# Inhibition of Glycosidases by Aldonolactones of Corresponding Configuration

## 2. INHIBITORS OF β-*N*-ACETYLGLUCOSAMINIDASE*

BY JUNE FINDLAY, G. A. LEVVY AND C. A. MARSH
*Rowett Research Institute, Bucksburn, Aberdeenshire*

(*Received* 23 *December* 1957)

Conchie & Levvy (1957) found that, in general, the enzymic hydrolysis of a simple glycoside was inhibited by the aldonolactone corresponding in configuration to the sugar residue of the substrate. This suggested that the structural requirements necessary for a specific inhibitor of β-*N*-acetylglucosaminidase would be fulfilled by the compound 2-acetamido-2-deoxygluconolactone (*N*-acetylglucosaminonolactone). Hitherto the most effective competitive inhibitor of this enzyme has been the hydrolysis product, *N*-acetylglucosamine, which has a considerably lower affinity than the substrates customarily used for assay (Pugh, Leaback & Walker, 1957*b*).

*N*-Acetylglucosaminonolactone has not previously been synthesized, although Hope & Kent (1955) stated that the crude product of the treatment of glucosaminic acid with keten had the characteristics of a lactone. Partial acetylation of glucosaminic acid with acetic anhydride yielded a product, which, while not crystalline, possessed the properties of the required compound. Another obvious route was by selective oxidation of *N*-acetylglucosamine, and this was performed successfully by two different methods. As was expected, *N*-acetylglucosaminonolactone proved a potent inhibitor of β-*N*-acetylglucosaminidase.

Ram testis and emulsin preparations with β-*N*-acetylglucosaminidase activity have been shown to hydrolyse *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide, and it has been tentatively concluded that the glucosaminidase is responsible (Heyworth, Borooah & Leaback, 1957). If this is so, 2-acetamido-2-deoxygalactonolactone (*N*-acetylgalactosaminonolactone) would also be expected to inhibit the enzymic hydrolysis of *N*-acetyl-β-D-glucosaminides; this has been found to occur. The complex structural changes in the inhibitory lactones under varying conditions of pH have been studied, largely by changes in their inhibitory power.

For most experiments, preparations of adult rat epididymis were used, since this tissue was found by Conchie, Findlay & Levvy (1956) to have much greater β-*N*-acetylglucosaminidase activity than any other studied: the action of the inhibitors on mammalian α-*N*-acetylglucosaminidase was also examined. The visceral hump of the common limpet (*Patella vulgata*) has been shown (Conchie & Levvy, 1957) to be a rich source of both α- and β-*N*-acetylglucosaminidase activity, and the effects of the lactones on the limpet enzymes have also been studied.

* The paper by Conchie & Levvy (1957) is to be regarded as Part 1.

## EXPERIMENTAL

### *Analytical methods*

Acetyl was determined by a modification of the method of Kuhn & Roth (1933).

Optical activity was measured on the Hilger Standard polarimeter, where possible with a 1 or 2 dm. tube; for measurements of $[\alpha]_D$ changes in *N*-acetylglucosaminonolactone solutions a 0·5 dm. microtube was employed.

All melting points are corrected. Measurement of pH was with the glass electrode.

### *Synthetic methods*

Evaporations of solutions were done on the water bath under reduced pressure.

*Phenyl* N-*acetyl-α- and -β-glucosaminides.* These were prepared as described by Conchie & Levvy (1957). The α-anomer had m.p. 244–246°, $[\alpha]_D^{22}$ +210° in water (*c*, 1) and the β-anomer m.p. 248–249°, $[\alpha]_D^{22}$ −3° in water (*c*, 1·3). Roseman & Dorfman (1951) give values of m.p. 246–247°, $[\alpha]_D^{25}$ +213° and m.p. 249–250°, $[\alpha]_D^{25}$ −10·3° respectively.

p-*Nitrophenyl* N-*acetyl-β-glucosaminide.* By preliminary experiment it was found more convenient to use β-glucosamine penta-acetate, and not the usual equilibrium mixtures of the anomers, for condensation with *p*-nitrophenol; other modifications of the original procedure (Westphal & Schmidt, 1952) were necessary to obtain a satisfactory yield of the β-glycoside tetra-acetate. Our attention was drawn by Dr D. H. Leaback to the use of $ZnCl_2$, which usually favours the formation of α-D-sugar acetates (see Conchie, Levvy & Marsh, 1957), as a catalyst for the preparation of β-hexosamine penta-acetates (Stacey, 1944).

Finely powdered glucosamine hydrochloride (20 g., L. Light and Co.) was added during 30 min. with frequent shaking to a solution of anhydrous granular $ZnCl_2$ (16·6 g., British Drug Houses Ltd.) in acetic anhydride (106 ml.); it was essential that the temperature should be maintained at 45–55°. Dissolution was complete after about a further 15 min. at 50°, and the solution was then poured with stirring into water (140 ml.) at 0°; NaOH (64 g.) in water (200 ml.) was added over 30 min. at a temperature of

40–50°. Separation of β-glucosamine penta-acetate began before the end of the alkali addition, and was completed by cooling the mixture for 1 hr. at 0°. The crystalline product (10 g., yield 28%) was filtered, freed from acid with ice-cold water, and used without further purification, m.p. 185–186°, $[\alpha]_D^{22}$ +1·5° in $CHCl_3$ (*c*, 4). Hudson & Dale (1916) give m.p. 188–189°, $[\alpha]_D^{20}$ +1·2° for β-glucosamine penta-acetate.

A melt at 130° of the β-glucosamine penta-acetate (20·6 g.), *p*-nitrophenol (24·6 g.) and toluene-*p*-sulphonic acid (0·14 g.) was maintained at 120° for 2 hr. with occasional stirring, and $CHCl_3$ (800 ml.) was then added. The solution was washed thoroughly with water at 0°, then with 0·1 N-NaOH (total 600 ml.) at 0° until the aqueous phase suddenly changed in colour from yellow to dark brown. The $CHCl_3$ solution was rewashed with water at 0° and dried over $CaCl_2$; evaporation to small bulk yielded a product which was recrystallized from ethanol–$CHCl_3$ (1:1, 130 ml.) to give almost pure *p*-nitrophenyl β-glucosaminide tetra-acetate (8·8 g., yield 35%); m.p. 233°, $[\alpha]_D^{20}$ −40° in acetone (*c*, 0·5). Westphal & Schmidt (1952) give m.p. 241°, $[\alpha]_D^{18}$ −46·75° in pyridine for this compound.

De-*O*-acetylation of the tetra-acetate (13·2 g.) in solution in methanol–$CHCl_3$ (1:1, 240 ml.) was done with 0·1 N-sodium methoxide in methanol (7·3 ml.), added to the boiling solution under reflux. The mixture was immediately left to cool and kept at 0° overnight, and the product (8·9 g., 92% of the theoretical), m.p. 204°, was filtered off and washed with methanol at 0°. Pure anhydrous *p*-nitrophenyl *N*-acetyl-β-glucosaminide, m.p. 210°, $[\alpha]_D^{22}$ −18° in aqueous acetone (1:1) (*c*, 1), was obtained after two recrystallizations from boiling water (the compound decomposes slowly in aqueous solution above 50° to liberate *p*-nitrophenol). According to Westphal & Schmidt (1952) the monohydrate has m.p. 204°, $[\alpha]_D^{16}$ −25·5° in pyridine.

