The method is capable of extension to the preparation of other nucleoside polyphosphates labelled with radioactive phosphorus.

SUMMARY

1. The reaction of orthophosphate with adenosine 5′-monophosphate, diphosphate and triphosphate in the presence of *NN′*-di*cyclo*hexylcarbodiimide has been used for the preparation of labelled adenosine polyphosphates.

2. The adenosine diphosphate so prepared is labelled exclusively in the terminal phosphate group. The adenosine triphosphate and adenosine tetraphosphate so prepared are labelled in the terminal phosphate group, and to a lesser extent in the penultimate phosphate group.

3. A feature of the method is that carrier-free labelled orthophosphate can be used without prior dilution with unlabelled orthophosphate.

4. The reaction of pyrophosphate with adenosine 5′-monophosphate in the presence of *NN′*-di*cyclo*hexylcarbodiimide has been used for the preparation of labelled adenosine triphosphate.

5. The adenosine triphosphate so prepared is labelled in the β- and γ-phosphate groups to an equal extent.

6. An improved method for the preparation of *NN′*-di*cyclo*hexylcarbodiimide is described.

I am indebted to Professor H. A. Krebs for the privilege and pleasure of working in his laboratory. I wish to thank Mr R. Hems for discussions and much helpful advice, Mr K. Jones for advice on the use of ultraviolet photography, Dr H. L. Kornberg for advice on autoradiography, Dr W. Bartley for a gift of potato apyrase and Professor J. Baddiley for pointing out to me the necessity of the refluxing procedure in the preparation of *N,N′*-di*cyclo*hexylthiourea.

My thanks go to the Sigma Chemical Co., St Louis, Missouri, for a most generous gift of adenosine tetraphosphate.

REFERENCES

Bell, R. N. (1950). *Inorg. Synth.* **3**, 98.
Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Crowther, J. (1954). *Analyt. Chem.* **26**, 1383.
Derache, R. & Lowy, R. (1955). *Bull. Soc. Chim. biol., Paris,* **37**, 1347.
Eggleston, L. V. (1954). *Biochem. J.* **58**, 503.
Hall, R. H. & Khorana, H. G. (1954). *J. Amer. chem. Soc.* **76**, 5056.
Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Hems, R. & Bartley, W. (1953). *Biochem. J.* **55**, 434.
Khorana, H. G. (1954). *J. Amer. chem. Soc.* **76**, 3517.
Khorana, H. G. & Todd, A. R. (1953). *J. chem. Soc.* p. 2257.
Knivett, V. A. (1954). *Biochem. J.* **56**, 602.
Krebs, H. A. & Hems, R. (1953). *Biochim. biophys. Acta*, **12**, 172.
Lee, K. H. & Eiler, J. J. (1951). *Science*, **114**, 393.
Lieberman, I. (1956). *J. biol. Chem.* **219**, 307.
Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). *Studies of Biosynthesis in* Escherichia coli. Carnegie Institution of Washington, publication no. 607.
Rosenberg, H. (1955). *Aust. J. expt. Biol. med. Sci.* **33**, part 1, 17.
Schmidt, E., Seefelder, M., Jennen, R. G., Striewsky, W. & Martius, H. (1951). *Liebigs Ann.* **571**, 83.
Skita, A. & Rolfes, H. (1920). *Ber. dtsch. chem. Ges.* **53**, 1242.
Thoai, N.-v., Roche, J. & An, T.-T. (1954). *Bull. Soc. Chim. biol., Paris*, **36**, 529.
Todd, A. R. (1955). *Ann. Acad. Sci. fenn.* Ser. A II, **60**, 19.
Veall, N. (1948). *Brit. J. Radiol., N.S.*, **21**, 347.
Webster, G. C. & Varner, J. E. (1954). *Arch. Biochem. Biophys.* **52**, 22.
Whittam, R., Bartley, W. & Weber, G. (1955). *Biochem. J.* **59**, 590.
Zetterström, R. & Ljunggren, M. (1951). *Acta chem. scand.* **5**, 291.

