6. The crystalline isomer obtained from tenuazonic acid, and previously called *iso*tenuazonic acid, is shown to be a mixture of tenuazonic acid and a diastereoisomer.

7. Tenuazonic acid is believed to be the first substituted tetramic acid isolated from natural sources.

I wish to thank Professor J. H. Birkinshaw for the use of a Hilger and Watts Uvispek spectrophotometer, purchased by means of a grant from the Central Research Fund of the University of London; Miss E. M. Tanner and Mr F. H. Oliver of Parke, Davis and Co. Ltd., Hounslow, Middlesex, for carrying out infrared analyses and microanalyses respectively; and Dr R. N. Lacey, of British Industrial Solvents, Hull, for supplying infrared curves.

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Oligosaccharide Synthesis in the Banana and its Relationship to the Transferase Activity of Invertase

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(*Received* 15 *August* 1958)

The banana fruit is normally picked when it is firm and green; ripening is then carried out under controlled conditions. It is well known that during such ripening the starch is largely converted into glucose, fructose and sucrose, and water is lost from the skin to the pulp (see Loesecke, 1949). In addition to these sugars, we have detected an oligosaccharide which increases in amount during the ripening process.

The invertase activity of banana pulp has been recognized for many years (Mierau, 1893*a*, *b*). Invertases from a variety of plant tissues have been shown *in vitro* to synthesize oligosaccharides by transglycosidation (see Edelman, 1956; Gottschalk, 1958). It therefore seemed probable that this action was responsible for the formation of banana oligosaccharide *in vivo*.

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This paper describes the separation and provisional identification of the above oligosaccharide, along with the partial purification of banana invertase and some studies of its hydrolytic and transferase activities.

MATERIALS AND METHODS

Bananas. Fruits of the variety *Musa cavendishii* (Lamb.), sent by rail from New South Wales, reached Melbourne within a few days of picking in a very firm and quite green condition. They were placed in single layers on wooden trays in a metal bin where ripening was accelerated by exposure for about 16 hr. to an atmosphere of coal gas. The trays were then transferred to a ventilated room which was maintained at 25° and ripening allowed to proceed in darkness. The first sample batch of thirty bananas was taken immediately upon arrival and before gassing; then at intervals of 4–5 days during ripening, similarly sized batches were withdrawn at random and held in a cold room

at 4–10° in separate, sealed containers in an atmosphere of carbon dioxide until analysis. The four stages of ripening selected for study were designated as green, green–yellow, yellow and speckled brown. The total ripening period was about 14 days, after which analysis was immediately commenced. Samples of 30 g. were taken after the bananas had been peeled, minced and the pulp thoroughly mixed. The samples were dehydrated in 97% (v/v) ethanol and then treated as described below.

Soluble sugars and starch. Soluble sugars were extracted by refluxing the dehydrated sample with the original ethanol adjusted to 80% (v/v), for 6 hr., after the method of Barnell (1941). Starch was estimated in the residue as by Pucher & Vickery (1936). Reducing sugars were estimated by the Lane & Eynon (1950) volumetric method. Non-reducing sugars were determined by the same procedure after hydrolysis in 0·1N-HCl at 100° for 15 min.

Sugars. The sample of 6^{G}-β-fructosylsucrose (for nomenclature see Allen & Bacon, 1956) was kindly supplied by Dr D. Gross, Tate and Lyle Research Laboratory, Keston, Kent. The other sugars were of A.R. quality and their purity, as checked by paper chromatography, was satisfactory.

Yeast invertase. A commercial preparation (British Drug Houses Ltd.) was used.

Banana invertase. A partially purified preparation was obtained as follows. About 1 kg. of pulp from ripe bananas was extracted with 1 l. of 0·05M-sodium pyrophosphate–HCl buffer, pH 7·8, for 2 min. in a Waring Blendor. The dispersion was centrifuged at 2700 *g* for 30 min. The supernatant was removed, brought to pH 7, then solid ammonium sulphate added to 30% saturation and again centrifuged at 2700 *g* for 30 min. The resulting supernatant was increased to 50% (w/v) saturation and centrifuged at 2700 *g* for 30 min. The precipitate was dissolved in about 50 ml. of 0·1M-sodium phosphate buffer, pH 7·2. The brown, somewhat turbid solution was then centrifuged at 1500 *g* for 30 min. and the clear, brown supernatant used as the source of invertase.