*2-Acetamido-2-deoxygluconolactone.* (*a*) By acetylation of glucosaminic acid. A modification of the method (Karrer & Mayer, 1937) of *N*-acetylation of chondrosaminic (galactosaminic) acid was used. Glucosaminic acid (3·1 g.), obtained by oxidation of glucosamine with freshly-precipitated yellow mercuric oxide (Hope & Kent, 1955), was dissolved in 10% (w/v) NaOH (26 ml.) and treated with acetic anhydride (8 ml.). The mixture rapidly warmed up, and after 15 min. was treated with dil. $H_2SO_4$ to give pH 2–3. Evaporation to dryness, followed by several evaporations with ethanol to remove the last traces of water, gave a resinous mass which was heated at 100° for 1 hr. *in vacuo*. This was then extracted twice with hot ethanol (total 300 ml.) and evaporated to dryness; the residue was re-extracted with hot ethanol (75 ml.), treated with charcoal and evaporated at minimum temperature to a clear gum. Extensive drying *in vacuo* yielded *2-acetamido-2-deoxygluconolactone* as a non-crystalline white powder (3·3 g., 95% of the theoretical) (Found: C, 42·8; H, 6·3; N, 5·8; $CH_3$·CO, 19·0. $C_8H_{13}O_6N$ requires C, 43·8; H, 6·0; N, 6·4; $CH_3$·CO, 19·6%).

This extremely deliquescent substance had initial $[\alpha]_D^{20}$ +81° in water (*c*, 2); the aqueous solution was acid (pH 3·5 at 25 mM) and reduced Fehling's solution slowly at 100°. Titration under $N_2$ with 0·01 N-NaOH to pH 7 gave an equivalent of 221 (theoretical value 219); it behaved almost entirely as a lactone with the ring opening readily at pH 6·4–6·5. The acetyl group was lost on treatment both with acid (see below) and with alkali; thus there was complete liberation of one equivalent of acetic acid in 0·5 N-NaOH at 100° after 1 hr.

(*b*) By oxidation of *N*-acetylglucosamine. Heyns & Koch (1953) found that glucosamine in the presence of a Pt catalyst was readily oxidized by gaseous $O_2$ to glucosaminic acid, but were unable to oxidize *N*-acetylglucosamine similarly. This was accomplished, however, by the procedure previously used to oxidize aliphatic hexosides to the conjugated hexuronic acids (Marsh, 1952), and also with freshly precipitated HgO at slightly alkaline pH.

(i) *N*-Acetylglucosamine (1 g., L. Light and Co.) in aqueous solution (25 ml.) with Pt catalyst (0·2 g.) was oxidized with gaseous $O_2$ at 40–45°, the mixture being kept neutral to litmus by frequent addition of 0·5 N-$NaHCO_3$ [see Marsh (1952) for details of catalyst preparation etc.]. When about one equivalent (9·0 ml.) of alkali had been added, the rate of change of pH was considerably decreased. At this point the solution was filtered and an insoluble lead salt was precipitated with basic lead acetate. Treatment with $H_2S$ yielded a gum which was extracted with hot acetone, evaporated, and precipitated with ether from cold butanol solution, to yield a very deliquescent solid (0·14 g.).

(ii) To $HgCl_2$ (12·5 g.) in water (400 ml.) at room temperature was added 3 N-NaOH (33 ml.) with shaking; the precipitated yellow HgO was removed, washed with water by centrifuging, and resuspended in water (130 ml.). *N*-Acetylglucosamine (3·0 g.) in water (33 ml.) was added and after heating to 100°, 0·5 N-NaOH (30 ml., 1 equiv.) was added dropwise with mechanical stirring during 2 hr. to keep the pH at 7·0–8·5; immediate reduction of HgO was observed. Mercuric ion was removed by centrifuging followed by treatment with $H_2S$; the aqueous solution after treatment with charcoal was acidified (pH 2–3) with dil. $H_2SO_4$ and treated as in (*a*) to give a highly deliquescent product (1·8 g.).

Products obtained by oxidation of *N*-acetylglucosamine appeared to be similar to that obtained by *N*-acetylation of glucosaminic acid, but had slightly lower inhibitory power for β-*N*-acetylglucosaminidase. These alternative methods of preparation afforded evidence of the identity of the product, but were less satisfactory and not investigated further.

*2-Acetamido-2-deoxygalactonolactone.* The method of Karrer & Mayer (1937) was modified as follows. An aqueous solution (30 ml.) of galactosamine hydrochloride (1·22 g.) was added to a suspension of freshly prepared HgO (5 g.) in water (120 ml.). The mixture was heated for 20 min. on the steam bath; there was obvious formation of free Hg. After being centrifuged, the solution was treated with $H_2S$, filtered and concentrated to small bulk and an equal volume of ethanol added. The galactosaminic acid was filtered off and recrystallized from water with the addition of a little ethanol; yield 276 mg. (26%).

A solution of galactosaminic acid (314 mg.) in 10% (w/v) NaOH (2·7 ml.) was treated with acetic anhydride (0·5 ml.). After 20 min. the liquid was adjusted to about pH 2·5 with dil. $H_2SO_4$; the residue after evaporation to dryness was heated at 100° *in vacuo* for 45 min., then extracted twice with hot ethanol (total 20 ml). The filtered ethanolic solution was again evaporated to give a glassy solid (304 mg.), which after recrystallization from ethanol gave the pure 2-acetamido-2-deoxygalactonolactone (*N*-acetylgalactosaminonolactone), m.p. 168–169° (decomp.) with

sintering at 165°, $[\alpha]_D^{22}$ −24° in water (*c*, 1). Yield 125 mg.; a second crop, m.p. 166–167° (decomp.) (28 mg.), was obtained from the mother liquors (total 39% of the theoretical). Karrer & Mayer (1937) give m.p. 165° for this compound (Found: C, 44·1; H, 5·9; N, 6·1. Calc. for $C_8H_{13}O_6N$: C, 43·8; H, 6·0; N, 6·4%).

An aqueous 20 mM solution of *N*-acetylgalactosaminonolactone had pH 6·1; it behaved entirely as a lactone with ring fission at pH 7·5. Titration with 0·01 N-NaOH under $N_2$ to pH 8·5, followed by back titration to pH 7 with standard acid, gave an equivalent weight of 227 (theoretical for $C_8H_{13}O_6N$, 219). The lactone in aqueous solution slowly reduced Fehling's solution at 100°.

Unfortunately, lack of material was a limiting factor in the experiments with the galactonolactone. In particular, it precluded measurements of changes in rotation during alterations in inhibitory power.

