Hence the proportional error in y resulting from a small proportional error e in E_t is

$$p_y = eEt/(E_t - E_p),$$

and substituting for E_t from (6)

$$p_y = e \left\{ 1 + \frac{E_p}{y(E_R - E_p)} \right\} = e \left(1 + \frac{r}{y} \right), \quad (7)$$

where $r = E_p/(E_R - E_p) = \epsilon_p/(\epsilon_R - \epsilon_p)$, that is, the ratio of the smaller extinction coefficient to the difference between them. Alternatively, if the wavelength chosen is such that $\epsilon_p > \epsilon_R$, it can be shown in a similar way from equation (4b) that

$$p_y = e \left(1 - \frac{1+r}{y} \right),$$

where r is again the ratio of the smaller extinction coefficient to the difference between them, in this case $\epsilon_R/(\epsilon_p - \epsilon_R)$.

For given substances R and P , r is determined solely by the wavelength. The error in determining y due to a given error in the initial reactant con-

centration therefore depends solely on the wavelength at which the measurements are made, and, in contrast to the precision of the measurements, is independent of the initial reactant concentration and the optical depth. The error increases with r , and is minimum when $r = 0$, that is when a wavelength is used which either the reactant or the product does not absorb. In Fig. 4, $p_y e$ is plotted against y for values of r of 0 and 2: the curves show the percentage error in y resulting from a 1% error in the initial reactant concentration, calculated from equations (7) and (8). It is evident that with high values of r errors in the initial reactant concentration are magnified considerably in the result, and in the flow method the constancy of the relative delivery rates will be critical. As in the case of the precision of the measurements, the condition $\epsilon_R > \epsilon_p$ gives the more uniform distribution of errors over the whole course of the reaction, and the smaller errors over the second half of the reaction, whilst the condition $\epsilon_p > \epsilon_R$ is better for the first half of the reaction.

REFERENCE

Twyman, F. & Lothian, G. F. (1933). *Proc. phys. Soc. Lond.* **45**, 652.

Mould 'Glucosaccharase': a Fructosidase

By F. J. BEALING

Department of Biochemistry, The University, Sheffield 10

(Received 5 December 1952)

Bealing & Bacon (1953) have shown that a trans-fructosidase is probably the only enzyme in extracts of *Penicillium spinulosum* that attacks sucrose. Their experimental data did not exclude the possibility that an additional enzyme attacking sucrose was present in extracts of *Aspergillus oryzae* and *A. niger*.

Further study of the extracts of *P. spinulosum* has shown that the transfructosidase is inhibited by glucose, i.e. it possesses a property previously attributed to a hypothetical α -glucosidase ('glucosaccharase': Kuhn, 1923) and accepted as a criterion to distinguish this enzyme from other invertases. The results presented here suggest that the inhibitory action of sugars and alcohols on mould invertase may be explained by their action as acceptors of fructose residues.

Evidence is also presented that the action of mould extracts on β -fructofuranosides other than sucrose, viz. raffinose, stachyose and melezitose, may be explained without assuming the presence of

a glucosaccharase capable of attacking α -glucosidic linkages similar to that found in sucrose.

These observations lead to the conclusion that there is now little reason to presume that mould extracts contain a glucosaccharase: whether or not enzymes of this type do exist remains to be established.

MATERIALS AND METHODS

The organisms, culture media, and method of preparation of extracts have been described previously (Bealing & Bacon, 1953). This procedure was modified in one instance, when a series of Czapek-Dox media was prepared in which the 5% (w/v) sucrose was replaced by raffinose, maltose, glucose, sorbose, fructose or galactose. Each solution was inoculated with spores of *P. spinulosum* (grown on a sucrose medium) and incubated at room temperature (18–24°). Mycelia were harvested at the beginning of active sporulation, after 8 days on the sucrose, raffinose, maltose and glucose media, and after 14 days on the sorbose and fructose media. Material was not obtained from the galactose media because

of the extremely slow rate of mycelial growth. Extracts were prepared in the usual way from the crushed mycelia.

Incubation procedure. Mixtures of buffer, substrate and enzyme were incubated in glass-stoppered tubes at room temperature. The buffers usually employed were sodium acetate-acetic acid (pH 5.0) and $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ (pH 6.8).

Samples of incubation mixtures were inactivated either by heating for 3 min. in tubes previously placed in a boiling-water bath, or by the addition of HgCl_2 solution to give a final concentration of 0.001 to 0.002 M- HgCl_2 .

Paper-partition chromatography and the estimation of sugars. Chromatograms were prepared by descending development in the butanol:acetic acid:water solvent described by Partridge (1948), using the procedures of Bacon & Edelman (1951), Bacon & Loxley (1952) and Bealing & Bacon (1953).

The methods employed for the estimation of sugars were those of Bealing & Bacon (1953).

