The Inhibition of *N*-Acetyl- β -D-Glucosaminase by the (1 $\rightarrow$ 4)- and (1 $\rightarrow$ 5)-Lactones of *N*-Acetylglucosaminic Acid

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The inhibition of *N*-acetyl- β -D-glucosaminase activity by 2-acetamido-2-deoxy-D-gluconolactone was examined. Separation of the (1 $\rightarrow$ 4)- and (1 $\rightarrow$ 5)-lactones was achieved by using paper chromatography or countercurrent distribution and identification was obtained by examination of the relative stability of the components of separated material. Quantitative measurement of the two lactones was by kinetic titration or by a colorimetric method based on their reaction with hydroxylamine. It was shown that only the (1 $\rightarrow$ 5)-lactone acted as an inhibitor of *N*-acetyl- β -D-glucosaminase.

Conchie & Levvy (1957) found that, in general, the enzymic hydrolysis of a simple glycoside was inhibited by the aldono-lactone corresponding in configuration to the sugar residue of the substrate. Findlay, Levvy & Marsh (1958) have shown that 2-acetamido-2-deoxy-D-gluconolactone acted as a powerful inhibitor of *N*-acetyl- β -D-glucosaminase and they originally assumed that the (1 $\rightarrow$ 4)- and (1 $\rightarrow$ 5)-lactones were equally inhibitory. Our findings were more consistent with that reported by Levvy, Hay & Conchie (1964), where, in contrast with earlier evidence, they report that glucono-(1 $\rightarrow$ 5)- and mannono-(1 $\rightarrow$ 5)-lactones are superior to the (1 $\rightarrow$ 4)-lactones in their enzyme inhibitory action. Conchie, Hay, Strachan & Levvy (1967) had also shown that more specific catalytic oxidation of pyranoses to the (1 $\rightarrow$ 5)-lactone resulted in greater inhibitory activity, which is consistent with evidence that we present here.

The enzyme under investigation and referred to above is EC 3.2.1.30 for which the I.U.B. trivial name is β -acetylglucosaminase. Consideration of carbohydrate nomenclature leads us to use the more correct designation *N*-acetyl- β -D-glucosaminase but care should be taken not to confuse this with EC 3.2.1.29 for which an alternative name is *N*-acetyl- β -glucosaminidase.

EXPERIMENTAL

Enzyme preparation. For all experiments the adult rat epididymis was used as a source of *N*-acetyl- β -D-glucosaminase since that tissue has been found by Conchie, Findlay & Levvy (1956) to have a high enzyme activity. The method of preparation was that described by Findlay *et al.* (1958). The fraction precipitated between 20 and 80% saturation with $(\text{NH}_4)_2\text{SO}_4$ was retained. The precipitate was dissolved in water (10 ml/g of tissue) to

give a stock solution, which was further diluted (usually 1:100) before use in the assay.

***p*-Nitrophenyl *N*-acetyl- β -D-glucosaminide preparation.** As described by Findlay *et al.* (1958) glucosamine hydrochloride was acetylated with acetic anhydride in the presence of anhydrous ZnCl_2 . Heating this with *p*-nitrophenol produced *p*-nitrophenyl β -D-glucosaminide tetraacetate. De-*O*-acetylation was achieved by treatment with sodium methoxide in methanol (Leaback, 1960) to give the required *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide. An overall yield of 15% was obtained for this preparation.

Inhibitor. The method used was a modification of that described by Findlay *et al.* (1958), the basis of which was the competitive enzymic inhibition of *N*-acetyl- β -D-glucosaminase by 2-acetamido-2-deoxy-D-gluconolactone. It has been stated (Findlay *et al.* 1958) that 50% inhibition was caused by a 1.3 μM solution of 2-acetamido-2-deoxy-D-gluconolactone. This was the most inhibitory solid preparation known at the commencement of our work. Our assumption that this consists of the pure inhibitory principle was incorrect but this value was used as a reference standard. The lactone samples were dissolved in water and diluted to give three concentrations such that the middle one gave approx. 50% inhibition when used in the assay. The assay procedure used was essentially that of Findlay *et al.* (1958). From the response of the three concentrations of lactone sample compared with that of a mixture incubated in the absence of lactone, the concentration of the lactone sample required to give 50% inhibition was calculated and inhibitory power was expressed as a percentage of the standard.