### *Buffer solutions*

Buffer solutions were prepared by titrating the calculated weight of the free acid (citric acid, glycine, $H_3PO_4$) to the required pH with NaOH solution.

### *Enzyme preparations*

*From rat epididymis.* The combined caput and cauda epididymis (total about 1 g.) of an adult rat was homogenized in water (8 ml.). According to Pugh, Leaback & Walker (1957*a*) complete solubilization of tissue β-*N*-acetylglucosaminidase requires the presence of NaCl; 1 ml. of 0·1 M-NaCl and 1 ml. of 0·5 M-citric acid–NaOH buffer, pH 4·3, was added and the mixture incubated at 38° for 1 hr. and centrifuged at 1500 *g* for 15 min. The supernatant was fractionated with ammonium sulphate and the precipitate obtained between 20 and 80% saturation with $(NH_4)_2SO_4$ was redissolved in water (10 ml./g. of tissue for assay of β-*N*-acetylglucosaminidase and 2 ml./g. of tissue for α-*N*-acetylglucosaminidase activity). The appropriate stock solution was further diluted 1:30 for assay with phenyl *N*-acetyl-β-glucosaminide, and 1:100 for assay with *p*-nitrophenyl *N*-acetyl-β-glucosaminide.

*From limpet.* The procedure was as described by Levvy, Hay & Marsh (1957). Before assay with the different substrates, the stock solution (2·5 ml./g. of tissue) was diluted 1:10 for phenyl *N*-acetyl-β-glucosaminide, 1:60 for *p*-nitrophenyl *N*-acetyl-β-glucosaminide and 1:3 for phenyl *N*-acetyl-α-glucosaminide.

### *Enzyme assay*

Preliminary experiments often showed a slight variable activation (not exceeding 10%) of epididymal β-*N*-acetylglucosaminidase activity in the presence of NaCl (cf. Pugh *et al.* 1957*a*). In general, therefore, all buffers contained NaCl to give a final concentration of 0·1 M in the enzyme assay.

*Liberation of phenol.* The hydrolysis of phenyl *N*-acetyl-α- and -β-glucosaminides was followed by the method of Kerr, Graham & Levvy (1948), free phenol being measured on the Spekker absorptiometer with Ilford no. 608 red filters (peak transmission 680 mμ). The incubation mixture (1 ml.) contained 0·1 ml. of 0·5 M citric acid–NaOH buffer, 0·5 ml. of substrate (0·02 M) and 0·2 ml. of enzyme solution. The buffer pH was adjusted to give a final value of 4·3 in the assay; this was shown by Pugh *et al.* (1957*b*) to be the pH optimum for rat-kidney β-*N*-acetylglucosaminidase, and was accepted arbitrarily for assay of rat-epididymis and limpet α- and β-*N*-acetylglucosaminidases.

In addition to the usual controls, it was necessary to correct for interference at concentrations greater than 0·1 mM of *N*-acetylglucosaminonolactone and *N*-acetylgalactosaminonolactone, owing to their reducing action.

*Liberation of p-nitrophenol.* The mixture incubated (4 ml.) contained 1 ml. of 0·2 M-citric acid–NaOH buffer, 2 ml. of 10 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide, and 0·5 ml. of enzyme solution, the final pH being 4·4 (Heyworth *et al.* 1957). After incubation for 1 hr. at 38°, 4 ml. of 0·4 M glycine–NaOH buffer, pH 10·5, was added, the solution centrifuged, and the liberated *p*-nitrophenol in the supernatant measured on the Spekker absorptiometer with Ilford no. 601 violet filters (peak transmission 430 mμ).

For studies of the changes in inhibitory power of the lactones and their derivatives, only rat-epididymis enzyme and 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide were used. Whereas the β-*N*-acetylglucosaminidase activity of stock epididymis preparations kept at 0° was constant over several weeks, when diluted for assay its activity decreased at a rate of about 5%/hr. at 0° (cf. Pugh *et al.* 1957*b*).

Experiments on changes in the inhibitory power of the lactones under varying conditions of pH were so arranged that dilution of the inhibitor solution before assay was great enough to preclude any effect on the pH of the incubation mixture in the enzyme assay.

## RESULTS

### *Comparison of α- and β-N-acetylglucosaminidase activities of rat epididymis and limpet*

The α- and β-*N*-acetylglucosaminidase activities of typical rat-epididymis and limpet-visceral-hump preparations are shown in Table 1. The ratios of β to α enzymic activities, with 10 mM-phenyl glycosides as substrates, were 615 and 4 for the epididymis and visceral preparations respectively. Rat epididymis was a much better source of β-*N*-acetylglucosaminidase activity, but the limpet was the more convenient source of the α-enzyme. Both preparations hydrolysed *p*-nitrophenyl *N*-acetyl-β-glucosaminide more readily than the phenyl β-glycoside, but whereas the ratio of μg. of *p*-nitrophenol to μg. of phenol liberated by epididymis was 2·4, identical with that for ram-testis preparations (Dr P. G. Walker, personal communication), the corresponding value for limpet was 10. We were unable to confirm the relatively high α-*N*-acetylglucosaminidase activity reported by Roseman & Dorfman (1951) for certain other rat tissues such as liver and testis. Epididymis displayed the highest activity observed for this enzyme in the rat.

The liberation of *p*-nitrophenol from the β-glycoside was found to be proportional to the amount of enzyme present and to the period of incubation for at least 1 hr., for both epididymis (cf. Fig. 2 below) and limpet preparations.

Table 1. *α- and β-N-Acetylglucosaminidase activities of rat-epididymis and limpet-visceral-hump preparations*

The figures represent μg. of aglycone liberated/hr./g. of tissue at 38° from 10 mM-phenyl *N*-acetyl-α- and -β-glucosaminides at pH 4·3, and from 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide at pH 4·4, in 0·05 M citric acid–NaOH buffer.

| Substrate | Enzyme activity (μg./g. of tissue) Epididymis | Limpet |
|---|---|---|
| Phenyl *N*-acetyl-α-glucosaminide | 102 | 1 200 |
| Phenyl *N*-acetyl-β-glucosaminide | 62 800 | 4 600 |
| *p*-Nitrophenyl *N*-acetyl-β-glucosaminide | 151 000 | 47 000 |

Table 2. *Inhibition by freshly prepared solutions of N-acetylglucosaminonolactone and N-acetylgalactosaminonolactone of the α- and β-N-acetylglucosaminidase activities in rat epididymis and limpet*

The substrates were 10 mM-phenyl *N*-acetyl-α- and -β-glucosaminides.

| Inhibitor | Concn. (μM) | Inhibition (%) Epididymis α-Enzyme | Epididymis β-Enzyme | Limpet α-Enzyme | Limpet β-Enzyme |
|---|---|---|---|---|---|
| *N*-Acetylglucosaminonolactone | 1000 | 47 | 100 | 22 | 96 |
| *N*-Acetylglucosaminonolactone | 2 | 0 | 67 | 0 | 0 |
| *N*-Acetylgalactosaminonolactone | 1000 | 0 | 98 | 0 | 57 |
| *N*-Acetylgalactosaminonolactone | 10 | — | 54 | — | 0 |

