactone.** The properties of this crystalline lactone, obtained after catalytic oxidation of a solution of mannose, have already been described (Levy *et al.* 1964). An aqueous solution of mannose (1.0 g.) was oxidized in the presence of 330 mg. of moist platinum catalyst. The solution was evaporated to dryness *in vacuo* at 40°, ethanol being added to assist removal of water, and the resulting syrup was left under vacuum for 2 hr. The dry residue was dissolved in hot ethanol (95%, v/v; 30 ml.), treated with charcoal, and, after filtration, kept at 25° for 2–3 days, when large heavy crystals were deposited. These were filtered off (yield, 300 mg.) and recrystallized from 95% ethanol to give pure mannono-(1→5)-lactone.

**Glucarolactones.** A solution of glucuronic acid was adjusted to pH 4 with sodium hydroxide. After oxidation, it caused 50% inhibition of rat preputial-gland  $\beta$ -glucuronidase activity at a concentration of 1.3  $\mu$ M (inhibitory concentrations are expressed throughout in terms of starting material). Evaporation yielded a solid that caused 50% inhibition at 2.3  $\mu$ M. Its major absorption peak at 1770  $\text{cm}^{-1}$  was consistent with a (1→4)-lactone (Barker, Bourne, Pinkhard & Whiffen, 1958). The much smaller peak at 1720  $\text{cm}^{-1}$  might well be attributed to the carboxyl group and makes detection of the (1→5)-lactone impossible. The same spectrum was seen with the (1→4)-lactone.

Table 1. *Optimum conditions for oxidation of different sugars, and changes observed in the solutions on oxidation*

Infrared spectra were done on the products from solution evaporated to dryness *in vacuo* at 40° with the aid of ethanol; only peaks in the lactone region are recorded.

Sugar	pH	Galacturonic acid	Galacturonic acid	N-Acetylglucosamine	N-Acetylgalactosamine	Galactose	L-Arabinose	D-Fucose
Oxidation conditions	Duration (min.)							
Optical rotation ( $[\alpha]_D^{20}$ ; c 0.5–1.5)	Before oxidation							
	After oxidation							
Conversion of sugar (%)	Loss by specific method							
	Loss of reducing power							
	Lactone by titration							
Infrared absorption of solid product ( $\text{cm}^{-1}$ )	Major peak							
	Other peaks							
pH of ring-fission	Fresh oxidation solution							
	Corresponding (1→4)-lactone							

\* Uronic acid determination.

† Boiled mucic acid solution.

‡ N-Acetylhexosamine estimation.

Glucurone yielded less efficient inhibitor solutions than glucuronic acid on oxidation. This is comprehensible when it is realized that the primary oxidation product will be an unstable dilactone that yields glucaro-(1→4)-lactone, among other products, on decomposition.

**Galactarolactones.** Before oxidation, potassium hydroxide was used to adjust the pH of galacturonic acid solutions. (This resulted in the most favourable pH for evaporation of the solution after oxidation.) After oxidation, the solution caused 50% inhibition of rat preputial-gland  $\beta$ -glucuronidase at  $1.6\mu\text{M}$ , whereas the evaporated product caused 50% inhibition at  $3.4\mu\text{M}$ . The same difficulties attend the interpretation of the infrared spectrum as with glucarolactone. Although a peak at  $1735\text{--}1740\text{cm}^{-1}$  is rather high for carboxyl, it is close to our value for galacturonic acid.

When a 'most inhibitory solution' was prepared by way of the dibasic salt from an oxidation solution, its inhibitory power was identical with that of a boiled mucic acid solution of similar concentration, at 100-fold less than the original oxidation solution.

Attempts were made to fractionate the residues obtained after evaporation of the glucuronic acid and galacturonic acid oxidation solutions. Extraction with various solvents such as ethanol, acetone and dioxan was tried, but in all cases both extract and residue showed considerable loss in inhibitory power, and on examination of the infrared spectra there was no apparent partition of (1→5)-lactone.

**2-Acetamido-2-deoxyglucosaminolactones.** After oxidation, a solution of *N*-acetylglucosamine caused 50% inhibition of boar epididymal *N*-acetyl- $\beta$ -glucosaminidase activity at a concentration of  $1.2\mu\text{M}$ . This solution could be evaporated *in vacuo* to give a product that caused 50% inhibition at  $1.6\mu\text{M}$ . The infrared spectrum of this product was consistent with the presence of a (1→5)-lactone only. Titration of the evaporated material indicated that it was 60% lactone and contained 20% free carboxyl.

For comparative purposes, use was made of the compound obtained when the evaporated product from a catalytic oxidation solution was heated *in vacuo* at  $100^\circ$  for 8 hr. This product, the infrared spectrum of which suggested that it was the (1→4)-lactone (peak at  $1770\text{cm}^{-1}$ ), caused 50% inhibition of boar epididymal *N*-acetyl- $\beta$ -glucosaminidase activity at  $8\mu\text{M}$  concentration: titration of a solution indicated about 55% purity. In the studies on the changes in inhibitory power of the lactone solutions under various conditions of temperature and pH, the above compound is referred to as *N*-acetylglucosaminono-(1→4)-lactone.

Oxidation of *N*-acetylglucosamine beyond the period specified caused an increase in the disappear-

Table 2. *Changes in inhibitory power, reducing power and N-acetylhexosamine content during a prolonged oxidation of a solution of N-acetylglucosamine*

The solutions were tested against boar epididymal  $\beta$ -*N*-acetylglucosaminidase. Reducing sugar was estimated by the method of Park & Johnson (1949) and acetylhexosamine by the method of Levvy & McAllan (1959).

Period of oxidation (min.)	Concn. for 50% inhibition ( $\mu\text{M}$ )	Reducing sugar remaining (%)	Acetylhexosamine remaining (%)
40	0.92	31	30
60	0.94	22	18
120	1.05	16	8
210	1.32	14	3

ance of *N*-acetylglucosamine, but also a fall in the inhibitory power of the solution. Losses of reducing sugar in the oxidation solution were not as great as losses in acetylhexosamine (Table 2). These results suggest that prolonged oxidation results in degradative changes, and that these changes rather than opening of the lactone ring are the explanation of falling inhibitory power, though the latter possibility cannot be excluded.

**2-Acetamido-2-deoxygalactosaminolactones.** A solution of *N*-acetylglucosamine (Sigma Chemical Co.) caused 50% inhibition of boar epididymal *N*-acetyl- $\beta$ -glucosaminidase activity at  $0.19\mu\text{M}$  concentration after oxidation. The product from the evaporated solution caused 50% inhibition at  $0.26\mu\text{M}$ , and the infrared spectrum was consistent with the presence of a (1→5)-lactone.