Stachyose preparation. A sugar considered to be stachyose, *O*- α -D-galactopyranosyl-(1 $\rightarrow$ 6)-*O*- α -D-galactopyranosyl-(1 $\rightarrow$ 6)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 2) β -D-fructofuranoside (Adams, Richtmyer & Hudson, 1943; Hrissey, Wickstrom, Courtois & Dizet, 1952), was isolated from rhizomes of *Stachys sylvatica* L. (hedge woundwort). The rhizomes, dug up in September, were washed, cut into sections 4–5 cm. long, and thrown into water maintained at boiling point for 10 min. An extract was obtained by treatment of this material in a Waring Blendor with the minimum amount of water: after squeezing through madapolam (a cotton cloth supplied by C. H. Baddeley and Co., 7 Buck Street, Leeds Road, Bradford; cf. Bawden & Pirie, 1942) the solution was deproteinized by the addition of 30% (w/v) lead acetate solution until no further precipitate appeared. Excess lead was removed by precipitation with 3% (w/v) sodium oxalate. The extract was concentrated to a tenth of its original volume, and the carbohydrates precipitated by the addition of 4 vol. of ethanol. Partition chromatography on cellulose (Whatman Cellulose Powder 'Standard Grade': *n*-butanol saturated with water as solvent; cf. Hough, Jones & Wadman, 1949) was used to separate the major constituent of this precipitate from other sugars.

The product obtained was an almost colourless glass melting at 165–175° and having $[\alpha]_D^{20} + 131^\circ$ (water, $c = 0.8$). Stachyose has m.p. 167° and $[\alpha]_D + 150^\circ$ (anhydrous) (Tanret, 1902).

Diazouracil (2 mg.) was mixed with 33 mg. of this material and 2 ml. 0.05 N-NaOH were added to give an opalescent solution which developed a green coloration after standing for 4–5 min. at 0°. Control experiments gave similar positive results with sucrose and raffinose but not with melezitose, nor in the absence of added carbohydrate. These results are in accordance with the reaction described by Raybin (1933, 1937) for sucrose, raffinose, gentianose and stachyose.

Paper chromatography showed that the glass contained a single oligosaccharide having R_F 0.02 (butanol:acetic acid; compared with sucrose, R_F 0.11). This substance did not reduce alkaline silver nitrate (Trevelyan, Procter & Harrison 1950); it contained fructose.

Partial hydrolysis was achieved by heating with 0.05 N-oxalic acid to 100° for 5 min., followed by rapid cooling, or by the action of yeast invertase. In both cases fructose was liberated. The residual oligosaccharide reduced alkaline silver nitrate and did not contain fructose. More complete

hydrolysis was obtained by treatment with 0.5 N- H_2SO_4 at 100°. After 1 hr., fructose had been liberated and the residual sugar partly broken down to a monosaccharide (galactose or glucose) and a second reducing sugar having the R_F value of melibiose. Further heating was accompanied by destruction of fructose so that, when hydrolysis was complete after about 8 hr., the solution contained only galactose and glucose; as judged qualitatively, the mixture contained a higher proportion of the former sugar.

These observations are in accordance with the idea that the carbohydrate isolated was stachyose. The R_F value of manninotriose (0.04 in butanol:acetic acid; Bradfield & Flood, 1950) corresponds with that of the oligosaccharide released by partial hydrolysis. The existence of the 6- α -D-galactosyl-glucose structure in stachyose and manninotriose (Hrissey *et al.* 1952) would indicate that melibiose (6- α -galactosyl-D-glucose) could appear as an intermediate in the hydrolysis of these substances.

Electrophoresis on filter paper. The apparatus used was based on that described by Durrum (1950). It consisted of two glass jars each holding 2 l. of buffer and linked by a strip of Whatman no. 3MM paper 6 in. wide and 24 in. long, the whole being enclosed in a glass tank sealed with a greased glass plate. A graphite electrode was suspended in each jar and 340 v applied between the terminals. Under the conditions used the current passing through the paper was 0.3–1.0 mA.

By the use of this technique a partial separation of the enzymes in mould extracts was accomplished. Volumes of 0.3–0.5 ml. of extract were placed as a band across the strip of paper and allowed to dry at room temperature; the paper was then soaked in the minimum amount of a buffer (10–15 ml.) delivered from a pipette drawn over the surface of the paper.

When 0.01 M-phosphate buffer (pH 6.8) was used, application of 340 v between the ends of the strip resulted in the movement of a sucrose-splitting enzyme at about 2 cm./hr. towards the cathode. Relatively small amounts of enzymic activity remained adherent to the paper at the base-line and behind the main band. Under these conditions maltase activity moved towards the anode, away from the invertase; when electrophoresis was carried out at pH 5.0 (0.01 M-acetate buffer) the invertase and maltase activities migrated together towards the anode.