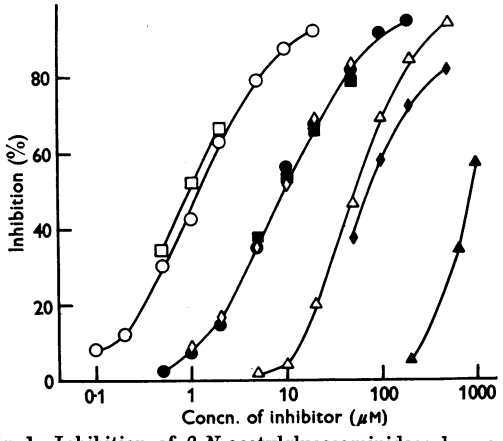


Fig. 1. Inhibition of β-*N*-acetylglucosaminidase by varying concentration of lactone inhibitors. Inhibitor, *N*-acetylglucosaminonolactone: substrate, 10 mM-phenyl *N*-acetyl-β-glucosaminide, enzyme from rat epididymis (□) and limpet (△); substrate 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide, enzyme from rat epididymis (○) and limpet (◇). Inhibitor, *N*-acetylgalactosaminonolactone: substrate 10 mM-phenyl *N*-acetyl-β-glucosaminide, enzyme from rat epididymis (■) and limpet (▲); substrate, 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide, enzyme from rat epididymis (●) and limpet (◆). Inhibitor concentrations are plotted on a logarithmic scale.

Prologation of incubation beyond this time often caused slight diminution of the rate of hydrolysis, presumably due to enzyme instability, even in the presence of substrate. These observations are in general agreement with those of Pugh *et al.* (1957*b*).

*Inhibition of epididymis and limpet α- and β-N-acetylglucosaminidase by the lactones*

With the phenyl glycosides as substrates, it was shown that both *N*-acetylglucosaminonolactone and *N*-acetylgalactosaminonolactone were very powerful inhibitors of epididymal β-*N*-acetylglucosaminidase and less effective towards the same enzyme from the limpet, whereas only the former lactone, and then at relatively high concentration, inhibited α-*N*-acetylglucosaminidase from either source (Table 2).

Further evidence for the non-identity of α- and β-*N*-acetylglucosaminidase was obtained in an experiment in which 5 mM-phenyl *N*-acetyl-α-glucosaminide had no effect on the hydrolysis of 2·5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide by the epididymal enzyme, whereas 5 mM-phenyl *N*-acetyl-β-glucosaminide caused 40% depression of nitrophenol liberation.

Inhibition of the β-glycosidase from both sources was studied at varying concentrations of both lactones (freshly prepared solutions), with 10 mM-phenyl and 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide as substrates. The results are shown in Fig. 1 and the derived values for the lactone concentrations giving 50% inhibition are given in Table 3.

Both lactones always acted as competitive inhibitors of β-glucosaminidase, and $K_m$ and $K_i$ were calculated by the method of Lineweaver & Burk (1934); mean values are given in Table 3. In the case of the epididymal enzyme, $K_i$ for either lactone was very similar with both substrates

employed, and N-acetylglucosaminonolactone had approximately five times the affinity ($1/K_i$) of N-acetylgalactosaminonolactone. Values of $K_m$ are of the same order as those given by Pugh *et al.* (1957*b*) for a rat-kidney preparation and by Heyworth *et al.* (1957) for the enzyme in ram testis.

Values of $K_m$ for the limpet enzyme were about ten times those for the epididymal enzyme. Both lactones were more powerful inhibitors for the hydrolysis of *p*-nitrophenyl than of phenyl N-acetyl-β-glucosaminide by the limpet enzyme, and this is reflected in the differing $K_i$ values for N-acetylglucosaminonolactone, again the more powerful of the two lactones. This variation in $K_i$ with differing substrates was previously encountered in studying the effects of saccharo-1:4-lactone and galactosaccharolactone on the hydrolysis of phenyl and *o*-nitrophenyl β-galacturonide by preparations from the limpet and the female rat preputial gland (Levvy, McAllan & Marsh, 1958). It was then considered more probable that the nitro group modified the mode of combination of substrate with enzyme, rather than that the effect was due to the presence of two enzymes of different aglycone specificity.

The results shown in Fig. 1 for the inhibition by N-acetylgalactosaminonolactone of the epididymal enzyme acting on both phenyl and *p*-nitrophenyl N-acetyl-β-glucosaminide, and for the inhibition by N-acetylglucosaminonolactone of the limpet enzyme acting on phenyl N-acetyl-β-glucosaminide fall on the same curve. This is an accidental phenomenon resulting from similarities in the ratio of $K_i$ to $K_m$, together with the selection of the substrate concentration employed in studying the effect of varying inhibitor concentration.

### *Nature of the inhibitory principle in solutions of the aldonolactones*

*General comments.* Before describing the diverse inhibitory effects of the lactones under different conditions of pH, it might be useful to mention the types of structural change which these compounds could feasibly undergo, namely (*a*) hydrolysis of the lactone ring to give the free acid or anion, (*b*) interconversion of one lactone form into another, (*c*) removal of the N-acetyl group and (*d*) any other change in structure. Conchie (1954) studied the changes of inhibitory power of glucono-1:4- and -1:5-lactones for β-glucosidase due to mutarotation in solution; in this case both lactones caused similar inhibition of the enzyme, but from considerations of the very specific requirements of lactone structure for glycosidase inhibition (Conchie & Levvy, 1957) this would not necessarily appear to be a general rule. The following hypotheses are therefore proposed in an attempt to reconcile the complex changes observed to occur in the experiments outlined below.

From its method of preparation and rotation the solid N-acetylglucosaminonolactone was probably an equilibrium mixture of the 1:4- and 1:5-lactones, both of which caused similar inhibition of β-N-acetylglucosaminidase. There was preferential formation of one species from the free acid in solution at low pH, followed by attainment of the equilibrium. Both lactones in the equilibrium mixture were very susceptible to alkaline hydrolysis and could not be distinguished by titration, nor by differential loss of acetyl in strongly acid solution. In addition to these changes, N-acetylglucosaminonolactone in solution showed a slow irreversible decomposition, largely independent of pH; Bergmann, Zervas & Silberkweit (1931) found that the product of partial acetylation of glucosaminic acid decomposed spontaneously to give an α-pyrone. The crystalline, sharply melting N-acetylgalactosaminonolactone, however, was almost certainly a single species, probably the 1:4-lactone from its relative stability to alkaline hydrolysis. In solution, particularly at about pH 5–6, there was formation of an equilibrium mixture containing the 1:5-lactone, which had a considerably greater

Table 3. *Dissociation constants for the complexes formed by rat epididymis and limpet β-N-acetylglucosaminidases with substrate ($K_m$) and lactone inhibitors ($K_i$), and the inhibitor concentration required for 50% inhibition of hydrolysis*

