Studies on Glucosaminidase

4. THE FLUORIMETRIC ASSAY OF *N*-ACETYL- β -GLUCOSAMINIDASE*

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Mead, Smith & Williams (1955) introduced a method for the fluorimetric assay of β -glucuronidase activity; the method consisted of the enzymic hydrolysis of methylumbelliferyl β -glucuronide, followed by fluorimetric estimation of the liberated methylumbelliferone. Robinson (1956) synthesized methylumbelliferyl β -glucoside and used it in the study of β -glucosidases from a variety of sources; like Mead *et al.* (1955), Robinson found the fluorogenic method convenient and extremely sensitive. Levvy, Hay & Marsh (1957) attempted to use methylumbelliferyl β -glucuronide in the study of limpet β -glucuronidase; they found methylumbelliferone to be unstable, and so considered the method to be too exacting for general use.

In the present paper, a preparation of *N*-acetyl- β -glucosaminidase from ram testis has been used to evaluate the fluorogenic methylumbelliferyl *N*-acetyl- β -glucosaminide as a substrate. A preliminary account of this work has appeared (Leaback & Walker, 1960).

MATERIALS AND METHODS

Methylumbelliferone. Calibration curves were constructed with 4-methylumbelliferone (British Drug Houses Ltd.) purified (m.p. 186–187°) according to Mead *et al.* (1955).

Methylumbelliferyl *N*-acetyl- β -glucosaminide. Methylumbelliferone (1.9 g.) and 5 g. of acetochloroglucosamine (Leaback & Walker, 1957) were dissolved in cold acetone (70 ml.) and 16.5 ml. of *N*-NaOH was added. After standing for 6 hr. at room temperature, followed by several days at 5°, the acetylated glycoside crystallized; only a small amount remained in solution. The solid was recrystallized from ethanol to give 2.9 g. (37% yield) of methylumbelliferyl tetra-acetyl- β -glucosaminide (4-methyl-2-oxo-1:2-benzopyran-7-yl-2'-acetamido-2'-deoxy-3':4':6'-tri-*O*-acetyl- β -D-glucopyranoside), m.p. 254–255°; $[\alpha]_D^{20}$ –18.9° in CHCl₃ (c, 1), (Found: C, 56.8; H, 5.5; N, 2.9. C₂₄H₂₇O₁₁N requires C, 57.0; H, 5.6; N, 2.8%).

The acetylated glycoside (1 g.) was suspended in 50 ml. of anhydrous methanol and on the addition of 1 ml. of *N*-sodium methoxide the compound went rapidly into solution; after a few minutes, the de-*O*-acetylated com-

pound started crystallizing. The mixture was kept overnight, and the solid filtered off. The solid was recrystallized once from hot water, and traces of methylumbelliferone were removed by stirring (magnetically) a suspension of the product in acetone (100 ml.) for 16 hr.; filtration gave 0.49 g. (62% yield) of methylumbelliferyl *N*-acetyl- β -glucosaminide (4-methyl-2-oxo-1:2-benzopyran-7-yl-2'-acetamido-2'-deoxy- β -D-glucopyranoside), m.p. 212–214°; $[\alpha]_D^{20}$ –14.5 in H₂O (c, 0.1), (Found: C, 57.1; H, 5.6; N, 3.6. C₁₈H₂₁O₈N requires C, 57.0; H, 5.5; N, 3.7%).

Tests were carried out for the presence of free methylumbelliferone in solutions of the glycoside. With the optical system described below, the fluorescence of a solution of the glycoside (66 μ M) was the same at pH 7 and pH 10.3. Mead *et al.* (1955) showed that the fluorescence of methylumbelliferone increased greatly with pH over this range. It was estimated that the presence of 0.002% of free methylumbelliferone would have been detectable by this test. The absence of free methylumbelliferone in fresh solutions of the glycoside was confirmed by chromatography on Whatman no. 1 paper, by using downward elution with the non-polar phase of butanol-ethanol-water (4:1:5, by vol.). Spots were rendered visible beneath a mercury lamp (Wood's-glass filter) by heating the chromatogram briefly to 105° and exposing it to ammonia vapour. Methylumbelliferyl *N*-acetyl- β -glucosaminide and (if present) methylumbelliferone showed as fluorescent spots of *R_F* 0.6 and 0.9 respectively.