The position of the invertase could be found by spraying the paper with a sucrose solution, incubating in a damp atmosphere for several hours at room temperature, and determining the position of reducing sugars by treatment with alkaline silver nitrate. More usually, however, the paper was cut into strips about 3 cm. wide; segments of the strips were incubated separately with solutions of sucrose or other sugars and the occurrence of enzymic activity tested by paper chromatography of the solution.

The breakdown of sucrose by the invertase band was qualitatively similar to that resulting from the action of entire mould extracts. Attempts to elute the enzyme were not successful; at pH 6.8 (phosphate) no activity was recovered, and at pH 4.5 (acetate) only 20% of the applied invertase activity was eluted. It appeared improbable that the latter value provided a true estimate of the amount of enzyme present, and it was therefore not possible to determine the percentage of the total sucrase activity which was represented by the separated enzyme; however, the occurrence of a second invertase band was not observed under any conditions.

RESULTS

Inhibitory effects of sugars

Effect of glucose. The effect of glucose on the rate of breakdown of sucrose was investigated by polarimetry.

An extract of crushed *P. spinulosum* mycelium (2.5 ml.) was added to each of two buffered sucrose solutions (12.5 ml.) to give a final concentration of 10.0% (w/v) sucrose in 0.005M-acetate buffer (pH 5.0). One solution contained in addition 5.0% (w/v) mutarotated glucose.

Fig. 1. Inhibitory effect of added glucose and fructose on the rate of inversion of sucrose. Samples of reaction mixtures inactivated by heating and left 12 hr. to permit mutarotation of glucose before measurement of optical rotations. Values for liberation of reducing sugars calculated from polarimetric data, assuming invert sugar to be liberated. ○—○, solution containing 10.0% (w/v) sucrose; ●—●, solution containing 10.0% (w/v) sucrose, 5.0% (w/v) glucose; ▲—▲, solution containing 10.0% (w/v) sucrose, 5.0% (w/v) fructose.

Successive samples of each solution were analysed by polarimetry; the addition of glucose was found to diminish the apparent rate of liberation of reducing sugars (Fig. 1).

The course of sucrose breakdown in solutions containing different amounts of glucose was followed by paper chromatography. Equal volumes (1.0 ml.) of an extract of crushed *P. spinulosum* mycelium were incubated with a series of buffered sucrose solutions (7.0 ml.) containing various amounts of mutarotated glucose (final concentrations, 7.5% sucrose and 0, 3.9, 5.8, or 7.8% glucose in 0.005M-acetate buffer, pH 5.0). Samples taken at intervals were analysed by paper chromatography.

The results of these analyses (Fig. 2) showed that the presence of glucose decreased the rate of sucrose disappearance, there being a corresponding decline in the rate of formation of 'upper spots' (Bealing & Bacon, 1953) but no effect on the liberation of fructose.

Effect of fructose. Fig. 1 shows the inhibitory effect of 5% fructose in the system used to study the inhibition by glucose.

In another experiment, an extract of *P. spinulosum* mycelium (0.4 ml.) was added to each of a series of buffered sucrose solutions (1.6 ml.) which contained different amounts of fructose (final concentrations, 7.5% sucrose and 0, 3.9, 7.8 or 11.7% fructose in 0.006M-acetate buffer, pH 5.0). Samples

Fig. 2. Effect of added glucose on the breakdown of sucrose by mould extracts. Mixtures incubated at room temperature and sample of each analysed after 1.5 hr. For details see text. Sucrose and upper spots expressed as mg. ketose/ml. On lines at about 4%: (upper line) sucrose, (middle line) upper spots, (bottom line) fructose.

taken after 90 min. were analysed by paper chromatography. The addition of fructose resulted in a decreased rate of disappearance of the ketose of the sucrose spot and a decreased rate of formation of upper spots (Fig. 3). No attempt was made to measure fructose liberation because of the relatively very great fructose 'blank'.

The apparently diminished rate of sucrose breakdown was associated with the appearance of a reducing sugar having an R_f value similar to that of sucrose. This substance was present in incubation mixtures after prolonged incubation of mould extracts with sucrose alone, but its formation was accelerated by the presence of fructose.

The production from raffinose of a reducing substance having approximately the same R_f value as sucrose but containing only fructose is described

below. In this case also, the addition of fructose had an accelerating effect on the rate of formation.

Effect of other sugars. The addition of galactose, mannose, arabinose or xylose to reaction mixtures decreased the rate of liberation of reducing sugars from sucrose by a Taka diastase solution. The solutions were examined by paper chromatography in an attempt to demonstrate the incorporation of the added sugars in oligosaccharides: this was successful only in the case of the mixture containing xylose, in which four new spots were detected. These substances appeared in order of decreasing R_f values.

Fig. 3. Effect of added fructose on the breakdown of sucrose by mould extracts. For details, see text. Mixtures incubated at room temperature. Sucrose (○—○) and upper spots (●—●) expressed as mg. ketose/ml.; fructose liberation not measured.