Attempts to purify the solid products obtained after oxidation of *N*-acetylglucosamine or *N*-acetylglucosamine were unsuccessful. Solids obtained by ethanol or acetone extraction showed little or no loss in inhibitory power, nor did they show any increase compared with the original solid.

The (1→5)-lactone present in the product obtained on evaporation of the oxidation solution of *N*-acetylglucosamine could be converted into the (1→4)-lactone. A solution of *N*-acetylglucosaminono-(1→5)-lactone was adjusted with sodium hydroxide to pH 9.5 to achieve ring-fission of the lactone, then adjusted to pH 2.5 with hydrochloric acid, evaporated to dryness *in vacuo* and heated at  $100^\circ$  *in vacuo* for 45 min. The residue was extracted with hot ethanol and the extract evaporated to dryness. This fraction had an infrared spectrum with a peak at  $1785\text{cm}^{-1}$ , consistent with the presence of a (1→4)-lactone. The product caused 50% inhibition of boar epididymal *N*-acetyl- $\beta$ -glucosaminidase activity at  $12\mu\text{M}$  concentration: authentic *N*-acetylglucosaminolactone (Findlay *et al.* 1958) causes 50% inhibition at  $8\mu\text{M}$ .

**Galactonolactones.** The solution from oxidation of galactose caused 50% inhibition of rat epididymal  $\beta$ -galactosidase activity at 58  $\mu$ M concentration, and of limpet  $\beta$ -galactosidase activity at 6.4  $\mu$ M. Prolonging the oxidation led to a fall in inhibitory power. The solution could not be evaporated to dryness without a considerable loss in inhibitory power (50% inhibition at 180  $\mu$ M and 19  $\mu$ M respectively for the two enzyme preparations), and titration of the evaporated product indicated that it contained only about 25% lactone. The major peak in the infrared could have been due to (1→5)-lactone or to carboxyl groups, and the minor one was probably due to (1→4)-lactone.

When the product from an evaporated galactose oxidation solution was heated *in vacuo* at 80–90° for 5–6 hr. and extracted with hot ethanol, the extracted product, which was very hygroscopic, had an infrared spectrum with a major absorption peak at 1760–1770  $\text{cm}^{-1}$ , consistent with the presence of a (1→4)-lactone, and a small peak at 1735  $\text{cm}^{-1}$ , indicating traces of (1→5)-lactone. The product caused 50% inhibition of limpet  $\beta$ -galactosidase at 1 mM concentration: pure galactono-(1→4)-lactone caused 50% inhibition at 1.3 mM.

Unexpected powerful inhibition of  $\beta$ -glucuronidase activity by the galactose oxidation solution was encountered (50% at 15  $\mu$ M). That this was not due to galactonolactone was shown by the isolation of mucic acid on concentrating the solution to small volume. The solid product that separated was boiled for 60 min. at pH 2 and tested against  $\beta$ -glucuronidase, when it gave an inhibitory value (50% inhibition at 0.24 mM) similar to that of an authentic specimen of mucic acid (50% inhibition at 0.18 mM). Pure mucic acid showed a peak in the infrared at 1720  $\text{cm}^{-1}$ .

A galactose oxidation solution treated for the production of the respective 'most inhibitory solutions' caused 50% inhibition of limpet  $\beta$ -galactosidase at 0.16 mM and of rat  $\beta$ -glucuronidase at 1 mM. This corresponded to about 30% transformation of galactose into galactonolactone and about 20% transformation into galactarolactone.

**L-Arabinolactones.** A solution of L-arabinose after oxidation caused 50% inhibition of limpet  $\beta$ -galactosidase activity at 320  $\mu$ M concentration and of rat epididymal  $\beta$ -galactosidase activity at 8.3 mM. As in the case of galactonolactone, there was a considerable loss in potency on evaporation to dryness (50% inhibition of limpet  $\beta$ -galactosidase at 1.8 mM). The infrared spectrum suggested that the solid product was a mixture of (1→5)- and (1→4)-lactones. Titration of the product indicated 80% lactone. It appears probable that in this case the loss in inhibitory power on evaporation was due more to (1→4)-lactone formation than to ring-opening.

By evaporating the oxidation solution and heating the residue *in vacuo* at 100° for 4 hr., a product was obtained which had an infrared spectrum with an absorption peak at 1775  $\text{cm}^{-1}$ , consistent with the presence of a (1→4)-lactone. The titration value corresponded to 82% lactone. This caused 50% inhibition of limpet  $\beta$ -galactosidase activity at 6.7 mM concentration.

**D-Fuconolactones.** A solution of D-fucose after oxidation caused 50% inhibition of limpet  $\beta$ -fucosidase activity at 8.4  $\mu$ M concentration. In this case also there was considerable loss in inhibitory power on evaporation of the oxidation solution (50% inhibition at 40  $\mu$ M). The infrared spectrum was similar to that of the galactose oxidation product. By titration the solid product contained about 50% lactone. As in the galactose oxidation, the fall in inhibitory power on evaporation appears to be mainly due to ring-opening.

**Oxidation of cellobiose.** Since the solution after oxidation of cellobiose was a very poor inhibitor of the enzymes tested, optimum conditions for oxidation could not be worked out. A solution of cellobiose was adjusted to pH 4 and oxidized for periods of 30, 60 and 90 min. before being tested against carboxymethylcellulase from *T. koningii* and from the limpet. In every case there was negligible inhibition of enzyme activity at 0.1 mM inhibitor concentration. Measurements of reducing sugar after 30, 60 and 90 min. oxidation gave values corresponding to 83, 80 and 77% disappearance of cellobiose.

**Oxidation of maltose.** As with cellobiose, optimum conditions for oxidation could not be worked out, since the product did not inhibit  $\beta$ -amylase activity from sweet potato or from limpet at a concentration of 2 mM. A solution of maltose, adjusted to pH 4, was oxidized for 30 min. before being tested against the two enzymes mentioned above. Measurements of reducing sugar indicated that 78% of the sugar had been oxidized.