Properties of Limpet β-Glucuronidase

By G. A. LEVVY, A. J. HAY and C. A. MARSH
Rowett Research Institute, Bucksburn, Aberdeenshire

(*Received* 16 *July* 1956)

The common limpet (*Patella vulgata*) was found to contain an α-glucuronidase (Conchie, Levvy & Marsh, 1956). This mollusc, like many others, was already known to possess β-glucuronidase activity (Dodgson, Lewis & Spencer, 1953), and may be one of the best sources of this enzyme for use in the hydrolysis of steroid glucuronides in urine. It was necessary to compare the properties of the two enzymes, and the present paper is concerned with the β-glucuronidase. Most of the experiments were done with phenolphthalein β-glucuronide, which has come to be the standard substrate for this enzyme. The opportunity was also taken to examine the fluorimetric method of Mead, Smith & Williams (1955), 4-methylumbelliferone β-glucuronide being used as substrate. Among other possible advantages is the fact that this substrate can be synthesized (Marsh & Levvy, 1956*a*).

EXPERIMENTAL

Preparation of the enzyme. Freshly dissected limpet visceral humps were suspended in water (10 ml./g. of moist tissue) by means of a glass homogenizer. According to Dodgson *et al.* (1953), most of the β-glucuronidase activity is extracted by homogenizing in water. The homogenate was frozen and thawed, and centrifuged at 10000 *g* for 15 min. The supernatant was fractionated with $(NH_4)_2SO_4$ at pH 5·2, with centrifuging as before, and the fraction separating between 20 and 80% saturation was retained and dissolved in water (2·5 ml./g. of tissue). This preparation kept indefinitely at −20° or 0°. There was slow separation of traces of insoluble material which could be removed without loss in activity. The stock preparation was usually diluted 400-fold before use (final concn. in terms of moist tissue, 0·1%); at this dilution, the activity fell about 10% daily at 0°.

Enzyme assay with phenolphthalein glucuronide. The assay with phenolphthalein glucuronide (Talalay, Fishman & Huggins, 1946) was done by the general procedure described previously (Levvy, 1952). The volume of the incubation mixture was 4 ml., and after 1 hr. at 37° the reaction was stopped and the phenolphthalein colour developed by the addition of 4 ml. of glycine–Na_2CO_3 buffer. In the pH–activity experiments, NaOH was then added to bring all the tubes to the same pH, and the final volume was made up to 10 ml. instead of the customary 8 ml. All results were read from the appropriate calibration curve.

Except where otherwise stated, the acetate buffer used in the experiments was made with NaOH. The phthalate buffer contained the potassium salt.

Fluorimetric measurements

The following modifications were made in the method of Mead *et al.* (1955).

Instrument. An H764 Spekker fluorimeter (with photomultiplier) was used. The 8 ml. cells contained exactly 6·5 ml. of solution and were used without covers. The primary filters were Wood's glass and the secondary filter was Wratten 47: the other types and combinations of filter which were tried were no better, and most of them were less satisfactory.

Glycine buffer. To develop the fluorescence in 4-methylumbelliferone solutions, 0·2 M glycine–NaOH buffer, pH 10·3, was employed. This had a blank somewhat higher than water, but the same as equimolar A.R. KCl solution. It was important to use A.R. glycine, since less pure material had a very high blank. The pure buffer gave a transmission of 33% (equivalent to 0·0008 μg. of 4-methylumbelliferone/ml.) when read against a quinine bisulphate standard containing 0·05 μg./ml., and this blank determined the lower limit of the method with our instrument.

Calibration curve for 4-methylumbelliferone. On exposure to sunlight or to an ultraviolet lamp, 4-methylumbelliferone solutions (at least up to 0·8 μg./ml.) were found to be unstable at neutral pH, and very unstable in the glycine buffer. At pH 4–5, the solutions were much more stable, but there was considerable decomposition in 0·05 M trichloroacetic acid, even in the dark. Attempts to stabilize the solutions in glycine buffer by adding ascorbic acid or sodium dithionite were unsuccessful.

Dilute solutions of 4-methylumbelliferone were prepared just before use and kept in the dark as much as possible. To 5 ml. of solution was added 5 ml. of glycine buffer, and the fluorescence was measured at once against a suitable quinine bisulphate standard in 0·1 N-H_2SO_4. Linear calibration curves were obtained with quinine bisulphate standards ranging from 0·05 μg./ml. (for 0·0002–0·0015 μg. of 4-methylumbelliferone/ml.) to 2 μg./ml. (for 0·01–0·075 μg. of 4-methylumbelliferone/ml.).