Spectra. Fluorescence and excitation spectra were determined with an Aminco-Bowman spectrofluorimeter; the instrument was standardized with mercury emission lines and by the method of Sprince & Radley (1956). The ordinates of the spectra (Figs. 1, 2) are arbitrary (dependent in part upon the characteristics of the instrument), but the 'mirror-image' character (see Bowen & Wokes, 1953) of the excitation and fluorescence peaks suggested that the curves were not seriously at fault. For the sake of clarity, the spectra in Figs. 1 and 2 were discontinued short of small scatter peaks.

Absorption spectra were measured in a Unicam SP. 500 spectrophotometer. The small difference in wavelength between the absorption and excitation maxima might be due to the characteristics of the spectrofluorimeter, or to uncertainties in the measurement of absorption spectra of fluorescent compounds (cf. White, Hoffman & Magee, 1957).

Estimation of methylumbelliferone

Spectrophotometric assay. Methylumbelliferone was estimated in alkaline solution with a Unicam SP. 500 spectrophotometer. The absorption at 360 μ of solution in

* Part 3: Woollen, Heyworth & Walker (1961).

0.15M-glycine buffer pH 10.3 was linear with concentration over the range (I) 0.5–11 $\mu\text{g.}$ of methylumbelliferone/ml.

Fluorimetric assay. Fluorimetric estimations of methylumbelliferone were carried out on a Locarte fluorimeter (see Laurence, 1957). The instrument was fitted with quartz optics and a 1 ml. cuvette holder. On the primary side, a Corning 5860 filter together with a Barr and Stroud 365 m μ interference filter were used: the secondary-side filter was a Corning 3389.

In 0.15M-glycine buffer, pH 10.3, linear calibration curves were obtained for methylumbelliferone concentrations of 0.01–0.45 $\mu\text{g./ml.}$ (range II) and 0.0025–0.10 $\mu\text{g./ml.}$ (range III) when read against a standard solution of quinine bisulphate (2 $\mu\text{g./ml.}$ in 0.1N-sulphuric acid).

The fluorescence of methylumbelliferone was unaltered in the presence of the enzyme preparation, albumin, citrate buffer and the substrate, at concentrations used in reaction mixtures. It was not necessary to take special precautions to exclude dust from the solutions.

Preparation and assay of enzyme

At high dilutions, the partially purified preparation from ram testis (Borooah, Leaback & Walker, 1961) showed erratic enzyme activity. J. W. Woollen (personal communication) overcame similar difficulties by the addition of 0.01% of crystalline bovine-serum albumin (Armour Products) to the reaction mixture; after the adoption of this procedure, no further difficulties of this kind were experienced. Unless otherwise stated, the enzyme was incubated at 37° for 30 min. with 0.05M-sodium citrate buffer pH 5.0, 0.01% albumin and 0.264 mM-methylumbelliferyl *N*-acetyl- β -glucosaminide; appropriate blanks were also incubated. The enzyme reaction was stopped, and liberated methylumbelliferone was converted into the anionic form by the addition of 3 ml. of 0.2M-sodium glycinate buffer, pH 10.65. Methylumbelliferone was then estimated spectrophotometrically (range I) or fluorimetrically (range II or III) according to the amount present.

The addition of 3 ml. of 0.2M-glycine buffer, pH 10.65 to reaction mixtures (1 ml.) buffered with 0.05M-citrate to values between pH 3.78 and pH 6.0, gave solutions of pH 10.2–10.6. No change in the fluorescence of methylumbelliferone was detectable within this range, so that adjustment of the final pH before estimation was not necessary (cf. Levvy *et al.* 1957).

RESULTS

Spectra. The excitation and fluorescence spectra of methylumbelliferone (Fig. 1) and methylumbelliferyl *N*-acetyl- β -glucosaminide (Fig. 2) were determined in 0.15M-glycine buffer, pH 10.3. It can be seen that both compounds fluoresce strongly, and that the excitation maximum of the methylumbelliferone anion falls close to the strong mercury emission line at 365 m μ . The fluorimetric estimation of methylumbelliferone in the presence of the glycoside therefore required a filter system which transmitted only the 365 m μ line on the primary side, and wavelengths above 405 m μ on the secondary side. The system chosen was satisfactory since the blanks due to scatter and to the

fluorescence of the glycoside (0.066 mm) and glycine buffer (0.15M) were equivalent to about 0.0015 $\mu\text{g.}$ of methylumbelliferone/ml. As Levvy *et al.* (1957) found, the glycine buffer itself showed a small but significant fluorescent blank; this blank was unaffected by recrystallizing the glycine and was of the expected order for the Raman effects described by Parker (1959).