The substrates were phenyl and *p*-nitrophenyl N-acetyl-β-glucosaminides (for details of assay, see Table 1). The amount of lactone required for 50% inhibition was read from Fig. 1.

| | | | Inhibitor | | | |
|---|---|---|---|---|---|---|
| | | | N-Acetylglucosaminonolactone | | N-Acetylgalactosaminonolactone | |
| Enzyme | Substrate aglycone | $K_m$ (mM) | $K_i$ (μM) | Concn. for 50% inhibition (μM) | $K_i$ (μM) | Concn. for 50% inhibition (μM) |
| Epididymis | Phenol | 0·71 | 0·072 | 1·0 | 0·40 | 8·3 |
| Epididymis | *p*-Nitrophenol | 0·40 | 0·090 | 1·3 | 0·41 | 8·3 |
| Limpet | Phenol | 6·4 | 27 | 54 | — | 850 |
| Limpet | *p*-Nitrophenol | 3·8 | 4·0 | 8·3 | 32 | 75 |

inhibitory effect upon β-*N*-acetylglucosaminidase, but which was much more unstable at neutral pH. *N*-Acetylgalactosaminic acid at low pH underwent preferential formation of the 1:5-lactone, followed by establishment of an equilibrium mixture, the composition of which depended upon the pH of the solution.

*Inhibition by* N-*acetylglycosaminate.* Solutions of *N*-acetylglucosaminonolactone and *N*-acetylgalactosaminonolactone showed no perceptible change in inhibitory power during 2 hr. incubation with enzyme and substrate at pH 4·4 and 38° (Fig. 2). In Fig. 2 (*a*), a linear curve was obtained in presence of *N*-acetylglucosaminonolactone, as in the control. In the experiment shown in Fig. 2 (*b*), the enzyme displayed significant inactivation in the control when incubation was prolonged beyond 1 hr., and this was reflected in the curve for inhibition by *N*-acetylgalactosaminonolactone, which was, however, linear over the first 90 min. After incubation for 1 hr. in the usual way, the *N*-acetylglycosaminic acids obtained by opening the lactone ring with alkali were found to give only feeble inhibition (5–10%) of β-*N*-acetylglucosaminidase at concentrations of 2 μM for the glucosamine derivative and 10 μM for the galactosamine derivative at which the untreated lactones caused 60% inhibition. This small residual effect was due, not to inherent inhibition by the *N*-acetylglycosaminate ion, but to partial closing of the lactone ring under the acid conditions of enzyme assay, as can be seen from Fig. 2, in which the anion concentration is 20 μM for the glucosamine derivative and 100 μM for that of galactosamine. The rate of enzymic hydrolysis, initially unchanged in the presence of the anion, progressively decreased during the period of incubation. Development of inhibitory power was more clearly seen with *N*-acetylgalactosaminate than with *N*-acetylglucosaminate (cf. also Fig. 6).

pH *stability of the lactones.* Incubation of the lactones at 37° and varying pH before assay in the usual way gave the results shown in Fig. 3. Neither inhibitor showed any change after 1 hr. at pH 3. After incubation for 1 hr. at a pH greater than 4, *N*-acetylglucosaminonolactone showed an increasing loss of inhibitory power with pH; after 1 hr. at pH 8, there was only a small residual inhibitory action, due no doubt to relactonization during the assay. Pretreatment of *N*-acetylglucosaminonolactone for 24 hr. caused an appreciable loss in activity even at pH 3.

In contrast, the maximum inhibitory power of *N*-acetylgalactosaminonolactone was displayed only after pre-incubation for 1 hr. at pH 5–6, indicating that a new and more inhibitory compound was formed. Inhibition was not completely abolished after 1 hr. at pH 9. Minimum inhibition,

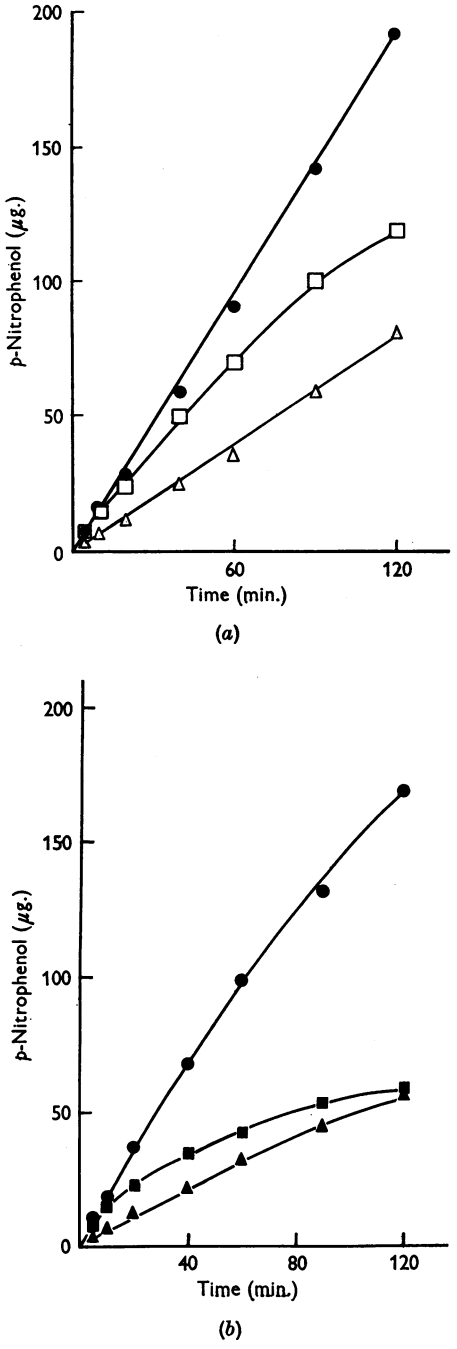


Fig. 2. Hydrolysis of 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide in the absence (●) and presence of (*a*) 2 μM-*N*-acetylglucosaminonolactone (△) and 20 μM-*N*-acetylglucosaminate (□), (*b*) 10 μM-*N*-acetylgalactosaminonolactone (▲) and 100 μM-*N*-acetylgalactosaminate (■).

corresponding to the figure for the anion, was reached after 24 hr. incubation at pH 7·5.

Solutions of *N*-acetylglucosaminonolactone in water (pH 3·2) slowly lost activity on standing at 0° (Fig. 4). Identical results were obtained in HCl (pH 2·0) at 37°, suggesting that neither loss of acetyl nor opening of the lactone ring was involved. In fact, it was not found possible to keep this inhibitor in aqueous solution without at least this fall in inhibitory power (about 12 % in 24 hr.). If the solid was exposed to moist air, deliquescence was accompanied by a similar and irreversible loss of inhibitory power.

Aqueous solutions of *N*-acetylgalactosaminonolactone (pH 6) kept at 0° increased in inhibitory power during the first 24 hr. (Fig. 4, cf. Fig. 3), by which time a stable equilibrium was apparently reached, with little further change of inhibitory power over an interval of 6 days.

