accomplished. Further, the use of a differential colour reaction is less time-consuming than alternative methods which involve separation of the two DNP-bases (cf. Axelrod *et al.* 1953).

In the rare instances where more than two DNP-bases are present, as, for example, in *Neurospora* lipids, which contain ethanolamine, serine and 1-amino-2-methylpropan-2-ol (Ellman & Mitchell, 1954), the procedure described by Levy (1954) for DNP-amino acids can be used. When this method was applied to a mixture of DNP-ethanolamine and DNP-serine the recovery was good (98%) but the colorimetric method was quicker.

The experimental results on the application of the colour test to the DNP-bases suggest that the acidic group in the DNP-serine is responsible for the difference in the dissociation constants of the coloured forms. This is confirmed by the fact that the ethyl ester of DNP-serine reacts like DNP-ethanolamine and that DNP-ethanolamine-*O*-phosphoric acid reacts like DNP-serine. These observations also explain why the colour reaction with DNP-lipids simulates DNP-serine and methylated DNP-lipids simulate DNP-ethanolamine, as in the first case the phosphoric acid groups are free and in the second case they are in the form of methyl esters.

The procedure described in this paper was developed as an adjunct to the study of DNP-lipids (Collins & Wheeldon, 1957) but, if used in conjunction with the method described by Wheeldon & Collins (1957) for total amino N, should be of wide applicability.

SUMMARY

1. A method depending on a differential colour reaction for the determination of ethanolamine and serine in phospholipids is described.

2. The advantages of the method, as compared with those of other methods, are discussed.

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Substrates for Rumen β-Glucosidase

By G. N. FESTENSTEIN
The Rowett Research Institute, Bucksburn, Aberdeenshire

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Substrates used for β-glucosidase include natural products such as cellobiose and salicin as well as synthetic compounds like the nitrophenyl glucosides (Aizawa, 1939; Conchie, 1954). Enzyme extracts from single micro-organisms have been shown to vary in their specificities. Jermyn (1952) separated eight components in an *Aspergillus oryzae* extract, with varying degrees of specificity towards different substrates, whereas a *Stachybotrys atra* preparation hydrolysed *p*-nitrophenyl β-glucoside but not *o*-nitrophenyl β-glucoside or cellobiose (Jermyn, 1955). Differences in relative cellobiase and salicinase activities in preparations from different sources were observed by Grassmann, Zechmeister, Tóth & Stadler (1933) and Reese & Levinson (1952). Barnett, Ingram & Swain (1956) studied the β-glucosidase activity of different yeasts and found that all split aesculin, but many would not split any one of arbutin, salicin or cellobiose. In the present investigation *o*-nitrophenyl β-glucoside, cellobiose and salicin have been used in a comparative study of β-glucosidase activity from cell-free extracts of mixed micro-organisms from sheep rumen.

EXPERIMENTAL

Materials. *o*-Nitrophenyl β-D-glucoside was kindly provided by Dr J. Conchie. Cellobiose (shown to be chromatographically pure) and glucono-1:4-lactone were obtained from L. Light and Co. Ltd., salicin from Hopkins and Williams Ltd., glucose oxidase from Sigma Chemical Co., and peroxidase from C. F. Boehringer und Soehne.

β-Glucosidase preparations. These were produced by butanol extraction of sheep-rumen micro-organisms centrifuged at 1500 *g*, as described previously (Festenstein, 1958).

Methods of enzyme assay

o-Nitrophenyl β-glucosidase. The procedure of Conchie (1954) was modified as follows: enzyme (0·5 ml.), substrate and 0·8 ml. of 0·1 M-citric acid–0·2 M-Na_2HPO_4 buffer, pH 5·8 (McIlvaine, 1921), in a total volume of 4 ml. (final pH 6·1) were incubated for 1 hr. and the reaction was stopped by adding 4 ml. of 0·25 M-glycine–Na_2CO_3 buffer, pH 10·0 (pH of mixture, 10·0). (This buffer was prepared by mixing 32·6 g. of glycine and 25·4 g. of NaCl in 800 ml. of water, adjusting to pH 9·6 with 50% NaOH, adding 400 ml. of 1·25 M-Na_2CO_3 and diluting to 2 l.)