The presence of xylose in each was indicated by a characteristic pink coloration produced by this sugar when sprayed with the benzidine-trichloroacetic acid reagent; confirmation was obtained by hydrolysis (with yeast invertase) of the two faster-running spots separated on a two-dimensional chromatogram.

Inhibitory effects of alcohols

Bacon (1952) has shown that methyl β -fructofuranoside is formed during the action of yeast invertase on sucrose in the presence of methanol. Addition of higher alcohols leads to the appearance of other substances having greater R_f values and considered to be homologous with the methyl fructoside.

Similar, probably identical, compounds were produced by Taka diastase or mould extracts incubated with sucrose (or raffinose) in the presence of methanol, ethanol, *n*-propanol, or *n*-butanol (Fig. 4). Each spot contained fructose (reaction

with phloroglucinol), and the R_f values increased with the size of the alkyl group expected to be present; the R_f value of the methanol spot was the same as that of methyl β -fructofuranoside.

The subsequent appearance of a second spot was observed in the solutions containing ethanol, *n*-propanol or *n*-butanol; in each case the R_f value of

Fig. 4. Formation of fructosides in presence of alcohols. Solutions containing 1.0 ml. 15% (w/v) sucrose, 0.4 ml. water or alcohol, 0.2 ml. 0.05M-acetate buffer (pH 5.0) and 0.4 ml. extract of *P. spinulosum* mycelium incubated for 8 hr. (Sprayed with benzidine-trichloroacetic acid; negative made by transmitted ultraviolet light.)

these substances was less than that of the first spot, and it is probable that a corresponding substance present in the methanol incubation would be obscured by the sugar spots. Traces of a third substance with a still smaller R_f value could be detected in solutions containing *n*-butanol.

Fructose-containing spots with high R_f values also appeared when ethylene or propylene glycols were added to incubation mixtures, but not when *isopropanol*, *sec.*- or *tert.*-butanol, *n*-amyl, or benzyl alcohols were added.

Hydrolysis of the fructosides occurred after a long incubation period; extracts of *P. spinulosum* were also capable of hydrolysing methyl β -fructo-

furanoside (obtained by the action of yeast invertase on a sucrose-methanol mixture).

Addition of alcohols resulted in a reduced rate of liberation of reducing sugar from sucrose, the effect of methanol in an extract of *A. oryzae* being shown in Fig. 5. Analyses of paper chromatograms showed that the rate of formation of upper spots decreased with increasing concentration of methanol. In the few experiments carried out the amount of methyl fructoside formed did not account for the progressive decrease in the rate of liberation of reducing sugar.

Fig. 5. Liberation of reducing sugar in presence of methanol. Equal volumes (1.2 ml.) of an extract of crushed *A. oryzae* mycelium added to a series of buffered sucrose solutions (2.8 ml.) containing different amounts of methanol to give final concentrations, 7.5% (w/v) sucrose and 0, 5, 10, 15, or 20% (v/v) methanol in 0.05 M-acetate buffer (pH 5.0). Reducing-sugar content of 10 or 20 μ l. quantities of each solution measured after approx. 30 and 60 min.

Effect of inorganic phosphate

Samples of a solution containing 1.0 ml. 32% sucrose, 0.5 ml. 5% (w/v) Taka diastase solution and 2.0 ml. 0.2 M-phosphate buffer (pH 6.8) were taken

after 3 and 6 hr. The mixtures were chromatographed on paper (previously washed with the tetrasodium salt of ethylenediaminetetraacetic acid in water), using ascending development and *tert*-amyl alcohol: formic acid as solvent (Hanes & Isherwood, 1949). When the chromatograms were sprayed with the perchloric acid-ammonium molybdate reagent of these authors, heated for 5 min. at 85° and treated with H_2S , blue spots indicating the position of inorganic phosphate appeared but the occurrence of sugar phosphates was not detected.

In a second experiment, two incubation mixtures were prepared, one solution containing veronal buffer (pH 7.0) and the other phosphate buffer adjusted to the same pH value. Sodium fluoride was added to the phosphate solution to decrease the hydrolysis by phosphatase of any sugar phosphates which might be formed, but analyses of samples by paper chromatography failed to show that the presence of phosphate influenced the course of the reaction (Table 1). It would thus seem that inorganic phosphate neither functions as an acceptor, nor has any inhibitory effect on sucrose breakdown.

Action of mould extracts on other sugars

Raffinose. All of the preparations examined (from *P. spinulosum*, *A. niger*, *A. oryzae*, *A. flavus*, and Taka diastase) were capable of hydrolysing raffinose. The course of the reaction depended on the presence or absence of melibiase. The adaptive formation of melibiase by *A. niger* has been described by Pottévin (1903); similar observations have been made in the present investigation with *P. spinulosum*. This mould, which had been subcultured on sucrose-containing media for 2 yr., produced insignificant amounts of melibiase when grown on sucrose media, but appreciable amounts of this enzyme when grown on raffinose or glucose media.