The greater retention of inhibitory power by *N*-acetylgalactosaminonolactone at physiological pH is shown also in Fig. 5. After incubation of this lactone in $H_3PO_4$–NaOH buffer, pH 7, at 38°, there was an initial rise in inhibitory power, followed by a fall, presumably due to opening of the lactone ring, but even after 3 hr. incubation the residual activity was only a little less than that of the starting material. In contrast with this, a solution of *N*-acetylglucosaminonolactone at pH 7 fell to its minimum inhibitory activity very rapidly.

Loss of inhibitory power due to loss of the acetyl group at acid pH is also shown in Fig. 5. After heating in 0·5N-HCl at 100° for 2 hr., *N*-acetylglucosaminonolactone solutions were non-inhibitory. Acetyl determinations showed that one equivalent of acetic acid was liberated by this treatment, and glucosaminic acid was isolated as follows. Treatment of *N*-acetylglucosaminonolactone (100 mg.) in 0·5N-HCl (5 ml.) at 100° for 2 hr., followed by adjustment of pH to 4·1 with NaOH, concentration to approximately 1 ml. and addition of an equal volume of methanol, yielded glucosaminic acid (58 mg., 65 % of the theoretical). After recrystallization from water with addition of methanol, this had $[\alpha]_D^{20} - 6°$ in water (*c*, 2) and $[\alpha]_D^{20} - 15°$ in 0·5N-HCl (*c*, 2); Hope & Kent (1955) give $[\alpha]_D^{23}$ 0 ± 2° in water (*c*, 0·83), $[\alpha]_D^{21} - 16·5°$ in 2·5 % HCl (*c*, 1·64). 10 mM-Glucosaminic acid, before or after heating at pH 2 for 1 hr. at 100° to effect any possible lactonization, had no effect on β-*N*-acetylglucosaminidase activity.

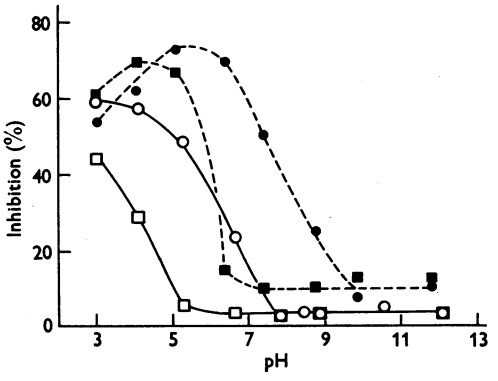


Fig. 3. Inhibition of β-*N*-acetylglucosaminidase by lactones pre-incubated in citrate buffer at 38° at varying pH; 2 μM-*N*-acetylglucosaminonolactone incubated for 1 hr. (○) and 24 hr. (□); 10 μM-*N*-acetylgalactosaminonolactone incubated for 1 hr. (●) and 24 hr. (■).

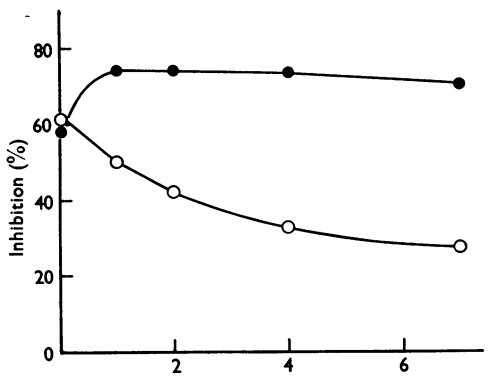


Fig. 4. Inhibition of β-*N*-acetylglucosaminidase by 2 μM-*N*-acetylglucosaminonolactone (○) and by 10 μM-*N*-acetylgalactosaminonolactone (●) kept at 0° in aqueous mM solution for varying periods.

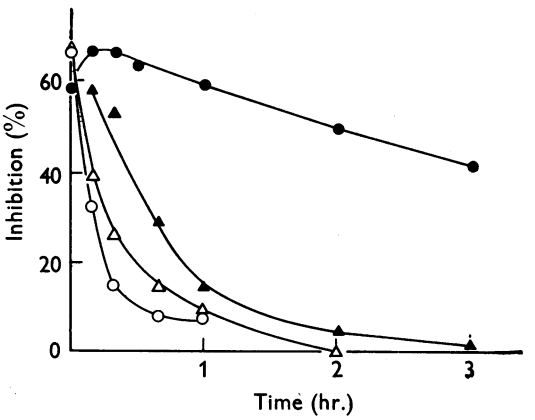


Fig. 5. Inhibition of β-*N*-acetylglucosaminidase by 2 μM-*N*-acetylglucosaminonolactone incubated in phosphate buffer, pH 7, at 38° (○) and in 0·5N-HCl at 100° (△), and by 10 μM-*N*-acetylgalactosaminonolactone incubated in phosphate buffer, pH 7, at 38° (●) and in 2N-HCl at 100° (▲).

The acetyl group of *N*-acetylgalactosaminonolactone was more stable to acid hydrolysis (Fig. 5). Heating in 2N-HCl at 100° caused almost complete loss of inhibitory power in 3 hr.; in 0·5N-HCl at 100° the loss was slow.

*Formation of lactone in N-acetylglycosaminate solutions.* As previously described (see Fig. 2) the inhibitory power of the lactones, completely lost by conversion into the sodium salts of the free acids, was slowly restored at the slightly acid pH of enzyme assay. After 2 hr. incubation at 38° and pH 2 solutions of *N*-acetylglucosaminate attained their original inhibitory power, and at 100° this value was reached in 20 min. at pH 3 (Fig. 6*a*). This maximum was followed by a steady loss of inhibitory power at pH 3 and 100°, probably due to loss of the acetyl group. Sodium *N*-acetylglucosaminate, calculated as the lactone, had $[\alpha]_D^{20} + 1°$ in water (*c*, 2); when maximum inhibitory power (61% inhibition at 2 μM) had been restored after 2 hr. at 38° and pH 2, the optical rotation was $[\alpha]_D^{20} + 37°$. The solution was then evaporated and the gum dried over anhydrous $CaCl_2$ *in vacuo* for 48 hr., whereupon the optical rotation had increased to $[\alpha]_D^{20} + 77°$, although the inhibitory power was unchanged. There was thus a species change that was without effect upon the inhibitory power, and the final product was apparently identical with the original *N*-acetylglucosaminonolactone ($[\alpha]_D + 81°$).

Bringing *N*-acetylgalactosaminate solutions to pH 2 or 3, however, produced very different effects (Fig. 6*b*). Maximum inhibitory activity was attained after the same period as with *N*-acetylglucosaminonolactone, but the inhibitory power was then much greater than anything yet displayed by the original lactone, and higher even than that of *N*-acetylglucosaminonolactone. At pH 3 and 100° subsequent incubation again caused loss of inhibitory power. At pH 4 and 100° a lower activity (comparable with that seen in Fig. 3) was attained after 30 min. incubation and was unaltered by further incubation.