Cellobiase. Conditions for assay and reducing-sugar determination (by the cuprimetric method only) were as described previously (Festenstein, 1958). Glucose was measured specifically by glucose oxidase (Huggett & Nixon, 1957); solutions were deproteinized as for reducing-sugar determination or alternatively the reaction was stopped by placing them in a boiling-water bath for 10 min. (the enzyme blank in either case was negligible). Cellobiose itself gave a small blank (2–3% of the value expected for an equal weight of glucose).

Salicinase. Incubation mixtures (1 ml.) were used for reducing-sugar determination without previous deproteinization, since the activity of the enzyme preparations permitted the use of very dilute solutions. Saligenin was determined by the method of Kerr, Graham & Levvy (1948) for phenol, modified as follows, to eliminate deproteinization: 1·5 ml. of reagent (Folin–Ciocalteu Reagent, British Drug Houses Ltd., diluted 1:4·2) was added, followed by 5 ml. of N-Na_2CO_3 before incubation for colour development; Ilford no. 608 filters (wavelength of peak transmission, 680 mμ) were used in the Spekker absorptiometer (Levvy & Marsh, 1952). Saligenin (British Drug Houses Ltd.), twice recrystallized from water, was used as a standard. Glucose did not interfere with colour production, nor did saligenin affect the reducing value of glucose.

RESULTS

Activities of enzyme preparations. Illustrative examples of enzyme activities are shown in Table 1.

In salicinase studies where both saligenin and reducing sugar were estimated, it was found that the ratio saligenin:reducing sugar increased with increasing substrate concentration. For salicin concentrations between 0·7 mM and 20 mM the reducing substances liberated increased only slightly, up to 2·8 mM, and then decreased, whereas the saligenin liberated increased throughout the range; the saligenin:reducing sugar ratio rose by over one-third between 2·8 mM and 20 mM. Reducing-sugar determination by the cuprimetric method was not possible for nitrophenyl glucosidase assays, since substantial amounts of substrate were hydrolysed during heating with copper reagent.

The glucose oxidase method was apparently unsuitable for glucose determination in the presence of liberated saligenin since a purple colour appeared, probably due to reaction of saligenin with oxidized *o*-dianisidine; phenol produced a similar colour. *o*-Nitrophenol liberated would likewise interfere, but in this case on account of its own colour quite apart from any possibility of further reaction.

Hydrolysis of substrate mixtures. The use of a chromogenic substrate enables other substances to be tested as possible competing substrates (Levvy & Marsh, 1954). Conchie (1954) established that 10 mM-cellobiose produced 30% inhibition of rumen *o*-nitrophenyl β-glucosidase; a detailed study of the nature of the inhibition showed that cellobiose acts chiefly as a competitive inhibitor.

Table 1. *Hydrolysis of different β-glucosides by rumen-enzyme preparations*

Enzyme activity was measured in 0·02 M-citric acid–0·04 M-Na_2HPO_4 buffer, pH 6·1, or 0·02 M-citric acid–NaOH buffer, pH 6·0 (for salicinase), with the following substrates: 5·8 mM-cellobiose, 2·5 mM-*o*-nitrophenyl β-glucoside, 1·4 mM-salicin. All values are expressed as μg. of substance liberated/ml. of reaction mixture/hr. at 37° and are within the range of linear hydrolysis of substrate with time. The original enzyme preparations were diluted five times in the cellobiase assay mixture and 400 times for assay with the other two substrates.