#### *Properties of the inhibitors*

Table 3 summarizes the percentage oxidation of the various sugars under optimum conditions, and gives the concentrations of oxidation solution required for 50% inhibition of the appropriate enzyme activity. Such figures provide a direct comparison of relative inhibitory efficiencies. All concentrations of inhibitors are based on the initial sugar concentrations and do not take into account the percentage oxidation. Galactono- and fuconolactones, in the form of their 'most inhibitory solutions', have already been shown to display cross-inhibitory effects against  $\beta$ -galactosidase and  $\beta$ -fucosidase from various sources (Levy & McAllan, 1963a,b), whether one is dealing with one

Table 3. Concentrations of sugar oxidation solutions required for 50% inhibition of the hydrolysis of substrates by enzymes from various sources

For description of oxidation and of methods of assay see the text. Concentrations of inhibitor are based on starting material, and percentage oxidations are derived from determinations of reducing sugar. Curves were drawn for percentage inhibition at varying inhibitor concentration and the 50% values found by interpolation.

Sugar oxidized	Oxidation (%)	Substrate	Enzyme	Concn. for 50% inhibition ( $\mu\text{M}$ )
Glucuronic acid	87	0.63 mM-Phenolphthalein $\beta$ -glucuronide	Rat preputial gland	1.3
Galacturonic acid	97	0.63 mM-Phenolphthalein $\beta$ -glucuronide	Rat preputial gland	1.6
Mannose	72	6 mM- <i>p</i> -Nitrophenyl $\alpha$ -mannoside	Rat epididymis	80
<i>N</i> -Acetylglucosamine	79	5 mM- <i>p</i> -Nitrophenyl <i>N</i> -acetyl- $\beta$ -glucosaminide	Boar epididymis	1.2
<i>N</i> -Acetyl-galactosamine	95	5 mM- <i>p</i> -Nitrophenyl <i>N</i> -acetyl- $\beta$ -glucosaminide	Boar epididymis	0.19
Galactose	50	2.5 mM- <i>o</i> -Nitrophenyl $\beta$ -galactoside	Rat epididymis	58
		2.5 mM- <i>o</i> -Nitrophenyl $\beta$ -galactoside	Limpet	6.4
		2.5 mM- <i>p</i> -Nitrophenyl $\beta$ -D-fucoside	Rat epididymis	11.5
		2.5 mM- <i>p</i> -Nitrophenyl $\beta$ -D-fucoside	Limpet	1600
L-Arabinose	82	2.5 mM- <i>o</i> -Nitrophenyl $\beta$ -galactoside	Rat epididymis	8300
		2.5 mM- <i>o</i> -Nitrophenyl $\beta$ -galactoside	Limpet	320
		2.5 mM- <i>p</i> -Nitrophenyl $\beta$ -D-fucoside	Rat epididymis	600
		2.5 mM- <i>p</i> -Nitrophenyl $\beta$ -D-fucoside	Limpet	32
D-Fucose	83	2.5 mM- <i>o</i> -Nitrophenyl $\beta$ -galactoside	Rat epididymis	9600
		2.5 mM- <i>o</i> -Nitrophenyl $\beta$ -galactoside	Limpet	600
		2.5 mM- <i>p</i> -Nitrophenyl $\beta$ -D-fucoside	Rat epididymis	4700
		2.5 mM- <i>p</i> -Nitrophenyl $\beta$ -D-fucoside	Limpet	8.4

enzyme or two. Table 3 shows some of the cross-effects observed with galactono-, L-arabinono- and fucono-lactone solutions, obtained by catalytic oxidation, against rat and limpet  $\beta$ -galactosidases and  $\beta$ -fucosidases.

**$\beta$ -Glucuronidase.** The solutions obtained by catalytic oxidation of glucuronic acid and galacturonic acid were in both cases even more powerful inhibitors of  $\beta$ -glucuronidase than glucaro-(1 $\rightarrow$ 4)-lactone, the most powerful  $\beta$ -glucuronidase inhibitor known (Levy, 1952). When tested in fresh solution against rat preputial-gland  $\beta$ -glucuronidase, the concentration of glucaro-(1 $\rightarrow$ 4)-, glucaro-(1 $\rightarrow$ 5)- and galactaro-(1 $\rightarrow$ 5)-lactones required for 50% inhibition were 4.5, 1.3 and 1.6  $\mu\text{M}$  respectively. 'Most inhibitory solutions' derived from potassium hydrogen saccharate and from mucic acid caused 50% inhibition at 9.7 and 180  $\mu\text{M}$  concentration respectively.

A study was made of the changes in the inhibitory powers of the various entities with temperature and pH, and the most important phenomena are shown in Figs. 1-3. With the glucarolactones (Fig. 1), the most stable pH range for all solutions (i.e. both lactones) was 4-5, but, whereas there was an overall fall in the inhibitory power of the (1 $\rightarrow$ 5)-lactone solution compared with that of an untreated control, both the (1 $\rightarrow$ 4)-lactone solution and the 'most inhibitory solution' showed a rise over their

controls in this pH range. This rise was also seen in other experiments with a glucaro-(1 $\rightarrow$ 4)-lactone solution momentarily exposed to buffers of various pH values, but in this case the greatest increase was observed at pH 7. A glucaro-(1 $\rightarrow$ 5)-lactone solution similarly exposed showed a considerable loss of inhibitory power at all pH values above 5. The two lactones thus showed interconversion at neutral pH.

The course of these changes with time is shown in Fig. 2. At pH 5 and 38°, a glucaro-(1 $\rightarrow$ 4)-lactone solution reaches its optimum inhibitory power in 30 min. and thereafter inhibition remains constant for at least 4 hr. At pH 7, on the other hand, the instantaneous rise in inhibitory power compared with that of an untreated control (43%) is followed by a slow decline at 38°. At both pH values and 38°, a glucaro-(1 $\rightarrow$ 5)-lactone solution, though initially more powerful an inhibitor than the (1 $\rightarrow$ 4)-lactone, steadily loses inhibitory power over the first hour, after which the inhibition remains constant for up to 4 hr. At pH 5 and 100°, the initial rise in the inhibitory power of the (1 $\rightarrow$ 4)-lactone solution reaches its maximum within 5 min., after which there is a rapid fall similar to that instantaneously begun by the (1 $\rightarrow$ 5)-lactone.