It was obviously desirable to study the properties of the very inhibitory entity initially formed in acidified solutions of *N*-acetylgalactosaminate; solutions heated for 20 min. at 100° and pH 3 were used. The resulting solution, at a concentration corresponding to 0·65 μM of the original *N*-acetylgalactosaminonolactone, caused 50% inhibition of hydrolysis of 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide by epididymis enzyme (cf. the figure of 8·3 μM given in Table 3). The inhibitory principle was quite stable for at least 2 hr. at 38° and pH 4·4, as shown by its effect in enzyme assays, but was very unstable at pH 7 in $H_3PO_4$–NaOH buffer (Fig. 7), as one would expect for the 1:5-lactone. Even addition of the buffer before immediate transfer to the assay caused appreciable

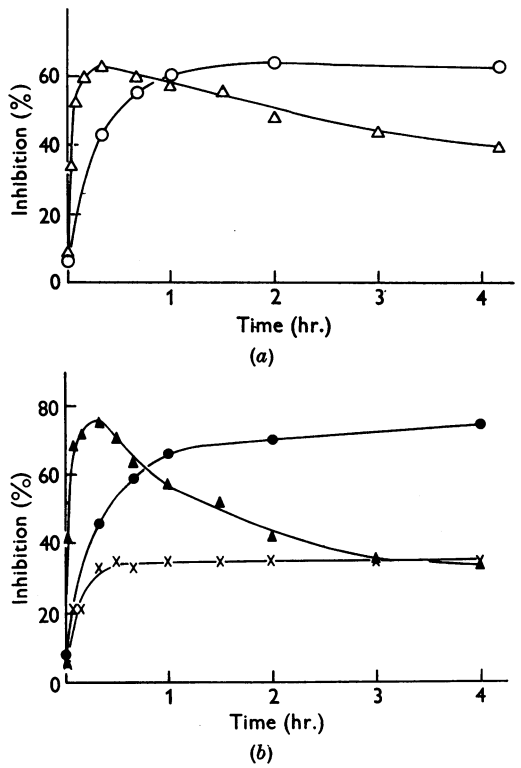


Fig. 6. Inhibition of β-*N*-acetylglucosaminidase by (*a*) 2 μM-*N*-acetylglucosaminate incubated at pH 2 and 38° (○) and at pH 3 and 100° (△); (*b*) 2 μM-*N*-acetylgalactosaminate incubated at pH 2 and 38° (●), pH 3 and 100° (▲) and at pH 4 (10 mM-citrate buffer) and 100° (×). The liquids were adjusted to pH 2 and 3 with HCl.

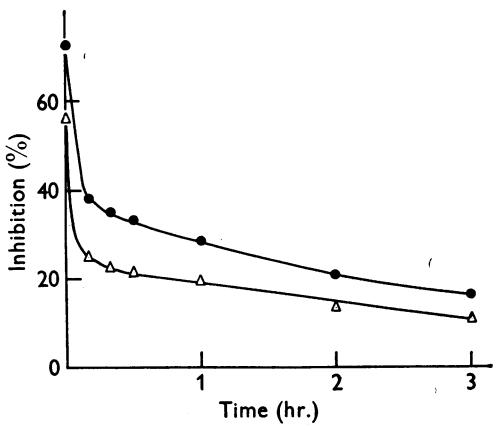


Fig. 7. Inhibition of β-*N*-acetylglucosaminidase by 5 μM-(●) and 2 μM- (△) -*N*-acetylgalactosaminate after 20 min. at pH 3 and 100°, followed by varying periods in phosphate buffer, pH 7, at 38°.

loss of inhibitory power. On incubation of the buffered solution at 38°, the inhibitory power fell rapidly to the normal values for *N*-acetylgalactosaminonolactone at this concentration, namely 38% inhibition at 5 μM and 25% inhibition at 2 μM (see Fig. 1). Thereafter there was a slow loss of inhibitory power, at the rate shown by the crystalline *N*-acetylgalactosaminonolactone at 38° and pH 7 (Fig. 5).

*Effects of other* N-*substituted glucosaminonolactones*

*N*-Benzoyl- and *N*-toluene-*p*-sulphonyl-glucosaminonolactone (Hope & Kent, 1955) at mM concentration had no effect on the hydrolysis of 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide by the epididymis enzyme. However, Neuberger & Pitt-Rivers (1939) observed similar hydrolysis of methyl *N*-formyl- and *N*-acetyl-β-glucosaminides by β-*N*-acetylglucosaminidase preparations from *Helix pomatia*, although other *N*-acyl β-glucosaminides were unattacked.

## DISCUSSION

This work provides further evidence on the one hand for the general nature of aldonolactone inhibition of glycosidases acting on substrates of the same configuration, and on the other hand for the value of such enzymic studies in the chemistry of sugar acid lactones. It has enabled us to isolate the hitherto unknown 2-acetamido-2-deoxygluconolactone, and has provided evidence for the existence in solution of a second 2-acetamido-2-deoxygalactonolactone, probably the 1:5-lactone. The isolation of this compound seems not impossible, since the slightly impure second crop obtained in the preparation of the 1:4-lactone was somewhat more inhibitory in freshly prepared solution than the main crop.

Since there was little to choose between 2-acetamido-2-deoxygluconolactone and the galactonolactone as inhibitors for β-*N*-acetylglucosaminidase, it would appear that, in agreement with Heyworth *et al.* (1957), a single enzyme is responsible for the hydrolysis of both *N*-acetyl-β-glucosaminides and -galactosaminides. The lactones, however, clearly distinguish between α- and β-*N*-acetylglucosaminidase, in agreement with the suggestion of Roseman & Dorfman (1951) that these are different enzymes. An analogous situation has already been described for enzymes hydrolysing glucuronides and galacturonides (Marsh & Levvy, 1958). In the same way α- and β-galactosidase could be distinguished by the action of galactonolactone, although gluconoand mannono-lactone inhibited their respective α- and β-glycosidases equally powerfully (Conchie & Levvy, 1957).

The *N*-acetylglycosaminonolactones are at least as powerful enzyme inhibitors as saccharo-1:4-lactone, the inhibitor for β-glucuronidase. The latter, as already noted by Conchie & Levvy (1957), is much more efficient than the simpler aldonolactones, such as gluconolactone, and it would seem that the presence of a second 'functional' group in the inhibitor molecule enhances its action.

Conchie & Levvy (1957) showed that β-*N*-acetylglucosaminidase showed no appreciable inhibition by any of the aldonolactones that they studied, and such other glycosidases as we have examined (β-glucuronidase, β-galactosidase, α-mannosidase) are not appreciably affected by mM-2-acetamido-2-deoxygluconolactone, proving that inhibition is highly specific for both enzyme and lactone.