Sheep no.	Cellobiase (μg. of glucose, estimated as reducing sugar)	*o*-Nitrophenyl β-glucosidase (μg. of *o*-nitrophenol)	Salicinase (μg. of saligenin)
44	800	19	24
91	360	8·6	8
107	213	10	—

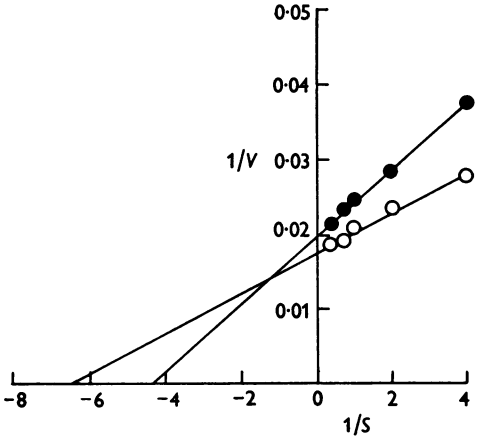

Fig. 1. Inhibition by 5·8 mM-cellobiose of the enzymic hydrolysis of *o*-nitrophenyl β-glucoside. Results for both the inhibited (●) and uninhibited (○) reactions are plotted according to Woolf (1932) and Lineweaver & Burk (1934): V, velocity of the reaction = μg. of *o*-nitrophenol liberated/75 min.; S, substrate concn. = mM-*o*-nitrophenyl β-glucoside. Kinetic constants obtained here are K_m, 0·16 mM; K_i (competitive component), 13 mM; K_i (non-competitive component), 50 mM. K_m determined directly for cellobiase was 0·9 mM.

but there is slight non-competitive inhibition (Fig. 1). This finding was consistently reproduced with eight different enzyme preparations, obtained from three sheep, the concentrations of cellobiose varying from 3·6 mM to 19 mM. Salicin (0·7 mM and 10 mM) was shown to be essentially a competitive inhibitor only, preparations from four different sheep being used. Glucose (11 mM) and gluconolactone (0·02 mM, 0·1 mM) were also competitive inhibitors, the latter substance behaving as established previously (Conchie, 1954).

Salicin could be used as a chromogenic substrate in the same way as nitrophenyl glucoside by estimating the saligenin liberated; cellobiose acted as a competitive inhibitor at a concentration of 3·6 mM, but a definite non-competitive component appeared for 19 mM. Gluconolactone (0·02 mM) was a competitive inhibitor.

DISCUSSION

The use with β-glucosidases of substrates such as nitrophenyl glucoside and salicin, where aglycone liberated is measured, provides more accurate results than measurement of reducing substances liberated. However, a study of both parameters is also useful as is observed from the increasing saligenin:reducing sugar ratio with increasing substrate concentration; this type of phenomenon may be due to transferase activity, as has been observed by Jermyn & Thomas (1953).

The specific glucose oxidase method has valuable potentialities in cellobiase studies, particularly since deproteinization is apparently unnecessary and the substrate blank is very small compared with that obtained by the best reducing methods. A complication is the coupling of the oxidized dye with saligenin in salicinase studies.

The results obtained with the substrate mixtures suggest that the substrates investigated act chiefly as competitors for the same site, or sites, of the enzyme molecule. The non-competitive component for the hydrolysis of nitrophenyl glucoside in the presence of cellobiose and also that obtained with 19 mM-cellobiose as inhibitor of salicinase, suggests a different or additional site of combination. Hofstee (1955), using almond extracts, showed cellobiose, glucose and salicin to be mainly non-competitive inhibitors of the hydrolysis of salicylic acid β-glucoside and claims that the finding may have a bearing on the theory of Pigman & Goepp (1948), that the glucose and aglycone moieties of the substrate combine with different groups on the enzyme molecule.

SUMMARY

1. β-Glucosidase activity of cell-free extracts of micro-organisms from sheep rumen has been measured with *o*-nitrophenyl β-glucoside, cellobiose and salicin as substrates.

2. Saligenin liberated from salicin has been shown to increase with substrate concentration even when the reducing substances liberated reach a constant value and decrease.

3. Cellobiose acts chiefly as a competitive inhibitor of the hydrolysis of *o*-nitrophenyl β-glucoside; salicin, glucose and gluconolactone are essentially competitive inhibitors.

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