In the galactarolactone series, the oxidation product is again extremely sensitive to changes in pH (Fig. 3). Only at pH 4 and 38° is most of the original inhibitory power retained. At 100°, there is

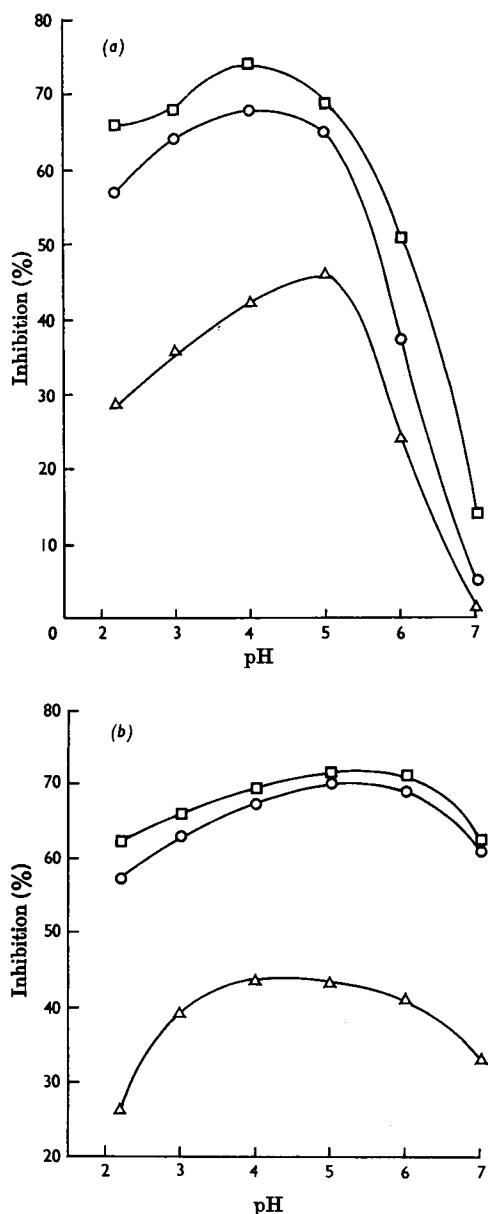


Fig. 1. Effect of varying the pH on the inhibitory power in buffered solution of  $4.9\mu\text{M}$ -glucaro-(1→4)-lactone (○),  $3.4\mu\text{M}$ -glucuronic acid oxidation solution (△) and  $18\mu\text{M}$ -potassium hydrogen saccharate as a 'most inhibitory solution' (□): (a) after heating for 5 min. at  $100^\circ$ , and (b) after heating for 1 hr. at  $38^\circ$ . All solutions were tested against rat preputial-gland  $\beta$ -glucuronidase, and the unbuffered unheated solutions caused 60, 66 and 66% inhibition respectively. Buffers were  $0.2\text{M}$ - $\text{Na}_2\text{HPO}_4$ - $0.1\text{M}$ -citric acid (McIlvaine, 1921), added to equal volumes of inhibitor solution. In the control  $35\mu\text{g}$ . of phenolphthalein was liberated.

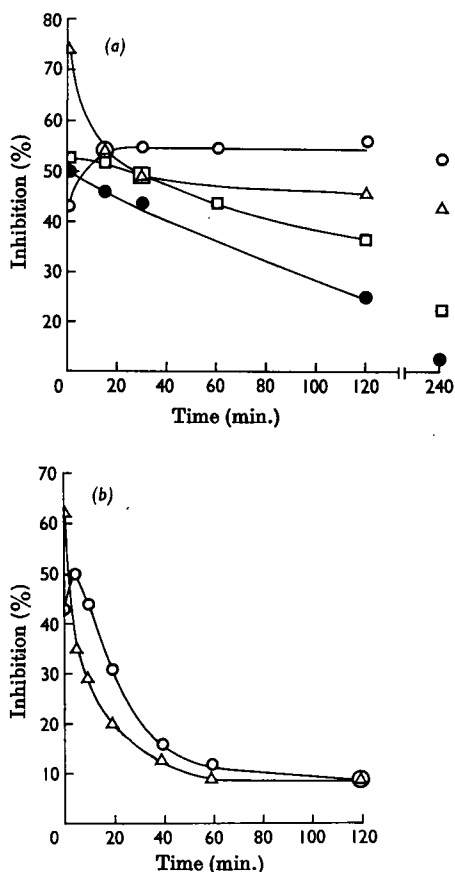


Fig. 2. Effect of time on the inhibitory power (a) of glucaro-(1→4)-lactone maintained in buffered solution at pH 5 and  $38^\circ$  (○), and at pH 7 and  $38^\circ$  (□); and of glucuronic acid oxidation solution maintained in buffered solution at pH 5 and  $38^\circ$  (△), and at pH 7 and  $38^\circ$  (●): (b) of glucaro-(1→4)-lactone (○) and of glucuronic acid oxidation solution (△) maintained in buffered solution at pH 5 and  $100^\circ$ . After exposure to the buffer for the required time, the inhibitor solution was rapidly diluted and tested. Both types of solution were tested against rat preputial-gland  $\beta$ -glucuronidase, at  $2.7\mu\text{M}$  concentration. The buffer was  $0.2\text{M}$ - $\text{Na}_2\text{HPO}_4$ - $0.1\text{M}$ -citric acid (McIlvaine, 1921). In the enzyme control  $40\mu\text{g}$ . of phenolphthalein was liberated.

a great loss in inhibitory power at all pH values. In contrast with this the 'most inhibitory solution' derived from mucic acid, though 100-fold less effective than the oxidation solution as an inhibitor, was stable over a wide range of pH values at  $38^\circ$ .

Fig. 4 shows the stability of various  $\beta$ -glucuronidase-inhibitor solutions during an enzyme assay at pH 4.5 and  $38^\circ$ . Over a period of 2 hr., the inhibitory power of the solution obtained by oxidation of glucuronic acid shows a decline, that of the glucaric

acid 'most inhibitory solution' maintains an unchanged level, and inhibition by the solution of glucaro-(1→4)-lactone rises considerably. In the last case, a more inhibitory entity is being formed during the assay. Over the same period, a solution of the galacturonic acid oxidation product showed a slight fall in inhibitory power, whereas a 'most inhibitory solution' derived from mucic acid showed no change.