RESULTS

Variation in activity with pH. Dodgson *et al.* (1953) observed a broad optimum at pH 4 for the hydrolysis of 0·045 M *p*-chlorophenyl glucuronide in 0·5 M acetate buffer by limpet β-glucuronidase. As shown in Fig. 1, the optimum for the hydrolysis of 0·00125 M phenolphthalein glucuronide in 0·05 M acetate buffer made with NaOH was at pH 3·8; replacement of NaOH by KOH had no effect on the curve. The position of the peak could be altered, without marked change in the optimum activity, by varying the concentration of buffer or substrate. Changes in pH optimum have been noted previously with β-glucuronidase from other sources (Smith & Mills, 1953; Levvy, 1954).

Fig. 1 also shows the pH–activity curve for the hydrolysis of 0·00125 M phenolphthalein glucuronide in 0·25 M phthalate buffer: the peak was at pH 4·6. The shape of the curve, as well as the position of the

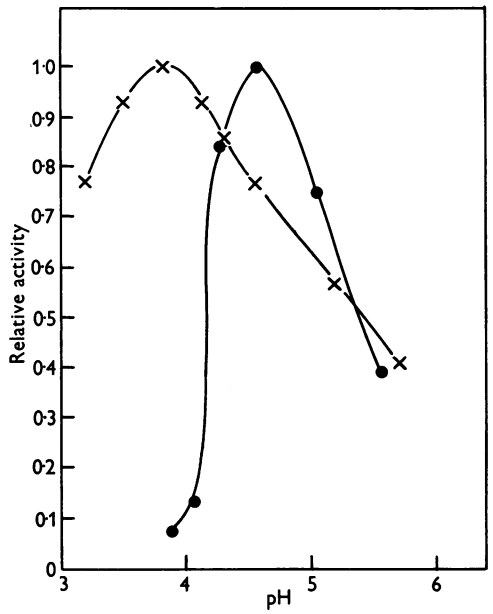

Fig. 1. Hydrolysis of 0·00125 M phenolphthalein glucuronide by limpet β-glucuronidase at varying pH in 0·05 M acetate (×) and 0·25 M phthalate buffer (●). (The optimum activity with each buffer is taken as 1·0.)

peak, could be altered by changing the buffer concentration. Optimum activity was only about 10% higher in phthalate than in equimolar acetate buffer, allowing for the effect of the buffer on the phenolphthalein colour. Smith & Mills (1953) noted considerable enhancement of animal β-glucuronidase activity by phthalate as compared with acetate buffer.

Variation in stability with pH *and temperature.* Measured samples of an enzyme preparation, initially at pH 6–7, were adjusted to the required pH with HCl or NaOH at 0°. After a predetermined period at 0° or 37°, the pH was brought back at 0° to the original figure, and the activities were compared with that of a control kept at 0°. Assays were done with 0·00125 M phenolphthalein glucuronide in 0·05 M acetate buffer, pH 4·14. In agreement with Dodgson *et al.* (1953), the enzyme was found to be rapidly inactivated below pH 3 at 0° (Fig. 2). At 37° it was slowly inactivated below pH 4. On the other hand, it displayed considerable stability at alkaline pH values. For purposes of comparison, experiments were also done with preparations of mouse-liver β-glucuronidase (Levvy, 1952), which showed a considerably narrower range of stability at varying pH. Rumen β-glucuronidase was stable only at pH 6–7 (Karunairatnam & Levvy, 1951). On the basis of the present experiments, a pH of 4·1–4·2 was adopted for routine assay of limpet β-glucuronidase.

Heating an unbuffered limpet preparation (approx. pH 5) for 5 min. at the usual dilution taken for assay caused 15% inactivation at 60° and 35% at 70°.