Hydroxylamine assay. A method for the determination of lactones was described by Kaye & Kent (1953) in which the lactone reacted with alkaline hydroxylamine and on acidification and addition of FeCl_3 a colour was produced. This was a modification of the original method of Hestrin (1949). Hilf & Castano (1958) have used a similar method for the determination of reducing sugars after conversion into the sugar acid lactone. To a 0.05% solution of the lactone (2 ml) was added alkaline hydroxylamine (4 ml), which was 1.0 M with respect to hydroxylamine and 1.75 M with respect to NaOH. After 4 min,

3.34M-HCl (2ml) and 0.37M-FeCl₃ (2ml) were added. After a further 10min the extinction of the solution at 505nm was read on a Unicam SP.500 spectrophotometer in a 1cm light-path cell against water as reference. The calculation was based on an experimentally determined value for the sum of the extinctions of the hydroxamates of both the lactone and the acetyl group of the lactone present.

Titration at constant pH. An E.I.L. automatic titrimeter was used with 0.1M-NaOH as titrant. The volume of NaOH added was plotted against time.

Chromatography. The lactone (25 μ l of a 1% solution) was spotted on Whatman no. 1 paper and developed by descending chromatography for 18h with butan-1-ol-acetic acid-water (12:2:5, by vol.) as developing solvent. Indication was carried out by dipping the chromatogram in a saturated solution of AgNO₃ in ethanol, then in ethanolic NaOH, followed by the removal of the unreduced AgNO₃ with sodium metabisulphite-sodium thiosulphate solution. As an alternative indication, Bromophenol Blue was used to indicate acid zones, or hydroxylamine hydrochloride followed by FeCl₃ to indicate lactone zones.

Countercurrent distribution. The apparatus consisted of 100 tubes constructed to take a 20 ml aqueous phase and an equal volume of solvent phase. The solvent system used consisted of water saturated with butan-1-ol and butan-1-ol saturated with water. The lactone sample was introduced into the first three tubes and 300 exchanges were carried out automatically with a 4 min shaking time and 12 min settling time.

2-Acetamido-2-deoxy-D-gluconolactone. The method of preparation was essentially that described by Findlay *et al.* (1958), in which glucosamine was oxidized with freshly precipitated yellow mercuric oxide and the resulting glucosaminic acid was acetylated with acetic anhydride.

RESULTS

Hydroxylamine assay. Many authors, including Kaye & Kent (1953), Cori & Lipmann (1952) and Brodie & Lipmann (1956), have described the estimation of lactones of sugar acids, including gluconolactone, by using a hydroxylamine reagent at a pH of about 11.0. Lien (1959) criticized the use of such conditions for the estimation of gluconolactone and galactonolactone on the grounds that, at the high pH, hydrolysis of the lactone prevented complete reaction with hydroxylamine, such complete reaction being obtained only in the range pH 6.0–10.0. Our work showed that at pH 11.0 rapid reactions with reproducible results were obtained when galactonolactone, ribonolactone and ethyl acetate were allowed to react. Almost identical molar extinction coefficients were obtained, and these were close to those reported in the literature. The reaction between alkaline hydroxylamine and 2-acetamido-2-deoxy-D-gluconolactone samples was also rapid as shown in timed experiments, and in addition it was shown that possible impurities including D-glucosamine, N-acetyl-D-glucosamine, D-glucosaminic acid, N-acetyl-D-

glucosaminic acid and D-arabinose did not react or otherwise interfere. The reaction between the lactone samples and alkaline hydroxylamine was examined chromatographically with the butan-1-ol-acetic acid-water (12:3:5, by vol.) developing solvent. Treatment with acidified ferric chloride showed that at a reaction pH in the range 7.0–9.0 two hydroxamic acids were produced, the faster-moving one being present only in small amounts. At pH 11.0, however, the intensity of the faster-running component was equal to that of the other component, which had maintained a steady intensity. The R_F value of the faster component was identical with that of acetylhydroxamic acid.

The results suggested that the reaction took place as follows. At pH 7.0–9.0 the lactone group reacted rapidly, and possibly completely, with hydroxylamine to give a hydroxamic acid. The acetyl group of the hydroxamic acid formed, unlike acetamide and N-acetyl-D-glucosaminic acid, which hardly react even at pH 11.0, then reacted slowly with further hydroxylamine to give acetylhydroxamic acid. The rate of the second reaction increased with increasing pH until at pH 11.0 both the first and the second reactions were complete within a few minutes. In the assay it was therefore necessary for the pH to be high enough (i.e. pH 11.0) to ensure that the N-acetyl group also reacted. The purities of lactone samples were assessed by using molecular extinction coefficients based on galactonolactone as standard, assuming that 2 equivalents of hydroxamic acid were formed.