The relative abundance of α-*N*-acetylglucosaminidase activity in the limpet is of particular interest since Neuberger & Pitt-Rivers (1939) could not detect this enzyme in *Helix pomatia*, although Utusi, Huzi, Matumoto & Nagaoka (1949) have reported trace activity in β-*N*-acetylglucosaminidase preparations from other snails. Herein lies yet another analogy between the glucuronidases and the *N*-acetylglucosaminidases, for the very high β-glucuronidase activity of the limpet is accompanied by an appreciable, but distinct, α-glucuronidase activity (Marsh & Levvy, 1958), whereas the snail possesses only the former enzyme (Utusi *et al.* 1949).

## SUMMARY

1. 2-Acetamido-2-deoxygluconolactone (*N*-acetylglucosaminonolactone) was synthesized; this compound and 2-acetamido-2-deoxygalactonolactone (*N*-acetylgalactosaminonolactone) were very powerful competitive inhibitors of β-*N*-acetylglucosaminidase from rat epididymis.

2. These lactones also inhibited the β-*N*-acetylglucosaminidase in the limpet, but less powerfully, and only *N*-acetylglucosaminonolactone, and then only at high concentration, had any effect upon α-*N*-acetylglucosaminidase.

3. The free acids obtained by alkali treatment of the lactones were not inhibitors of β-*N*-acetylglucosaminidase, but some reconversion into the lactones occurred at the acid pH of the enzyme assay.

4. *N*-Acetylgalactosaminonolactone was more stable than *N*-acetylglucosaminonolactone in pure aqueous solution or in buffers at physiological pH.

5. The lactones and the acids derived from them underwent conversion in aqueous solution into equilibrium mixtures containing more than one species, the composition depending on the pH. In particular, there was some conversion of the known *N*-acetylgalactosaminonolactone into a more powerful but less stable inhibitor, probably the 1:5-lactone.

We are greatly indebted to Dr J. W. Minnis for C, H and N analyses, to Dr P. G. Walker for a gift of D-galactosamine hydrochloride and to Dr P. W. Kent for specimens of *N*-benzoyl- and *N*-toluene-*p*-sulphonyl-glucosaminonolactones.

One of us (J.F.) is in receipt of a grant from the Medical Research Council.

## REFERENCES

Bergmann, M., Zervas, L. & Silberkweit, E. (1931). *Ber. dtsch. chem. Ges.* **64**, 2428.
Conchie, J. (1954). *Biochem. J.* **58**, 552.
Conchie, J., Findlay, J. & Levvy, G. A. (1956). *Nature, Lond.*, **178**, 1469.
Conchie, J. & Levvy, G. A. (1957). *Biochem. J.* **65**, 389.
Conchie, J., Levvy, G. A. & Marsh, C. A. (1957). *Advanc. Carbohyd. Chem.* **12**, 157.
Heyns, K. & Koch, W. (1953). *Chem. Ber.* **86**, 110.
Heyworth, R., Borooah, J. & Leaback, D. H. (1957). *Biochem. J.* **67**, 21P.
Hope, D. B. & Kent, P. W. (1955). *J. chem. Soc.* p. 1831.
Hudson, C. S. & Dale, J. K. (1916). *J. Amer. chem. Soc.* **38**, 1431.
Karrer, P. & Mayer, J. (1937). *Helv. chim. acta*, **20**, 407.
Kerr, L. M. H., Graham, A. F. & Levvy, G. A. (1948). *Biochem. J.* **42**, 191.
Kuhn, R. & Roth, H. (1933). *Ber. dtsch. chem. Ges.* **66**, 1274.
Levvy, G. A., Hay, A. J. & Marsh, C. A. (1957). *Biochem. J.* **65**, 203.
Levvy, G. A., McAllan, A. & Marsh, C. A. (1958). *Biochem. J.* **69**, 22.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Marsh, C. A. (1952). *J. chem. Soc.* p. 1578.
Marsh, C. A. & Levvy, G. A. (1958). *Biochem. J.* **68**, 610.
Neuberger, A. & Pitt-Rivers, R. V. (1939). *Biochem. J.* **33**, 1580.
Pugh, D., Leaback, D. H. & Walker, P. G. (1957*a*). *Biochem. J.* **65**, 16P.
Pugh, D., Leaback, D. H. & Walker, P. G. (1957*b*). *Biochem. J.* **65**, 464.
Roseman, S. & Dorfman, A. (1951). *J. biol. Chem.* **191**, 607.
Stacey, M. (1944). *J. chem. Soc.* p. 272.
Utusi, M., Huzi, K., Matumoto, S. & Nagaoka, T. (1949). *Tohoku J. exp. Med.* **50**, 175.
Westphal, O. & Schmidt, H. (1952). *Liebigs Ann.* **575**, 84.

# Crystalline *p*-Aminobenzylpenicillin: Preparation and some Properties

BY A. L. TOSONI, D. G. GLASS AND L. GOLDSMITH*
*Connaught Medical Research Laboratories, University of Toronto*

(*Received* 25 *November* 1957)

Attempts to prepare *p*-aminobenzylpenicillin were made shortly after the role of precursors in penicillin fermentations became known. These attempts apparently failed (see Behrens, 1949, for review). The formation of *p*-aminobenzylpenicillin was reported by Corse, Jones, Soper, Whitehead & Behrens (1948), but no material was isolated. Brewer & Johnson (1953) described the preparation of a partially purified *p*-aminobenzylpenicillin by using *p*-aminophenylacetic acid as precursor. The levels of penicillin obtained in the fermentation were not given, the units/mg. of the material obtained were not indicated and it was stated that attempts at further purification had failed. It was reported, however, that the rate of decrease in concentration of *p*-aminobenzylpenicillin in the blood of dogs was slower than that of benzylpenicillin (penicillin G), that the rate of renal excretion was only one-third as fast as that for benzylpenicillin and that the material was somewhat more stable to acid than benzylpenicillin.

Owing to the presence of its aromatic amino group, *p*-aminobenzylpenicillin would be amenable to treatments yielding several types of derivatives. This fact, together with the properties indicated by Brewer & Johnson (1953), initiated, in our Laboratories, a search for a method of preparing pure *p*-aminobenzylpenicillin in sufficient quantities for a systematic study.

The work of Brewer & Johnson (1953), using *p*-aminophenylacetic acid as precursor, was repeated. Penicillin titres of about 1000 units/ml. were obtained, and the active material when isolated was a yellow-to-brown amorphous powder which assayed at about 300 units/mg. Attempts to further purify the material were not successful.

*p*-Aminobenzylpenicillin is more hydrophilic than *p*-hydroxybenzylpenicillin (penicillin X) and hence cannot be separated from impurities found in the fermentation liquors by the conventional methods of solvent extraction commonly used with benzylpenicillin. Similar difficulties have been encountered in the purification of another hydrophilic penicillin, aminocarboxybutylpenicillin (cephalosporin N, or synnematin B; Newton & Abraham, 1954).

Since *p*-aminobenzylpenicillin is intractable to purification by solvent extraction of fermentation

* Present address: Fermentation Products, Newaygo, Michigan, U.S.A.