Effect of varying concentrations of substrate and inhibitors. Fig. 3 shows the variation in enzyme activity with the phenolphthalein glucuronide concentration, and the effects of saccharo-1:4-lactone and boiled mucic acid solution (Levvy, 1952), both of which acted as competitive inhibitors. When the results were analysed by the method of Lineweaver & Burk (1934), this particular experiment gave values of $2{\cdot}7 \times 10^{-5}$ M for K_m, the dissociation constant of the enzyme–substrate complex, and $0{\cdot}89 \times 10^{-7}$ M and $9{\cdot}8 \times 10^{-6}$ M for K_i, the dissociation constant of the enzyme–inhibitor complex, for saccharo-1:4-lactone and boiled mucic acid respectively. Mean values for the dissociation constants obtained in a number of experiments were: phenolphthalein glucuronide, $2{\cdot}4 \times 10^{-5}$ M; saccharo-1:4-lactone, $1{\cdot}05 \times 10^{-7}$ M; boiled mucic acid, $6{\cdot}7 \times 10^{-6}$ M. The affinities of the first two compounds were thus five times greater than those with mouse-liver β-glucuronidase (Levvy, 1952), but the relative affinities were the same (affinity = 1/dissociation constant). Boiled mucic acid had a somewhat higher relative affinity for the limpet than

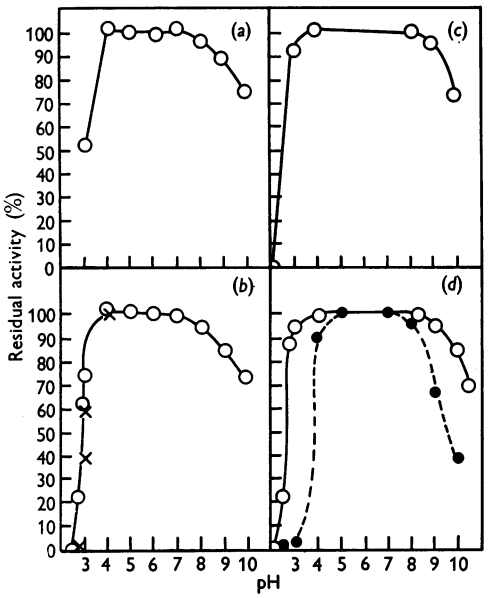

Fig. 2. Stability of limpet and mouse-liver β-glucuronidase at varying pH: (*a*) 1 hr. at 37°; (*b*) 15 min. at 37°; (*c*) 1 hr. at 0°; (*d*) 1 min. at 0°. ○, Limpet enzyme adjusted with NaOH and HCl; ×, limpet enzyme adjusted with NaOH and acetic acid; –●–, mouse-liver enzyme adjusted with NaOH and HCl.

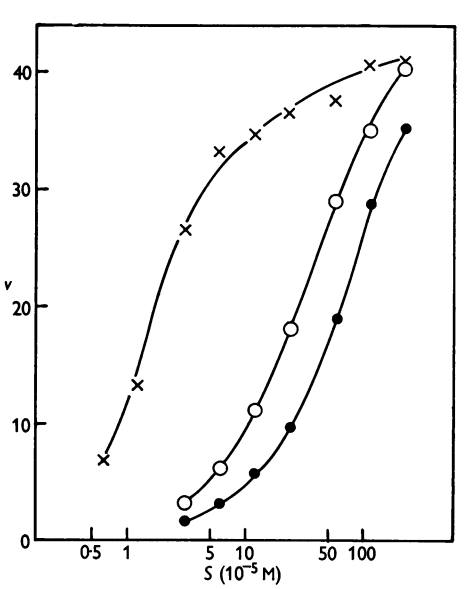

Fig. 3. Effect of varying the substrate concentration (*S*) on the rate of hydrolysis (*v*) of phenolphthalein glucuronide by limpet β-glucuronidase in the absence and the presence of inhibitors: ×, no inhibitor; ○, $1{\cdot}25 \times 10^{-4}$ M mucic acid (boiled); ●, $2{\cdot}5 \times 10^{-6}$ M saccharo-1:4-lactone. Acetate buffer, pH 4·14, final concn. 0·05 M.

for the mouse-liver enzyme: in this instance the term affinity has no theoretical significance since inhibition is probably due to galactosaccharolactone present in the boiled solution.