*N-Acetyl-β-glucosaminidase.* In their original studies on inhibitors of *N*-acetyl-β-glucosaminidase, Findlay *et al.* (1958) found that the lactone of *N*-acetylglucosaminic acid (2-acetamido-2-deoxygluconolactone), obtained by oxidation of glucosamine with mercuric oxide followed by acetylation, was a very powerful inhibitor. This product has been further examined during the present investigations. It caused 50% inhibition of boar epididymal *N*-acetyl-β-glucosaminidase activity at 2.3 μM concentration. From its infrared spectrum it appears to have only the (1→5)-lactone present. Another specimen, obtained by essentially the same method, has been subjected to intensive purification by Mr N. M. Cross (personal communication) (Eli Lilly Research Laboratories Ltd., Wirral, Cheshire)

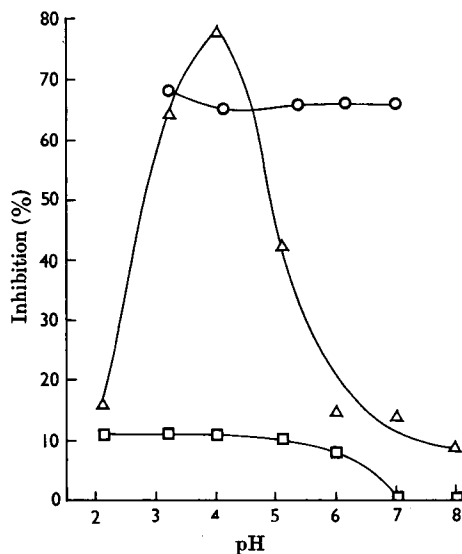


Fig. 3. Effect of varying the pH on the inhibitory power in buffered solution of 0.5 mM-mucic acid as a 'most inhibitory solution' after heating for 1 hr. at 38° (○), and of 6.7 μM-galacturonic acid oxidation solution after heating for 1 hr. at 38° (Δ) and 5 min. at 100° (□). All solutions were tested against rat preputial-gland β-glucuronidase, and the unbuffered unheated solutions caused 66 and 87% inhibition respectively. Buffers were 0.2 M-Na<sub>2</sub>HPO<sub>4</sub>-0.1 M-citric acid (McIlvaine, 1921). In the control 35 μg. of phenolphthalein was liberated.

and caused 50% inhibition of boar epididymal *N*-acetyl-β-glucosaminidase activity at 0.6 μM concentration: examination of its infrared spectrum

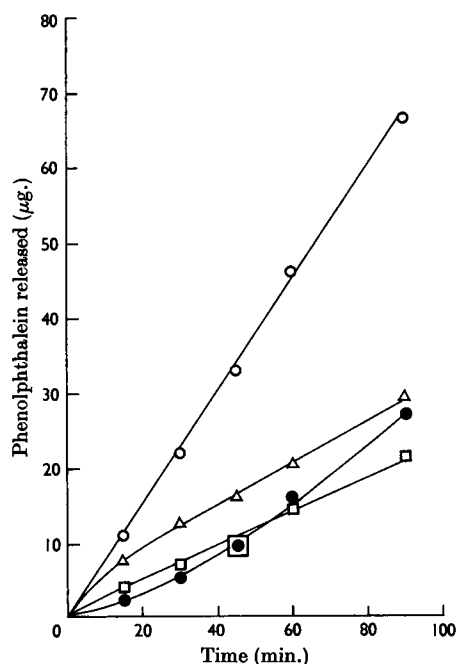


Fig. 4. Hydrolysis of 0.63 mM-phenolphthalein β-glucuronide by rat preputial-gland β-glucuronidase alone (○) and in the presence of: 5.95 μM-glucaro-(1→4)-lactone (Δ), 18.2 μM-potassium hydrogen saccharate as a 'most inhibitory solution' (□), and 2.7 μM-glucuronic acid oxidation solution (●).

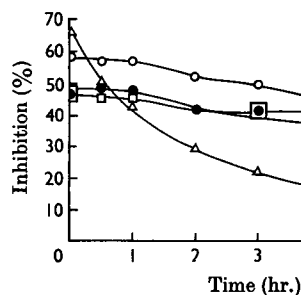


Fig. 5. Effect of time on the inhibition of boar epididymal acetyl-β-N-glucosaminidase by 2 μM-N-acetylglucosamine oxidation solution maintained at room temperature and pH 4 (○), and pH 6 (Δ); and by 6 μM-N-acetylglucosaminono-(1→4)-lactone (see the Experimental and Results section) maintained at room temperature and pH 4 (□), and pH 6 (●). Buffers were 0.2 M-Na<sub>2</sub>HPO<sub>4</sub>-0.1 M-citric acid (McIlvaine, 1921). In the enzyme control 145 μg. of *p*-nitrophenol was liberated.

suggests that it also is the (1→5)-lactone. The aqueous product obtained by catalytic oxidation of *N*-acetylglucosamine had an inhibitory power intermediate in value to the above specimens. It is compared with the (1→4)-lactone in Fig. 5.

On standing at room temperature and pH 4, the solution of the impure (1→4)-lactone prepared by heat treatment retained its inhibitory activity for several hours, whereas the (1→5)-lactone solution prepared by catalytic oxidation lost a little. Increasing the pH to 6 caused no change in the behaviour of the (1→4)-lactone, but greatly exaggerated the fall in inhibitory power of the (1→5)-lactone. When studied under the conditions of assay in the same way as in Fig. 4, the (1→5)-lactone of *N*-acetylglucosaminic acid showed a slight loss of inhibitory power, whereas inhibition by the (1→4)-lactone remained unchanged.

With the *N*-acetylgalactosaminolactones (2-acetamido-2-deoxygalactonolactones), the inhibitory action of pure *N*-acetylgalactosaminono-(1→4)-lactone against rat epididymal and limpet *N*-acetyl- $\beta$ -glucosaminidase had already been studied (Findlay *et al.* 1958), and evidence had been obtained that on preincubation at 38° or maintenance at 0° for about 24 hr. a new and more inhibitory compound was formed at pH 5–6. It was therefore not surprising that the product obtained by catalytic oxidation of a solution of *N*-acetylgalactosamine was about 40 times as powerful an inhibitor of boar epididymal *N*-acetyl- $\beta$ -glucosaminidase as *N*-acetylgalactosaminono-(1→4)-lactone.

The marked difference in the stability of the two lactone solutions under various conditions of temperature and pH is shown in Fig. 6. As with glucaro-(1→4)-lactone, there was an increase in inhibitory power when the (1→4)-lactone was momentarily subjected to a buffer of pH 7. At higher temperatures this change occurred at lower pH. Above pH 4–5 the (1→5)-lactone solution showed a dramatic loss of inhibitory power at all temperatures. The two lactones displayed greatest stability below pH 4 and interconversion as the solutions became more alkaline.

The behaviour of the two lactone solutions during incubation with enzyme and substrate under the conditions of assay is illustrated in Fig. 7. Over a period of 2 hr. there was a slight loss in the inhibitory power of the (1→5)-lactone and a considerable increase in the inhibitory power of the (1→4)-lactone. The formation of lactone from *N*-acetylgalactosaminine, which initially has no inhibitory power, is also illustrated.