When melibiase activity was absent, raffinose was hydrolysed to a mixture of fructose and melibiose. The reaction was accompanied by the appearance of a fructose-containing substance ('first upper spot') having R_f less than that of raffinose; the formation of a second upper spot having even smaller R_f was observed when extracts were incubated with high

Table 1. Effect of inorganic phosphate on the course of sucrose breakdown by a mould extract

(0.5 ml. 30.0% (w/v) sucrose, 0.5 ml. water, 0.6 ml. 0.03 M-sodium veronal buffer (pH 7.0) and 0.4 ml. extract of crushed *P. spinulosum* mycelium were incubated at room temperature; samples taken at intervals were analysed by paper chromatography. 0.5 ml. 30.0% sucrose, 0.6 ml. M-phosphate (pH 7.0), 0.5 ml. M-NaF solution and 0.4 ml. of the same mould extract were incubated under the same conditions and a sample was analysed after 30 min., the chromatogram being run in solvent saturated with NaH_2PO_4 . Composition of the mixtures compared when the sucrose spots contained equal amounts of ketose.)

Buffer	Time (min.)	Composition of solution (mg. ketose/ml.)			Recovery of fructose (%)
		Upper spots	Sucrose	Fructose	
Veronal	25	7.6	27.6	2.0	95
Phosphate	30	7.8	27.6	1.8	95

concentrations (10–15%) of raffinose. In addition to these substances a reducing sugar appeared in the sucrose position of chromatograms; its formation was accelerated by the addition of fructose to the reaction mixture and it was probably the same as the spot, considered to be a fructose disaccharide, which appeared when sucrose was used as substrate.

Hydrolysis of the sugars (with yeast invertase) after separation by the first development of a two-dimensional chromatogram showed that the disaccharide spot was composed entirely of fructose; the 'first upper spot' was hydrolysed to a mixture of fructose and melibiose.

Prolonged incubation with mould extracts resulted in the disappearance of fructose-containing oligosaccharides, the disaccharide remaining longer than the other sugars.

Extracts containing appreciable amounts of melibiase hydrolysed raffinose completely, but traces of melibiose could usually be detected in the reaction mixture, indicating that the liberation of fructose by these extracts was still independent of galactosidase activity.

Addition of methanol (10%, v/v) completely inactivated the melibiase. At the same time, fructosidase activity, although diminished, resulted in the formation of a non-reducing fructose-containing substance having the same R_f value as methyl β -fructofuranoside.

Stachyose. Incubation of stachyose at pH 5.0 with the extracts examined (*P. spinulosum*, *A. oryzae*, *A. niger* and Taka diastase) resulted in a slow liberation of fructose and the appearance of manninotriose. Subsequently, manninotriose was hydrolysed to a mixture of glucose and galactose; a reducing oligosaccharide having the same R_f as melibiose appeared as an intermediate in the reaction. Relatively small amounts of a reducing, fructose-containing sugar having R_f less than that of stachyose were also produced.

Addition of fructose to reaction mixtures resulted in the formation of a reducing sugar which ran in the sucrose position of chromatograms. This substance was probably the same as that considered to be a fructose disaccharide, which was formed when sucrose or raffinose was used as substrate; it gave the same yellowish colour as the difructose when sprayed with benzidine, in contrast to the reddish colour of the aldose-containing sugars.

The presence of methanol (20%, v/v) suppressed the breakdown of manninotriose and decreased the rate of liberation of fructose from stachyose; at the same time, traces of a fructoside running on chromatograms in the same position as methyl β -fructofuranoside could be detected in the reaction mixture.

Electrophoresis of mould extracts in 0.01M-phosphate buffer at pH 6.8 resulted in the loss of

α -galactosidase activity and the separation of a single fructosidase band. The fructosidase separated in this way from an extract of *A. oryzae* mycelium liberated fructose from sucrose, raffinose and stachyose; there was thus no indication that more than one fructosidase was involved in the breakdown of these sugars.

Melezitose. Melezitose was completely hydrolysed at pH 5.0 by the mould extracts examined (*P. spinulosum*, *A. niger*, *A. oryzae*). Electrophoresis of mould extracts in 0.01M-phosphate (pH 6.8) resulted in a slow migration of melezitase activity (i.e. ability to produce monosaccharide from melezitose) towards the anode, away from the invertase. An enzyme capable of hydrolysing turanose to a mixture of glucose and fructose, and relatively small amounts of the fructosidase were present in the same position as the melezitase. Turanase activity was not found on any other region of the paper strip, but the fructosidase probably represented small amounts of invertase which had remained adherent to the paper at the base-line; the main invertase band did not attack melezitose. Incubation of melezitose with sections of paper carrying the separated melezitase resulted in liberation of glucose and fructose; a fructose-containing spot in the disaccharide position of the chromatogram appeared as an intermediate. The disaccharide was present only in small amounts and, because of difficulty in distinguishing between sucrose and turanose on chromatograms, its nature was not established.