Fig. 3 shows the absorption spectra of methylumbelliferone ($\lambda_{\text{max.}}$ 360 m μ , ϵ 16 720) and the *N*-acetyl- β -glucosaminide ($\lambda_{\text{max.}}$ 317 m μ , ϵ 13 620) in glycine buffer, pH 10.3; the absorption of the glycoside resembles that of methylumbelliferone in neutral solution ($\lambda_{\text{max.}}$ 320 m μ , ϵ 12 800). Thus, in

Fig. 1. Fluorescence and excitation spectra of methylumbelliferone (0.1 $\mu\text{g./ml.}$) in 0.15M-glycine buffer, pH 10.3. —, Fluorescence spectrum, $\lambda_{\text{max.}}$ 448 m μ ; ---, excitation spectrum, $\lambda_{\text{max.}}$ 364 m μ .

Fig. 2. Fluorescence and excitation spectra of methylumbelliferyl *N*-acetyl- β -glucosaminide (25 $\mu\text{g./ml.}$) in 0.15M-glycine buffer, pH 10.3. —, Fluorescence spectrum, $\lambda_{\text{max.}}$ 378 m μ ; ---, excitation spectrum, $\lambda_{\text{max.}}$ 325 m μ .

common with other phenols (Robinson, Smith, Spencer & Williams, 1952), the methylumbelliferyl anion shows an absorption peak sufficiently different from that of the glycoside to enable the absorption maximum to be used to determine methylumbelliferone in presence of the glycoside.

Stability of methylumbelliferone and the glycoside. Satisfactory recoveries of methylumbelliferone were demonstrated by Mead *et al.* (1955), when the compound was incubated at 37° and pH 4.6 with various tissue preparations. Levvy *et al.* (1957) found methylumbelliferone to be unstable under a variety of conditions, but particularly when irradiated by sunlight or ultraviolet light. Robinson (1956) found that, even in the cold, methylumbelliferyl β -glucoside decomposed appreciably overnight.

In view of these reports, the stabilities of methylumbelliferone and the *N*-acetyl- β -glucosaminide were investigated by incubation under a variety of

conditions followed by fluorimetric assay for methylumbelliferone.

Table 1 shows that methylumbelliferone was stable under conditions likely to be encountered during enzyme assay. However, since solutions of methylumbelliferone showed decreased fluorescence on prolonged storage (one aqueous solution showed a 6.5% loss after 21 days at room temperature), fluorescent measurements were carried out against a quinine sulphate standard; the quinine sulphate solution had excellent storage properties.

Solutions of methylumbelliferyl *N*-acetyl- β -glucosaminide slowly liberated methylumbelliferone (confirmed by paper chromatography) on storage at 0–5°; all substrate solutions were therefore prepared freshly before use. The results also showed that small amounts of methylumbelliferone were liberated on incubation under the conditions of enzyme assay; this non-enzymic hydrolysis was linear with time and substrate concentration, but did not vary greatly over the pH range studied (3.8–6.0).

Effect of time of incubation. The effect of time of incubation on the enzymic and non-enzymic hydrolysis of 0.264 mM-methylumbelliferyl *N*-acetyl- β -glucosaminide is shown in Fig. 4. It can be seen that at pH 4.65 and 5.0 the enzymic and non-enzymic liberation of methylumbelliferone was linear with time for at least 90 min. The maximum amount of hydrolysis of the substrate was 0.9%.

Effect of enzyme concentration. The fact that methylumbelliferone could be conveniently estimated (i.e. with the same volumetric technique) in solutions containing 0.0025–11 μ g./ml. permitted the enzyme concentration-activity curve to be examined over a wide range of dilution.

Fig. 5 shows that over a 1000-fold range the enzyme concentration-activity curve was linear.

Effect of pH. With methylumbelliferyl *N*-acetyl- β -glucosaminide as substrate, the activity of the testicular enzyme varied with pH as shown in

Fig. 3. Absorption spectra of methylumbelliferone and methylumbelliferyl *N*-acetyl- β -glucosaminide in 0.15M-glycine buffer, pH 10.3. —, Methylumbelliferone, λ_{max} 360 m μ ; ---, *N*-acetyl- β -glucosaminide, λ_{max} 317 m μ .

Table 1. *Stability of methylumbelliferone and methylumbelliferyl N-acetyl- β -glucosaminide*

Solutions of methylumbelliferone (0.22 μ g./ml. in water or 0.05M-citrate buffer, 0.055 μ g./ml. in 0.15M-glycine buffer) and methylumbelliferyl *N*-acetyl- β -glucosaminide (0.264 mM in water or 0.05M-citrate buffer, 0.066 mM in 0.15M-glycine buffer) were assayed fluorimetrically (for method see text) before and after incubation under the conditions specified.