**$\beta$ -Galactosidase.** A comprehensive study of inhibitors of  $\beta$ -galactosidase from various sources (Levy *et al.* 1962; Levy & McAllan, 1963b) led to the conclusion that galactono-(1→4)- and fucono-(1→4)-lactone could, under certain condi-

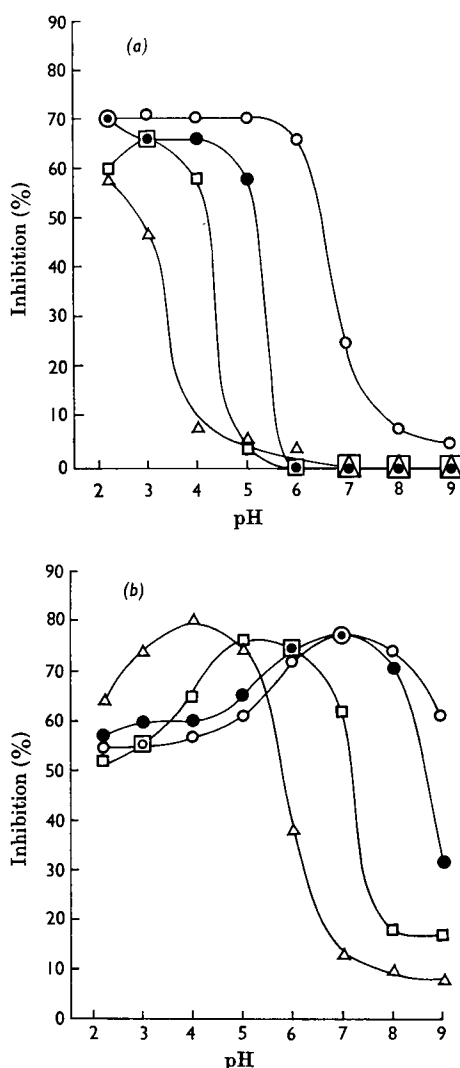


Fig. 6. Effect of varying the pH on the inhibitory power (a) of 0.44 μM *N*-acetylgalactosamine oxidation product; and (b) of 9 μM *N*-acetylgalactosaminono-(1→4)-lactone, tested at once (○), after 5 min. at 100° (Δ), after 1 hr. at 38° (□), and after 2 hr. at 0° (●). All the solutions were tested against boar epididymal  $\beta$ -*N*-acetylglucosaminidase. Buffers were 0.2M-Na<sub>2</sub>HPO<sub>4</sub>-0.1M-citric acid (McIlvaine, 1921). In the enzyme control 155 μg. of *p*-nitrophenol was liberated.

tions, give rise to more inhibitory entities that were almost certainly the corresponding (1→5)-lactones. The 'most inhibitory solution' from galactono-(1→4)-lactone caused 50% inhibition of rat epididymal  $\beta$ -galactosidase activity at 0.6mM concentration. A similarly treated solution of fucono-(1→4)-lactone caused no inhibition of rat

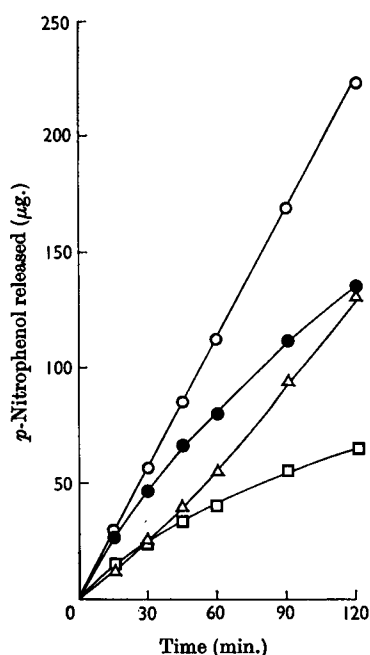


Fig. 7. Hydrolysis of 5mm-*p*-nitrophenyl *N*-acetyl- $\beta$ -glucosaminide by a boar epididymal preparation alone (○), and in the presence of 0.36  $\mu$ M-*N*-acetylgalactosamine oxidation product (Δ), 14  $\mu$ M-*N*-acetylgalactosaminono-(1→4)-lactone (□), and 14  $\mu$ M-sodium galactosaminatate (●).

$\beta$ -galactosidase activity, though it was a potent inhibitor of ox liver  $\beta$ -galactosidase activity. Both these lactone solutions were inhibitors of limpet  $\beta$ -galactosidase activity, galactonolactone causing 50% inhibition at 46  $\mu$ M and fuconolactone at 2.8 mm.

The lactone solution obtained by catalytic oxidation of galactose was about 10 times as powerful an inhibitor of rat and limpet  $\beta$ -galactosidase activity as the solutions described above, causing 50% inhibition of the rat enzyme at 58  $\mu$ M concentration and of the limpet enzyme at 6.4  $\mu$ M. On incubation at pH 3.0 with limpet  $\beta$ -galactosidase and substrate at 38° over a period of 2 hr., the oxidation solution showed a loss in inhibitory power, whereas a solution of galactono-(1→4)-lactone maintained a constant level of inhibition (Fig. 8). The oxidation solution was stable for at least 2 hr. at 0°.

L-Arabinono-(1→5)-lactone, prepared by catalytic oxidation of L-arabinose, was found to be an inhibitor of rat and limpet  $\beta$ -galactosidase (Table 3). When tested against the latter enzyme after treatment under varying conditions of temperature and pH, it proved to be very unstable (Fig. 9). The

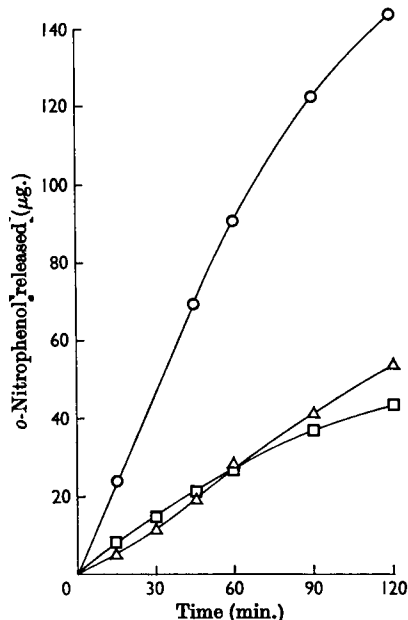


Fig. 8. Hydrolysis of 2.5mm-*o*-nitrophenyl  $\beta$ -galactoside by a limpet preparation alone (○), and in the presence of 23  $\mu$ M-galactose oxidation solution (Δ), and 4mm-galactono-(1→4)-lactone (□).