Chromatographic examination of 2-acetamido-2-deoxy-D-gluconolactone. Samples of lactones that were chromatographed with the butan-1-ol-acetic acid-water solvent system followed by detection with silver nitrate showed that three components were present. These had R_X values of 1.7, 2.7 and 3.3 relative to D-glucosaminic acid (R_X 1.0). The components at R_X values of 1.7 and 2.7 were detected by acid indicators whereas the zone at 3.3 was not detected. Hydroxylamine-ferric chloride reagent detected two zones with R_X values of 2.7 and 3.3. Elution of a chromatogram in horizontal segments and assay of the various zones by the enzyme and hydroxylamine assay methods showed the enzyme-inhibitory activity to be associated with the zone of R_X 2.7 whereas that at R_X 3.3 was responsible for the hydroxylamine assay.

Chromatograms on which the silver nitrate indicator had been used were scanned with the reflectance densitometer. When the density of each zone was expressed as a percentage of the total reactive material in all the zones, then for a series of samples of differing composition the zones at R_X 3.3 always corresponded quite closely to the value obtained by the hydroxylamine assay method.

A test was carried out on the stability of the various components in the chromatography solvent. This consisted of the development of a chromatogram in one direction followed by rechromatography at right-angles to the original direction. Only single zones were detected in the second direction, and it was therefore probable that none of these components split into separate components during the chromatography.

The zone at R_x 3.3 was eluted from a number of chromatograms, refluxed with dilute hydrochloric acid and then concentrated in vacuo. Chromatography of the concentrate showed some decomposition to D-glucosaminic acid at R_x 1.0 together with zones at R_x 1.7 and 2.7 and some unchanged material at R_x 3.3.

Since the zone at R_x 2.7 was indicated by the hydroxylamine-ferric chloride indicator and by acid indicators it was assumed to be a lactone of low stability. This component, when eluted, was shown to be enzyme inhibitory, but was not measured to any extent under the conditions of the hydroxylamine assay, although it had been indicated by the neutral hydroxylamine-ferric chloride indicator on the chromatogram. This component could be produced from the component of R_x 3.3 by treating with acid, and since the component at R_x 3.3 responded to the hydroxylamine assay it appeared that these two components represented the (1 $\rightarrow$ 4)- and (1 $\rightarrow$ 5)-lactones. Of these two isomers the (1 $\rightarrow$ 5)-lactone is the less stable (Levene & Simms, 1925; Charlton, Haworth & Peat, 1926) and this must therefore be the component at R_x 2.7. The component with R_x 1.7 was assumed to be *N*-acetyl-D-glucosaminic acid.

Titration at constant pH. Chromatographic experiments had shown evidence of three components present in samples of 2-acetamido-2-deoxy-D-gluconolactone that would be the (1 $\rightarrow$ 4)- and (1 $\rightarrow$ 5)-lactones together with the free acid. In an attempt to determine these components the rate of hydrolysis of samples was studied on the assumption that the (1 $\rightarrow$ 5)-lactone would be more readily hydrolysed (Charlton *et al.* 1926). A constant pH value of 7.0 was found to give a convenient hydrolysis time. The initial rapid addition of alkali was taken as equivalent to the free acid, the second slower stage would represent the hydrolysis of the (1 $\rightarrow$ 5)- and (1 $\rightarrow$ 4)-lactones together and the final slow stage represented the hydrolysis of the (1 $\rightarrow$ 4)-lactone only. It was thus possible by extrapolating the constant rate portions of the curve, representing either hydrolysis of (1 $\rightarrow$ 4)-lactone or (1 $\rightarrow$ 4)- with (1 $\rightarrow$ 5)-lactone, to zero time and, by taking the final constant titration value, it is possible to calculate the percentage of each component present in the sample. A typical titration curve is shown in Fig. 1.