Medium	Time (hr.)	Temperature	Loss of fluorescence of methylumbelliferone (%)	Methylumbelliferone liberated from glycoside (μ g./ml.)
Citrate, pH 3.83	1.5	37°	<1.4	0.02
Citrate, pH 4.96	1.5	37	<1.4	0.03
Citrate, pH 6.02	1.5	37	<1.4	0.015
Water	18	20–22	<1.4	0.005
Water	18	0–5	<1.4	<0.0025
Glycine, pH 10.3	1.0	20–22	<1.4	<0.0025
Glycine, pH 10.3	18	20–22	2.5	<0.0025

Fig. 6. The pH optimum depended upon substrate concentration, being 5.0 and 4.6 at substrate concentrations of 0.264 and 2.11 mM respectively. Using other substrates for this enzyme, Borooah *et al.* (1961) found pH optima of 4.4–4.8.

Effect of substrate concentration. No direct measurement of the Michaelis constant of an enzyme for a methylumbelliferyl glycoside has been reported. Fig. 7 shows the results of a typical experiment on the effect of various substrate concentrations on the activity of the testicular enzyme. A 256-fold range of substrate concentration (0.0165–4.22 mM) was used, but at no level was the amount of substrate hydrolysed greater than 0.6 %.

It can be seen from Fig. 7 that the curves are different at pH 4.65 and 5.0; both gave straight-line Lineweaver & Burk (1934) plots and the constants derived therefrom were: maximum velocities 1.0 and 0.84 m-moles/30 min., and Michaelis constants 1.22 and 0.89 mM at pH 4.65 and 5.0 respectively. In view of the non-ionic nature of the substrate, these substrate-concentration–pH effects indicate the ionization of an im-

portant group at the active site of the enzyme (cf. Dixon & Webb, 1958).

DISCUSSION

Since many glycosidases show little specificity towards the aglycone part of the molecules on which they act, the factors to be considered in the choice of a substrate are its ease of synthesis, stability, solubility and rate of hydrolysis by the enzyme. The stability of the liberated aglycone, and the sensitivity and convenience of the estimation technique are also important. Borooah *et al.* (1961) compared an alkyl and several aryl *N*-acetyl- β -glucosaminides as substrates for testicular *N*-acetyl- β -glucosaminidase; they concluded that the *p*-nitrophenyl glycoside was the most suitable. The high sensitivity of the fluorimetric estimation of methylumbelliferone has been demonstrated by Mead *et al.* (1955); the present work was undertaken to assess methylumbelliferyl *N*-acetyl- β -glucosaminide as a substrate for enzyme assay.

Special routes for the preparation of the β -glucuronide were used by Mead *et al.* (1955), and by Marsh & Levvy (1956); the most general method for the synthesis of methylumbelliferyl 1:2-*trans*-

Fig. 4. Effect of time of incubation on degree of hydrolysis. Methylumbelliferyl *N*-acetyl- β -glucosaminide (0.264 mM) was incubated for various periods at 37° in 0.05M-citrate buffer, pH 4.65 (●) and pH 5.0 (○), in the presence (—) and in the absence (---) of *N*-acetyl- β -glucosaminidase.

Fig. 5. Effect of enzyme concentration on reaction velocity. Various dilutions of a preparation of *N*-acetyl- β -glucosaminidase were incubated at 37° in 0.05M-citrate buffer, pH 4.65, for 30 min. Substrate was 2.11 mM-methylumbelliferyl *N*-acetyl- β -glucosaminide; final vol. of reaction mixture was 1.0 ml.; additions of enzyme are expressed as ml. of undiluted preparation. The insert shows a portion of the curve in the region of the origin magnified 40 times.

glycosides seems to be the condensation of an alkaline solution of methylumbelliferone with an acetone solution of the appropriate 1:2-*cis*-aceto-halogeno sugar. Under these conditions, yields of the glucoside (Robinson, 1956) and the *N*-acetyl-glucosaminide were comparable to those of other aryl β -glycosides (cf. Leaback & Walker, 1957).