An extract of *A. niger* in acetate buffer (pH 5.0) which had been passed through a column of aluminium hydroxide (British Drug Houses Ltd., 'Dry') was incapable of attacking either melezitose or turanose, although invertase activity was not greatly diminished.

Attempts to confirm that the mould fructosidase was concerned in the breakdown of melezitose by extracts of *A. oryzae* or *A. niger* were not successful: it was not found possible to demonstrate the transference of fructose residues to added fructose or to methanol (20%, w/v).

Maltose. The breakdown of maltose by mould extracts at pH 7.0 resulted in the liberation of glucose, and the formation of two other reducing oligosaccharides having R_f values less than that of maltose. Of these sugars, one was possibly 6- α -glucosyl-D-glucose (isomaltose) and the other 4- α -isomaltosyl-D-glucose ('panose'), formed from maltose by the action of an α -glucosidase from *A. niger* (Pan, Andreasen & Kolachov, 1950; French, 1951) and *A. oryzae* (Pazur & French, 1952).

Further investigation of the course of maltose breakdown was not attempted because maltase activity separated by electrophoresis of mould extracts did not attack sucrose.

Methyl α -glucoside. The hydrolysis of methyl α -glucoside at pH 5.0 resulted from the action of a mould enzyme which could not be detected after electrophoresis of the extract, and therefore appeared to be distinct from the maltase.

The majority of the extracts examined contained relatively small amounts of methyl α -glucosidase, although greater amounts were usually present in mycelia grown on glucose media. Glucose was the only detected product of the hydrolysis of methyl α -glucoside.

Trehalose. Mould extracts hydrolysed trehalose at pH 5.0; in this case, also, glucose was the only observed product of the reaction. Comparison of the various ' α -glucosidase' activities of extracts of mycelia grown in different sugars showed that maltose activity was greatest in extracts of material grown on maltose or raffinose media; methyl α -glucosidase in an extract of material from a glucose medium, and trehalase in extracts of mycelia from maltose and sorbose media.

DISCUSSION

The identification of mould invertase with an α -glucosidase ('glucosaccharase') was suggested originally by Kuhn (1923) in order to account for the inhibitory action of α -glucose, presumed to result from competitive adsorption of the sugar on the enzymic surface. In contrast, yeast invertase was inhibited by fructose rather than by α -glucose and was considered to be a fructosidase.

Subsequent reports (Kuhn & Münch, 1927) that mould invertase preparations attacked melezitose but not raffinose provided support for the glucosaccharase theory, since it was not expected that the enzyme would attack a substituted α -glucosidic residue.

However, later workers found that mould extracts commonly, if not always, contained a fructosidase, usually demonstrated by the ability of such preparations to attack raffinose under conditions where melibiose was not attacked (Hofmann, 1934; Hestrin, 1940). The preparation used by Hestrin failed to attack melezitose, and therefore could not contain a glucosaccharase, but in other cases it was assumed that both glucosaccharase and fructosidase were present. Despite these observations, the idea that mould invertase is typically an α -glucosidase has been restated in recent reviews (Neuberg & Mandl, 1950; Gottschalk, 1950).

In previous publications (Bealing & Bacon, 1951, 1953) it was reported that mould extracts contained a transfructosidase. This was also discovered independently by Wallenfels (1951) and Pazur (1952). It was shown that the action of this enzyme on sucrose resembled that of the mould invertase described by earlier authors, i.e. it possessed

properties hitherto attributed to a glucosaccharase, thus raising the question whether the latter enzyme did, in fact, exist.

The experiments described here go further than those previously reported (e.g. by Hestrin) in showing (a) that the inhibitory effects of glucose on the breakdown of sucrose by extracts of *P. spinulosum* are manifested not only in a decreased rate of change of optical rotation, but also in an apparent diminution of fructosidase activity, and (b) that these extracts attack raffinose, stachyose, and melezitose in a manner which can be explained by the action of the fructosidase and of other glycosidases (melibiase, turanase) known to be present.

The action of mould extracts on sucrose leads to the formation of fructose-containing oligosaccharides ('upper spots') by the transference of fructose residues to sucrose (Bealing & Bacon, 1953). The inhibitory action of glucose, which is exerted mainly on oligosaccharide formation, could thus be explained by the supposition that this sugar also acts as an acceptor for fructose, in competition with sucrose.

If the product (fructose - glucose) of reaction (2) were sucrose the net effect would be a slower rate of progress of reaction (1).

Under these circumstances, free radioactive glucose should become incorporated in sucrose. This has been demonstrated by Edelman & Bealing (1953), the rate of incorporation being much greater than that required to account for the inhibitory action of added glucose and indicating the existence of a continuous interchange of glucose between the free state and combination in sucrose. The fact that sucrose, rather than a β -glucoside, is formed in the reaction is in accordance with the observations of earlier workers that mould invertase is inhibited by α -glucose rather than by the β -form.