Similar experiments were done in which the fluorimetric substrate, 4-methylumbelliferone glucuronide (Mead *et al.* 1955), prepared by chemical synthesis (Marsh & Levvy, 1956*a*), was treated as a competing substrate in the hydrolysis of phenolphthalein glucuronide, and a value of $1{\cdot}75 \times 10^{-5}$ M was obtained for K_i. Control experiments showed that fluorescence due to liberated 4-methylumbelliferone did not pass the filter in the phenolphthalein estimation.

Inhibition by excess of phenolphthalein glucuronide was not seen with limpet β-glucuronidase. Maximal velocity of hydrolysis was reached with this substrate at a concentration of 0·00125 M.

Effects of varying the period of hydrolysis and the enzyme concentration. The rate of hydrolysis of 0·00125 M phenolphthalein glucuronide in 0·05 M acetate buffer, pH 4·14, remained unchanged during incubation for 4 hr. at 37°. Increasing the concentration of the enzyme preparation during incubation for 1 hr. under the same conditions caused a slight net increase in activity per unit of enzyme (Fig. 4). Effects of the same order (10 %) could be produced by addition of a filtrate from a boiled, relatively concentrated preparation to the usual concentration of enzyme (1 'unit'). A diffusate from an unboiled preparation did not produce this effect.

Hydrolysis of 4-methylumbelliferone β-glucuronide. Fig. 5 shows that the peak in the pH–activity curve obtained for the hydrolysis of 10^{-5} M 4-methylumbelliferone glucuronide (synthetic) by limpet β-glucuronidase in 0·05 M acetate buffer was at pH 3·8. The stock enzyme preparation had to be diluted 80 000-fold before use (corresponding to a final tissue concentration of 0·0005 %), and 0·5 ml. of this diluted enzyme was employed in a final incubation volume of 4 ml. Incubation was carried out for 1 hr. at 37° in the dark, and 5 ml. of 0·2 M glycine buffer was added. All tubes were brought to the same final pH by adding a predetermined volume of NaOH, and the final volume was brought to 10 ml. The tubes were stored in the dark at 0° until read against a quinine bisulphate standard containing 0·2 μg./ml. At the concentration of substrate employed, the enzyme was by no means saturated, the dissociation constant determined indirectly having a value of $1{\cdot}75 \times 10^{-5}$ M (see above), but the maximum hydrolysis was only 0·2 %.

There was measurable fluorescence in the 10^{-5} M substrate control, corresponding to a figure of 0·001–0·003 μg. of 4-methylumbelliferone/ml. after adding glycine buffer. This value places an upper limit on the substrate concentration that may be employed, depending on the strength of the quinine standard, and thus introduces difficulties into more detailed kinetic studies. The substrate was freed

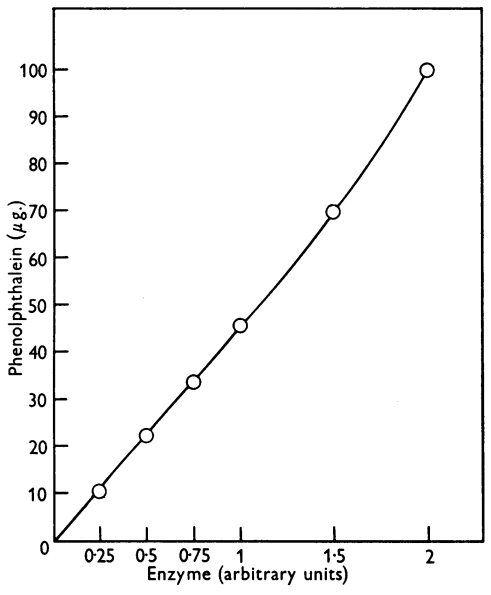

Fig. 4. Phenolphthalein liberated from 0·00125 M phenolphthalein glucuronide in 0·05 M acetate buffer, pH 4·14, by varying amounts of limpet β-glucuronidase after 1 hr. at 37°.