In preliminary experiments where the activities of enzymes from different sources are to be compared, it is preferable (in the hope of saturating the enzymes present) to use a high substrate concentration. The upper limit of usable substrate concentrations may be determined by the contribution the substrate preparation makes to the blank (cf. Levvy *et al.* 1957). A high fluorescence blank could be due to the fluorescence of the glycoside itself, to the presence of free methylumbelliferone in the preparation or to the non-enzymic release of methylumbelliferone from the glycoside. The information provided by the excitation and fluorescence spectra of methylumbelliferone and the *N*-acetyl- β -glucosaminide permitted the selection of filters which effectively decreased the blank due to scatter and to the fluorescence of the glycoside. Robinson (1956) had difficulty in removing free methylumbelliferone from the glucoside; an effi-

cient method of doing this for the *N*-acetyl- β -glucosaminide has been devised. Under the conditions used in the present work, the non-enzymic liberation of methylumbelliferone from the substrate was not a serious handicap; this effect may, however, limit the use of high substrate concentrations with extremely low enzyme activities.

In kinetic experiments, the use of a wide range of substrate concentration has the advantage of avoiding large graphical extrapolations. Provided that the preparation gives satisfactory blanks, the upper limit of substrate concentration is dependent upon the solubility of the substrate. The glycosides of large aromatic aglycones are usually sparingly soluble, so it is not surprising that the solubility of methylumbelliferyl *N*-acetyl- β -glucosaminide (about 8 mm at 20°) is smaller than that of other aryl *N*-acetyl- β -glucosaminides (cf. Borooah *et al.* 1961). Given the condition that only a small proportion of the substrate should be hydrolysed, the lower limit of substrate concentration is dependent upon the sensitivity of the assay procedure. Owing to the high sensitivity of the fluorimetric method, the methylumbelliferyl glycoside can be used over a wider concentration range than is possible with other, slightly more soluble, aryl glycosides. Thus, in the experiments described in Fig. 7, a 256-fold range of substrate concentration was used: this is in contrast to the 20- to 30-fold range which can be achieved with the phenyl or *p*-nitrophenyl *N*-acetyl- β -glucosaminides (cf. Pugh, Leaback & Walker, 1957; Borooah *et al.* 1961).

The choice of substrate can be influenced by the maximum velocity of enzymic hydrolysis of the substrates concerned; in this respect, there was

Fig. 6. Effect of pH on reaction velocity. Incubation in 0.05 M-citrate buffer for 30 min. at 37°. Substrate concentration: ○, 0.264 mM; ●, 2.11 mM.

Fig. 7. Effect of substrate concentration on reaction velocity. Various concentrations of methylumbelliferyl *N*-acetyl- β -glucosaminide were incubated with the enzyme for 30 min. at 37° in 0.05 M-citrate buffer, pH 4.65 (●) and pH 5.0 (○). The insert shows portions of the curves in the region of the origin magnified 5 times.

little to choose between the *p*-nitrophenyl and methylumbelliferyl *N*-acetyl- β -glucosaminides, since, with the testicular enzyme, the ratio of the respective maximum velocities was 1:0.95.

The Laurence (1957) fluorimeter proved to be particularly suitable for the present kind of study. In agreement with the findings of Mead *et al.* (1955) and Robinson (1956), the fluorimetric estimation of methylumbelliferone was found to be simple and convenient.

Robinson (1956) pointed out that the fluorimetric method permitted the use of very dilute enzyme preparations and that the high dilution would tend to eliminate the effect of interfering substances. Before assaying very dilute preparations, it may be advisable to establish the form of the enzyme activity-concentration curve; it has been shown here that, with a methylumbelliferyl glycoside, this can be done conveniently with a combination of spectrophotometric and fluorimetric techniques.

The fluorimetric method permits the use of low substrate concentrations with the consequent advantage of conserving substrate; however, the advisability of using low substrate concentrations should be investigated, since results presented here show that characteristic properties of an enzyme (e.g. Michaelis constant, pH optimum) may change under these conditions. Further applications of the fluorogenic method for the estimation of glycosidase activity are being investigated.

SUMMARY

1. 4-Methylumbelliferyl *N*-acetyl- β -glucosaminide has been prepared, and its use as a substrate in the assay of *N*-acetyl- β -glucosaminidase has been described.

2. The range and sensitivity of spectrophotometric and fluorimetric estimations of methylumbelliferone gave the procedure advantages over existing methods.

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Purification and Specificity of Pancreatic Elastase

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For the determination of the amino acid sequences in proteins, the most useful reagents for the initial breakdown of the molecules are the proteolytic enzymes. Trypsin and chymotrypsin

are the most generally used because of their high degree of specificity and because they have different specificities from one another. It would be useful to have other enzymes having complementary specificities which could be used in conjunction with trypsin and chymotrypsin. During work on the structure of insulin (Ryle, Sanger, Smith & Kitai, 1955) it was found that, when insulin was treated with crude pancreatic extracts,

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