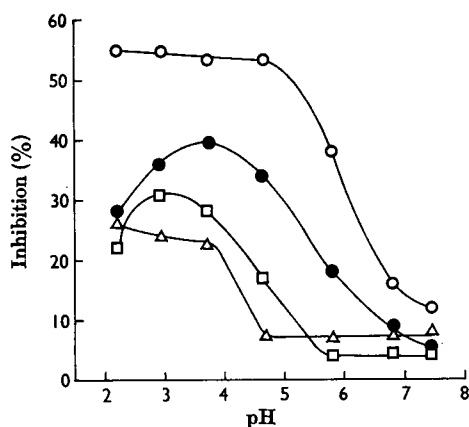


Fig. 9. Changes in inhibitory power of an L-arabinose oxidation solution at varying pH in buffered solution: tested at once (○), tested after 5 min. at 100° (Δ), tested after 1 hr. at 38° (□), and tested after 2 hr. at 0° (●). All tests were at 0.45 mm concentration against limpet  $\beta$ -galactosidase. Buffers were 0.2M- $\text{Na}_2\text{HPO}_4$ -0.1M-citric acid (McIlvaine, 1921). In the enzyme control 86  $\mu$ g. of o-nitrophenol was liberated.

solution was most stable at about pH 4, but even at 0° there was some loss of inhibitory power at this pH value.

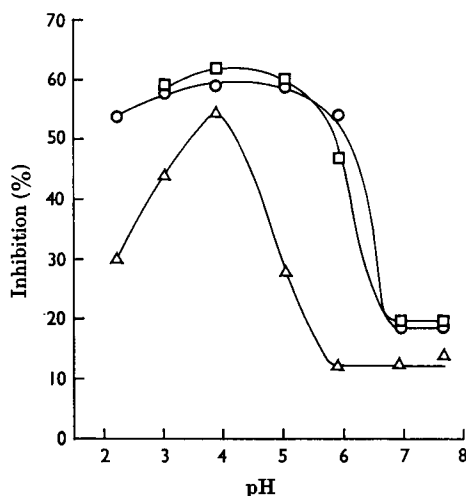


Fig. 10. Changes in the inhibitory power of a D-fucose oxidation solution at varying pH in buffered solution: tested at once (○), tested after 1 hr. at 38° (Δ), and tested after 2 hr. at 0° (□). All solutions were tested at 1.5 mM concentration against limpet  $\beta$ -galactosidase. Buffers were 0.2 M- $\text{Na}_2\text{HPO}_4$ -0.1 M-citric acid (McIlvaine, 1921). In the enzyme control 90  $\mu\text{g}$ . of *o*-nitrophenol was liberated.

Lactone solution produced by catalytic oxidation of D-fucose inhibited rat and limpet  $\beta$ -galactosidase. Though more stable than L-arabinono-(1→5)-lactone solutions under varying conditions of temperature and pH, fucono-(1→5)-lactone solutions were still unstable at all pH values when incubated at 38°, with minimal loss at pH 4 (Fig. 10). Previous work has shown that D-fucono-(1→4)-lactone is most stable at pH 4 and rapidly converted into the (1→5)-lactone at pH 6–7 (Levy *et al.* 1962).

With both rat and limpet  $\beta$ -galactosidase, galactono-(1→5)-lactone was a much more powerful inhibitor than either L-arabinono-(1→5)-lactone or D-fucono-(1→5)-lactone (Table 3).

**$\beta$ -D-Fucosidase.** As shown in Table 3, the galactose oxidation solution was a potent inhibitor of rat epididymal and limpet  $\beta$ -D-fucosidase activities. It was a more powerful inhibitor of the rat enzyme than the corresponding lactone from L-arabinose, which was in turn more powerful than the oxidation product from D-fucose. With limpet  $\beta$ -D-fucosidase the lactones arranged themselves in reverse order of inhibitory power.

## DISCUSSION

It is evident that the main product produced by catalytic oxidation of an aldose or uronic acid at an acid pH is the (1→5)-lactone of the same configura-

tion. This is the only molecular species other than the (1→4)-lactone and free acid that could be present in an aqueous solution of an aldonic acid and yet readily undergo transformation into those other species. The (1→5)-lactone is also, on chemical grounds, the most probable initial product of catalytic oxidation of a pyranose. Although the (1→5)-lactone has only been isolated in the pure state in the case of the mannose oxidation, inferential evidence for the nature of the product has been obtained in other instances.

The yield of (1→5)-lactone varies considerably from sugar to sugar. Oxidations continued for periods longer than are quoted in Table 1 result in a decrease in the amount of lactone present, as determined by inhibition tests, with or without a continued fall in reducing sugar. A fall in inhibitory power accompanied by a fall in reducing power could result from isomeric or degradative changes in the lactone. When there is little or no fall in reducing power, degradative changes presumably result in the production of other reducing material (cf. Table 2). The composition of solutions after over-oxidation has not, however, been extensively examined. With galactose, oxidation at acid pH and optimum conditions, though by no means complete, led to transformation at C-6 as well as at C-1, with the production of D-galactaro-(1→5)-lactone in addition to galactono-(1→5)-lactone: mucic acid (galactaric acid) could be isolated from the reaction product.

The stability of the different (1→5)-lactones in solution and on evaporation to dryness *in vacuo* varied considerably: solutions of the (1→5)-lactones obtained by oxidation of *N*-acetylglucosamine and *N*-acetylgalactosamine could be evaporated to dryness without much transformation occurring, whereas those obtained by oxidation of galactose, L-arabinose and D-fucose were transformed to a major extent into the (1→4)-lactone or the acid on evaporation, and lost much of their inhibitory power on standing at 0° for a few hours. The glycaric acid derivatives are intermediate in character.

Studies on the pH-stability of glucaro-(1→4)-lactone show that at pH 4.5, one of the two alternative pH values normally employed in the assay of  $\beta$ -glucuronidase, this lactone is at least partially transformed into a more inhibitory entity. In view of this transformation, it is not possible to make an accurate measurement of the inhibitory power of glucaro-(1→4)-lactone. Measurements of the rate of hydrolysis of phenolphthalein  $\beta$ -glucuronide at pH 4.5 in the presence and absence of glucaro-(1→4)-lactone indicate that the initial inhibitory power of the lactone is considerably lower than our 1 hr. value, but it is not possible to say how much of this initial value is due to immediate conversion

into the (1→5)-lactone. The changes in glucaro-(1→4)-lactone solutions cannot be explained in terms of intermediate formation of the dicarboxylic acid. Rapid interconversions of lactones at acid and neutral pH are known. Takahashi & Mitsumoto (1963) have shown that glucono-(1→5)-lactone in solution at pH values of 4.6 or higher undergoes immediate partial transformation into the (1→4)-lactone. The reverse was also noted in enzyme experiments with these lactones (Levy *et al.* 1964). In general, for the sugars dealt with here, both the (1→4)- and (1→5)-lactones seem to be most stable at acid pH and interconvertible at or near neutrality.