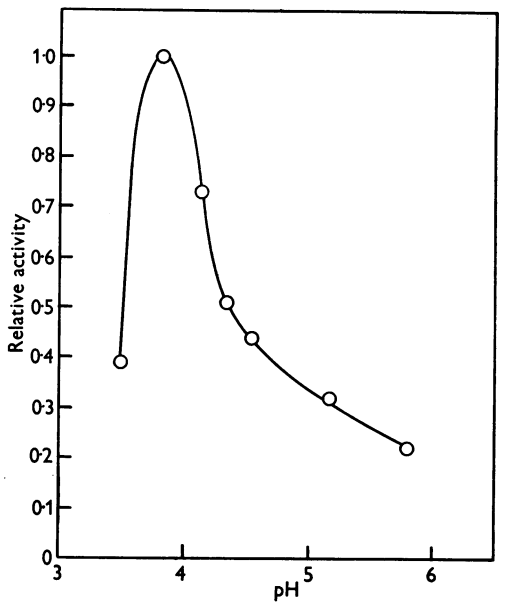

Fig. 5. pH–Activity curve for the hydrolysis of 10^{-5} M 4-methylumbelliferone glucuronide by limpet β-glucuronidase in 0·05 M acetate buffer.

from last traces of 4-methylumbelliferone before use by precipitation from ethanol solution with ether.

Effects of metal ions. Doyle, Katzman & Doisy (1955) have described the inhibition of the β-glucuronidase from *Escherichia coli* by Cu^{2+}, Ag^{+} and Hg^{2+} ions in low concentration, and the reversal of inhibition by chelating agents and by L-cysteine. As shown in Table 1, limpet β-glucuronidase was inhibited by Ag^{+} and Hg^{2+} ions, but only very slightly by Cu^{2+} ions. Inhibition by both Ag^{+} and Hg^{2+} ions was reversed by ethylenediaminetetra-acetic acid (EDTA) and by L-cysteine, the latter being much more efficient in overcoming inhibition. The action of silver could also be overcome very efficiently by adding Cl^{-} ions. No other metal ion tested (Zn^{2+}, Co^{2+}, Ba^{2+}, Ca^{2+}, Mn^{2+}, Ni^{2+}) was any more effective than Cu^{2+} ions, nor were sodium arsenite and sodium borate. Unlike the *E. coli* preparation, which is activated by EDTA and L-cysteine in absence of added metal ions (Doyle *et al.* 1955), limpet β-glucuronidase was unaffected by EDTA alone and was slightly inhibited by L-cysteine.

Identification of free glucuronic acid in the enzyme hydrolysate. This was done chromatographically as described in an earlier paper (Marsh & Levvy, 1956*b*), and the only spots obtained were those for D-glucurone and D-glucuronic acid. There was no trace of pentose.

For the enzymic hydrolysis, 5 ml. of conc. stock limpet enzyme was incubated with 5 ml. of 0·02M phenolphthalein glucuronide, pH 4·1, for 4 hr. at 37°. The mixture was then dialysed against 100 ml. of water for 72 hr. at 0°, and the diffusate, after adjustment to pH 2·5 with H_2SO_4, was extracted in a separating funnel with ethyl acetate until free from phenolphthalein and unhydrolysed glucuronide. Glucuronic acid was determined in a portion by measuring the reducing power (Levvy, 1946), and the total corresponded to 17·4 mg. (90% hydrolysis of the substrate), after correction for the enzyme blank. Substrate incubated alone showed no liberation of reducing substance or phenolphthalein at this stage.

The aqueous solution was shaken with $BaCO_3$ to remove H_2SO_4, filtered, concentrated *in vacuo* to 20 ml., and treated with Amberlite IR-120 (H) for 1 hr. The filtrate was maintained at 100° for 15 min. and evaporated to dryness *in vacuo*, and the resulting gum was dissolved in water for chromatography. As chromatographic control, a solution of sodium glucuronate of similar concentration was treated likewise after deionization.

DISCUSSION

The main object of this work was to lay a foundation for distinguishing between limpet β-glucuronidase and the other hexuronidases present in the preparation, namely, α-glucuronidase and α- and β-galacturonidase (Conchie *et al.* 1956), as well as for purifying the β-glucuronidase. From our present results, the limpet visceral hump is exceptionally high in β-glucuronidase activity in comparison with most animal tissues (cf. Fishman, 1955), giving a figure of approximately 100 000 phenolphthalein units/g. wet wt. Billet (1954) found that most of the β-glucuronidase activity in the Roman snail (*Helix pomatia*) was confined to the digestive gland.