The inhibitory action of glucose on the rate of sucrose breakdown may therefore be attributed to its participation in a reaction catalysed by mould transfructosidase, and it becomes unnecessary to presume the existence of a glucosaccharase to account for the effect.

The inhibitory action of fructose resembles that of glucose in that oligosaccharide formation is decreased, but differs in giving rise to a new substance, apparently a reducing disaccharide composed of two fructose residues. A substance of this type might be expected to appear if fructose, like glucose, inhibits the reaction by functioning as an alternative acceptor for fructose residues.

The inhibitory actions of other sugars seem to be explicable in similar terms; whether any 'true' competitive inhibition occurs cannot be decided with existing information. With alcohols it seems possible that there is some inactivation of the enzyme distinct from the effect of the transfer reaction whereby alkyl fructosides are produced.

The experiments with raffinose and stachyose as substrates confirmed the presence of a fructosidase in all the extracts examined. The liberation of fructose was accompanied by fructose transfer, thus strengthening the tentative conclusion of Bealing & Bacon (1953) that the transfructosidase and fructosidase (invertase) activities are due to the same enzyme.

All the enzyme preparations attacking melezitose could also attack turanose, suggesting that the first stage in the hydrolysis of the trisaccharide is the formation of glucose and sucrose, the latter subsequently being acted upon by the fructosidase. However, the turanase was not obtained entirely free from invertase activity, and it was not possible to identify the disaccharide formed as an intermediate when the partly purified enzyme acted on melezitose. It might therefore be argued that the slight invertase activity of turanase preparations was an indication that turanase itself (an α -glucosidase) is capable of attacking sucrose, but this is made less likely by the fact that the course of the action on sucrose resembled qualitatively the action of the transfructosidase.

The variations in maltase, methyl α -glucosidase, and trehalase activities when the mould was cultured on different sugars suggest strongly that these are three distinct enzymes, confirming the observation of Hestrin (1940) and at the same time refuting the theory of Weidenhagen (1932). The hypothetical glucosaccharase could be one of these, or distinct from all three.

The maltase was separable by filter-paper electrophoresis, and had no action on sucrose. This confirms the results of Hestrin (1940), who inactivated the invertase, but not the maltase, of Taka diastase by treatment with heat or acid. Methyl α -glucosidase was lost during electrophoresis, but the effect of this loss on the total invertase activity was not investigated.

There are therefore no grounds for postulating the existence of a glucosaccharase in extracts of *P. spinulosum*, although the general properties of the invertase present are those of the mould invertases previously described. Although it is not excluded that a glucosaccharase may occur in other moulds it

would seem better to abandon the use of the term until some positive evidence for its existence is produced. Some unpublished experiments of ours suggest that the invertase extracted from the viscera of bees acts as a transfructosidase and attacks raffinose without the preliminary action of a melibiase. A detailed investigation of this and other enzymes hitherto classified as glucosaccharases seems desirable.

SUMMARY

1. The inhibition by free sugars and alcohols of sucrose breakdown by extracts of *Penicillium spinulosum* has been studied by paper chromatography.
2. The rate of formation of oligosaccharides (transfructosidation) from sucrose was decreased by the addition of glucose or fructose. In the presence of the latter a reducing fructose disaccharide was formed.
3. In the presence of various alcohols the breakdown of sucrose was accompanied by the appearance of substances presumed to be the corresponding alkyl β -fructofuranosides.
4. No evidence could be obtained for the participation of inorganic phosphate in the action of the fructosidase.
5. The action of extracts of *P. spinulosum* and other moulds on raffinose and stachyose could be explained by the presence of melibiase and of a fructosidase possessing transfructosidase activity.
6. The mould extracts examined hydrolysed melezitose completely, but fractionation by adsorption on aluminium hydroxide, or by filter-paper electrophoresis, yielded preparations inactive towards melezitose, but still possessing the characteristic transfructosidase activity towards sucrose. All preparations capable of attacking melezitose could also attack turanose.
7. None of the observations made requires the postulation of an α -glucosidase capable of attacking sucrose or sucrose derivatives. It is therefore suggested that the concept of a 'glucosaccharase' (Kuhn, 1923) should be abandoned until positive evidence for the existence of the enzyme has been obtained.

The author wishes to express his gratitude to Dr J. S. D. Bacon for helpful advice, criticism, and suggestions, and to Dr J. Edelman for allowing him to see the results of unpublished experiments. He is also indebted to the Medical Research Council for grants during the course of the investigation.