It seems probable that the inhibitory product formed when a potassium hydrogen saccharate solution is boiled for 30 min. (Levy, 1952) is a mixture of glucaro-(1→4)- and glucaro-(1→5)-lactone. Temperature-stability experiments show that such a solution behaves like glucaro-(1→4)-lactone rather than the (1→5)-lactone: on the other hand, the relative stability of the inhibitory power when the effect of the solution on the rate of hydrolysis of substrate is studied is characteristic of the behaviour of the (1→5)-lactone. Nevertheless, boiling a solution of potassium hydrogen saccharate for 30 min. is still the most rapid and convenient method of preparing a powerful inhibitor of  $\beta$ -glucuronidase activity.

A boiled mucic acid solution, which also inhibits  $\beta$ -glucuronidase, shows no sign of transformation when preincubated at 38° at varying pH values, in marked contrast with the behaviour of the oxidation product from galacturonic acid, which under such conditions is extremely unstable, except in the region of pH 4. It seems likely that 'most inhibitory solutions' prepared from mucic acid contain mainly the D- and L-(1→4)-lactone, but that inhibition is due entirely to a trace of D-(1→5)-lactone in the equilibrium mixture. Lactonization in a solution of a glycaric acid is much more complex than in an aldonic acid solution, since both carboxyl groups take part.

The enzyme *N*-acetyl- $\beta$ -glucosaminidase is known to be inhibited by both *N*-acetylglucosaminono- and *N*-acetylgalactosaminono-lactones (Findlay, *et al.* 1958). Catalytic oxidation of *N*-acetylglucosamine results in the production of the most powerful inhibitor of boar epididymal *N*-acetyl- $\beta$ -glucosaminidase yet known. The oxidation solution can be evaporated to dryness *in vacuo*, with little or no loss in inhibitory power, to give a hygroscopic solid. The formation of this powerful inhibitor confirms the observations of Findlay *et al.* (1958) that solutions prepared from pure crystalline *N*-acetylgalactosaminono-(1→4)-lactone could give rise to an even more inhibitory compound when preincubated at 38° and pH 5–6 for 1 hr.

Catalytic oxidation of *N*-acetylglucosamine gives a product 1½–2 times as powerful an inhibitor of boar enzyme activity as that obtained by the usual mercuric oxide oxidation procedure, though both products are undoubtedly far from pure entities. The impurities to be expected in a preparation derived from mercuric oxide oxidation of glucosamine, followed by acetylation of glucosaminic acid, are quite different from those one would look for after the platinum-catalysed oxidation of *N*-acetylglucosamine. Specimens of the (1→4)-lactone prepared from the (1→5)-lactone by prolonged heating of the catalytic oxidation product at 100° had about one-seventh of the latter's inhibitory power.

Cross-inhibitory effects have already been observed with 'most inhibitory solutions' derived from galactono-(1→4)-lactone and D-fucono-(1→4)-lactone acting on the  $\beta$ -galactosidase and  $\beta$ -D-fucosidase activities of rat and limpet preparations (Levy & McAllan, 1963*a,b*). When allowance is made for the greater inhibitory power of the solutions obtained by catalytic oxidation of the pyranose sugars, present findings are in agreement with the previous results. Since it has the same configuration of substituent groups in the lactone ring as galactono- and fucono-(1→5)-lactone, it is hardly surprising that L-arabinono-(1→5)-lactone inhibits both types of enzyme activity from both sources. Wallenfels & Malhotra (1960) have noted that  $\beta$ -galactosidase from calf intestine and *Escherichia coli* will hydrolyse  $\alpha$ -L-arabinosides as well as  $\beta$ -D-fucosides.

It was previously concluded that the inhibitory power of galactono-, fucono- and 2-acetamido-2-deoxygalactono-lactone towards the appropriate glycosidases was dependent on the proportion of (1→5)-lactone formed in the solution of the crystalline (1→4)-lactone (Levy *et al.* 1962). This was explained by the reversal of the configuration of the ring compared with the pyranosyl substrate that occurs in the galactose and derived series when one passes from the (1→5)-lactone to the (1→4)-lactone. This argument did not apply to the glucose or the mannose series. The inhibitory power of glucono-(1→5)-lactone was found to be relatively only slightly greater than that of the (1→4)-lactone, and both were considered to be inhibitors. Mannono-(1→5)-lactone was, on the other hand, a very much more powerful inhibitor towards mannosidases than mannono-(1→4)-lactone, and no satisfactory explanation on configurational grounds could be offered for this finding (Levy *et al.* 1964). Nevertheless, the possibility had to be entertained that inhibition by the latter lactone was always due to traces of the former produced in solution.

As a speculative theory which would embrace all

the lactones that have been studied, including those newly described in the present paper, it is suggested that the difference in inhibitory power between a pair of isomeric lactones measures the stability of the lactones in solution, and that only the (1→5)-lactone ever inhibits. This theory is admittedly more difficult to accept in the case of glucose derivatives, where the difference in inhibitory power between pairs of isomeric lactones is always relatively small, than in the case of the mannonolactones and the galactonolactones. One must postulate that the latter are relatively stable in water, whereas glucono-(1→4)-lactone and, to a smaller extent, glucaro-(1→4)-lactone, are envisaged as undergoing very rapid and extensive interconversion (without ring-opening) with the respective inhibitory (1→5)-lactones.

The only evidence we have to support the theory is derived from experiments with limpet  $\beta$ -glucosidase. The relative inhibitory power of glucono-(1→5)-lactone compared with the (1→4)-lactone rose from 4 in assays done at pH 5 to 80 in assays at pH 3. It is concluded that interconversion of the isomeric lactones was retarded at the more acid pH. Since the inhibitory power of the (1→5)-lactone remained unaltered on changing the pH of assay, it appears that, in this instance at least, the stability of the (1→4)-lactone was the major factor in determining the inhibitory ratio. For further evidence on this point it would be desirable to study the relative effectiveness under comparable conditions of a (1→4)- and (1→5)-lactone at different stages of interconversion towards a related pyranosidase and furanosidase.

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