For most purposes, the fluorimetric method for β-glucuronidase assay (Mead *et al.* 1955) is probably too exacting to displace the phenolphthalein method (Talalay *et al.* 1946). The fluorimetric method may, however, be useful when extreme sensitivity is required as, for example, in the assay of normal body fluids. By the use of the Spekker absorptiometer and the H764 Spekker fluorimeter (quinine bisulphate standard 0·2 μg./ml.), the difference in sensitivity between the two methods was about

Table 1. *Effects of copper sulphate, silver nitrate and mercuric chloride on limpet β-glucuronidase activity, and the reversing actions of EDTA and L-cysteine*

Residual activity is expressed as percentage of control. Substrate: 0·00063M phenolphthalein glucuronide. Buffer: 0·05M acetate, pH 4·14.

		Reversing agent				
			EDTA		Cysteine	
Metal ion	Final concn. (M)	None	0·002M	0·0002M	0·002M	0·0002M
None	—	100	100	100	75	95
Cu^{2+}	0·001	88	—	—	—	—
Ag^{+}	0·001	0	—	—	—	—
	0·0001	26	43	35	84	100
	0·000025	84	—	88	—	—
Hg^{2+}	0·001	0	—	—	—	—
	0·0001	10	—	26	—	47
	0·000025	18	41	38	70	69

1000-fold; this was reduced to 100-fold, in terms of total aglycone released, when the micro-scale modification (Levvy, 1954) of the phenolphthalein method was employed. The greater sensitivity of the fluorimetric method was due almost entirely to the greater sensitivity of fluorimetric as compared with colorimetric measurements, since the rates of hydrolysis of 4-methylumbelliferone glucuronide and phenolphthalein glucuronide by a limpet preparation were much the same. Since many of the difficulties associated with the fluorimetric method were due to the instability of the aglycone at alkaline pH values, the fluorimetric assay might be improved by seeking a new substrate of suitable structure.

SUMMARY

1. Some of the properties of β-glucuronidase from *Patella vulgata* have been studied, including its action on phenolphthalein glucuronide and 4-methylumbelliferone glucuronide, and the effects of various inhibitors.

2. In dilute acetate buffer the pH optimum for both substrates was at pH 3·8.

3. The following dissociation constants (at 37°) were obtained for complexes formed by the enzyme: phenolphthalein glucuronide, $2{\cdot}4 \times 10^{-5}$ M; 4-methylumbelliferone glucuronide, $1{\cdot}75 \times 10^{-5}$ M; saccharo-1:4-lactone, $1{\cdot}05 \times 10^{-7}$ M; boiled mucic acid solution, $6{\cdot}7 \times 10^{-6}$ M.

REFERENCES

Billet, F. (1954). *Biochem. J.* **57**, 159.
Conchie, J., Levvy, G. A. & Marsh, C. A. (1956). *Biochem. J.* **62**, 24P.
Dodgson, K. S., Lewis, J. I. M. & Spencer, B. (1953). *Biochem. J.* **55**, 253.
Doyle, M. L., Katzman, P. A. & Doisy, E. A. (1955). *J. biol. Chem.* **217**, 921.
Fishman, W. H. (1955). *Advanc. Enzymol.* **16**, 361.
Karunairatnam, M. C. & Levvy, G. A. (1951). *Biochem. J.* **49**, 210.
Levvy, G. A. (1946). *Biochem. J.* **40**, 396.
Levvy, G. A. (1952). *Biochem. J.* **52**, 464.
Levvy, G. A. (1954). *Biochem. J.* **58**, 462.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Marsh, C. A. & Levvy, G. A. (1956*a*). *Nature, Lond.*, **178**, 589.
Marsh, C. A. & Levvy, G. A. (1956*b*). *Biochem. J.* **63**, 9.
Mead, J. A. R., Smith, J. N. & Williams, R. T. (1955). *Biochem. J.* **61**, 569.
Smith, E. E. B. & Mills, G. T. (1953). *Biochem. J.* **54**, 164.
Talalay, P., Fishman, W. H. & Huggins, C. (1946). *J. biol. Chem.* **166**, 757.