REFERENCES

- Adams, M., Richtmyer, N. K. & Hudson, C. S. (1943). *J. Amer. chem. Soc.* **65**, 1369.
- Bacon, J. S. D. (1952). *Biochem. J.* **50**, xviii.
- Bacon, J. S. D. & Edelman, J. (1951). *Biochem. J.* **48**, 114.
- Bacon, J. S. D. & Loxley, R. (1952). *Biochem. J.* **51**, 208.
- Bawden, F. C. & Pirie, N. W. (1942). *Brit. J. exp. Path.* **23**, 314.
- Bealing, F. J. & Bacon, J. S. D. (1951). *Biochem. J.* **49**, lxxv.
- Bealing, F. J. & Bacon, J. S. D. (1953). *Biochem. J.* **53**, 277.
- Bradfield, A. E. & Flood, A. E. (1950). *Nature, Lond.*, **166**, 264.
- Durum, E. L. (1950). *J. Amer. chem. Soc.* **72**, 2943.
- Edelman, J. & Bealing, F. J. (1953). *Biochem. J.* **53**, ii.
- French, D. (1951). *Science*, **113**, 352.
- Gottschalk, A. (1950). In *The Enzymes*, vol. 1, pt. I, p. 577. Ed. by Sumner, J. B. & Myrback, K. New York: Academic Press.
- Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
- Hérissey, H., Wickstrom, A., Courtois, J. E. & Dizet, P. (1952). *2nd Int. Congr. Biochem. Abstr.* p. 311.
- Hestrin, S. (1940). *Enzymologia*, **8**, 193.
- Hofmann, E. (1934). *Biochem. Z.* **273**, 198.
- Hough, L., Jones, J. K. N. & Wadman, W. H. (1949). *J. chem. Soc.* p. 2511.
- Kuhn, R. (1923). *Hoppe-Seyl. Z.* **129**, 57.
- Kuhn, R. & Münch, H. (1927). *Hoppe-Seyl. Z.* **163**, 1.
- Neuberg, C. & Mandl, I. (1950). In *The Enzymes*, vol. 1, pt. I, p. 542. Ed. by Sumner, J. B. & Myrback, K. New York: Academic Press.
- Pan, S. C., Andreasen, A. A. & Kolachov, P. (1950). *Science*, **112**, 115.
- Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
- Pazur, J. H. (1952). *Fed. Proc.* **11**, 267.
- Pazur, J. H. & French, D. (1952). *J. biol. Chem.* **193**, 265.
- Pottevin, H. (1903). *C. R. Acad. Sci., Paris*, **136**, 169.
- Raybin, H. W. (1933). *J. Amer. chem. Soc.* **55**, 2603.
- Raybin, H. W. (1937). *J. Amer. chem. Soc.* **59**, 1402.
- Tanret, C. (1902). *C. R. Acad. Sci., Paris*, **134**, 1586.
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
- Wallenfels, K. (1951). *Naturwissenschaften*, **38**, 306.
- Weidenhagen, R. (1932). *Ergebn. Enzymforsch.* **1**, 168.

Separation of Uroporphyrin Esters I and III by Paper Chromatography

BY J. E. FALK AND AMY BENSON

Nuffield Unit for the Investigation of Pyrrole Pigment Metabolism, Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

(Received 17 January 1953)

The separation of free porphyrins according to the number of carboxyl groups on their substituent side chains has been achieved by paper chromatography (Nicholas & Rimington, 1949, 1951a). This method is very sensitive, and has already revealed the presence of hitherto unknown porphyrins in pathological urines (McSwiney, Nicholas & Prunty, 1950; Nicholas & Rimington, 1951a; Rimington & Miles, 1951); it does not, however, separate the position isomers coproporphyrins I and III or uroporphyrins I and III. The method of Chu, Green & Chu (1951), in which the porphyrin esters are used, allows the identification of protoporphyrin, coproporphyrin I, coproporphyrin III and uroporphyrin I in mixtures. But these workers did not study uroporphyrin III and we have found that the uroporphyrin isomers do not separate under their conditions.

The identification of a uroporphyrin isolated from natural sources as the series I or series III isomer has relied in the past mainly on comparison of the melting point of the esterified specimen with

the melting points of the supposedly pure isomers. Only since the isolation by Nicholas & Rimington (1951b) of unequivocal uroporphyrin III from turacin, has the melting point (264°) of pure uroporphyrin III ester been established beyond doubt. Even so, reliable estimates of the composition of a specimen from natural sources cannot be made simply from melting-point comparisons, because of the unexpected form of the mixed melting-point curve recently established in this laboratory for uroporphyrin esters I and III (Nicholas & Rimington, 1953). A powerful tool has been provided, however, by the use of this curve coupled with the specific decarboxylation of uroporphyrin mixtures to the corresponding coproporphyrins, with subsequent determination of the proportions of the latter (cf. Rimington & Miles, 1951).

In this paper we describe a paper-chromatographic method which gives good separation of the uroporphyrin esters I and III. By a combination of this method with that of Chu *et al.* (1951) all five main natural porphyrins (uroporphyrins I and III,