Fig. 1. Typical curve for the titration of 2-acetamido-2-deoxy-D-gluconolactone at a constant pH of 7.0. Volume Z represents the volume of alkali utilized by the slowest hydrolysing lactone (1 $\rightarrow$ 4). Volume Y represents that used by the lactone of lower stability (1 $\rightarrow$ 5). The volume X is utilized in neutralizing the free acid. X:Y:Z represents the molar proportions of acid, (1 $\rightarrow$ 5)-lactone and (1 $\rightarrow$ 4)-lactone respectively.

Fig. 2. Components formed by the hydrolysis of 2-acetamido-2-deoxy-D-gluconolactone at pH 7.0 studied by using various analytical methods. ■, Enzyme assay; ○, (1 $\rightarrow$ 5)-lactone by titration; □, (1 $\rightarrow$ 4)-lactone by titration; △, hydroxylamine assay.

The hydrolysis of a lactone sample at a controlled pH of 7.0 was followed by the enzymic and hydroxylamine assay methods. Calculations were also made for (1 $\rightarrow$ 4)- and (1 $\rightarrow$ 5)-lactone content based on the titration curve that was produced. The results shown in Fig. 2 indicated that the (1 $\rightarrow$ 5)- (or readily hydrolysed) lactone was probably solely responsible for enzyme inhibition, whereas only the (1 $\rightarrow$ 4)-lactone was determined by the hydroxylamine method and the values obtained were almost coincident with the result obtained by titration. The experiment also showed that the results by titration for (1 $\rightarrow$ 5)-lactone and the enzyme assay results had similar slopes but did not coincide, and this demonstrated that the enzyme assay measured (1 $\rightarrow$ 5)-lactone but was numerically biased high. A satisfactory direct relationship between enzyme assay and titration was not found.

Countercurrent enrichment. Preliminary experiments had shown that at least 300 exchanges would

Fig. 3. Countercurrent extraction of 2-acetamido-2-deoxy-D-gluconolactone in a butan-1-ol/water system. Results were obtained on studying the composition of the aqueous phase by hydroxylamine assay (○) and enzyme assay (×) after 300 exchanges.

have to be carried out so that reasonable enrichment could be expected. A run was therefore carried out in which, after 300 exchanges, the aqueous phase was examined by chromatography, hydroxylamine assay and enzyme assay. The results displayed in Fig. 3 showed that the hydroxylamine assay had a peak at about tube 44 whereas the enzyme inhibitor occurred about tube 33. Chromatography revealed that the separation was not complete, but it was evident that the component at R_F 3.3 was determined by the hydroxylamine assay whereas that at R_F 2.7 was enzyme inhibitory. The aqueous phases of those tubes around the two peaks were separated and then, after the butan-1-ol had been removed under reduced pressure, freeze-dried. The resulting solids were shown chromatographically to have reverted to a mixture of components at R_F 1.7, 2.7 and 3.3, but it is noteworthy that one solid that was obtained from tubes 31–35 had an assay of 158% by the enzymic method and 32% by the hydroxylamine assay, whereas the other solid obtained from tubes 40–50 had an assay of 51% by the hydroxylamine method and 83% by the enzyme assay.

DISCUSSION

The work of Findlay *et al.* (1958) had shown that 2-acetamido-2-deoxy-D-galactonolactone acted as an inhibitor of *N*-acetyl- β -D-glucosaminase, and they were also able to show that in this instance the

(1 $\rightarrow$ 5)-lactone was at least of greater inhibitory power if not completely responsible for the inhibition. These workers were, however, unable to distinguish between the two lactone forms of 2-acetamido-2-deoxy-D-gluconolactone by inhibitory activity and therefore concluded that they were of equal activity. By selective catalytic formation of the (1 $\rightarrow$ 5)-lactone, Conchie *et al.* (1967) were able to produce a greater inhibitory activity.

It has now been shown by chromatography and kinetic hydrolysis studies that the (1 $\rightarrow$ 5)-lactone of 2-acetamido-2-deoxy-D-gluconolactone was the inhibitory isomer. It is therefore evident that the activity is not solely controlled by the configuration of the C-4 and C-5 hydroxyl groups, which are *cis* in glucose but *trans* in galactose. It would appear more likely that the ring size of the inhibiting lactone is critical. Leaback (1968) has considered in some detail the mechanism of inhibition of this enzyme by the (1 $\rightarrow$ 5)-lactone in relation to its activity towards various alkyl and aryl 2-acetamido-2-deoxy- β -D-glucopyranoside substrates.

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