Synthesis of oligosaccharides. Yeast invertase was usually incubated for 30 min. with sucrose (10%, w/v) in 0·01M-sodium acetate–acetic acid buffer at pH 5·0 and 25°. Banana invertase was also incubated as described above with sucrose concentrations up to 20% (w/v) for varying times either at room temperature or at 25°. Chromatographic tests of the above solutions, inactivated by boiling, showed no detectable amounts of glucose, fructose or oligosaccharides at zero time of incubation.

Paper chromatography. Whatman no. 3 paper was used throughout. The most generally useful solvent system was butanol–acetic acid–water (5:1:4, by vol.). The other solvent systems used were: propanol–water–aq. NH_3 soln. (16:3:1, by vol.); butanol–ethanol–water (57:13:32, by vol.); butanol–pyridine–water (6:4:3, by vol.).

Spray reagents. The following were used: naphtharesorcinol (Bryson & Mitchell, 1951); aniline hydrogen phthalate (Partridge, 1949); benzidine–trichloroacetic acid (Bacon & Edelman, 1951) and a saturated solution of *p*-anisidine phosphate in moist butanol.

Diazouracil test. The Raybin (1933) test as modified by Feingold, Avigad & Hestrin (1956) was carried out on quantities of sugar about 0·5 mg.

5-Aminouracil was diazotized and stored at 4°; this retained full activity over the 4 weeks' period of test.

Measurement of hydrolysis. The total reducing sugar released by invertase action on sucrose was determined according to King (1951) with the modified Harding & Downs copper reagent, and the cuprous oxide formed was estimated by the blue colour produced with the phosphomolybdic acid solution of Folin & Wu (1920). The inhibitory action of copper on invertase was used to stop the enzymic action at the required time.

EXPERIMENTAL AND RESULTS

In agreement with the findings of earlier workers using other varieties of bananas (see Loesecke, 1949), experiments over several years have shown that there is a substantial decline in the starch content and concurrent increase in both non-reducing and reducing sugars during the ripening process (e.g. see Table 1).

Oligosaccharide in the ripening banana

Fig. 1 shows a chromatogram of the ethanolic extracts of bananas at the four stages of ripening, together with marker spots of glucose, fructose and sucrose. It is seen that these three sugars are present at all stages but increase substantially even at very early stages of ripening when the banana is still quite firm. In addition, there is present a component with R_F less than that of sucrose and whose colour reactions with *p*-anisidine and naphtharesorcinol led to tentative identification as an oligosaccharide consisting predominantly of fructose. This component appears at an early stage and increases during the later stages of ripening.

It seemed likely that the unknown spot was a product formed by invertase action. The reaction products of yeast invertase and sucrose were therefore compared chromatographically with

Table 1. *Reducing and non-reducing sugars in bananas*

Stage of ripeness of bananas	Percentage of fresh pulp			
	Starch	Reducing sugar	Non-reducing sugar	Moisture content
Green	12·1	<0·5	<0·5	71
Yellow–green	8·8	5·0	5·1	74
Yellow	1·9	8·4	8·9	74
Speckled brown	0·9	10·3	8·2	75

ethanolic extracts of banana and it was observed that there was an oligosaccharide formed by the yeast invertase in a position identical with that of the unknown component obtained from the banana.

Separation and crystallization of the oligosaccharide

The ethanolic extract of banana was applied to the paper as a streak and then run in the usual butanol–acetic acid solvent. The positions of the sugars were defined by spraying narrow strips cut down the long edges of the paper. The paper was then cut across just behind the sucrose streak to obtain a strip containing the unknown saccharide which was eluted with water into about 0·3 ml. of solution and concentrated *in vacuo* at room temperature; on being taken slowly to dryness a crystalline residue was obtained. This residue was rechecked by paper chromatography and one spot only was obtained (see Fig. 2).

Identification of the oligosaccharide

The banana oligosaccharide was hydrolysed in a sealed tube for 1 hr. at 100° in 0·6N-HCl. Tested chromatographically, the hydrolysate showed only fructose and glucose. The ratio of these two sugars was then determined on paper, chromatographically, by the micromethod of Gottschalk & Ada (1956). For estimation of fructose against fructose standards the naphtharesorcinol-spray reagent was used, and, for glucose, aniline hydrogen phthalate was used. The ratio of fructose to glucose was found to be 2:1 (±10%).

The Raybin (1933) diazouracil test on the compound gave a deep yellow–green (positive reaction). Addition of magnesium sulphate to this coloured solution produced a stable blue precipitate.

Chromatographic comparison of the oligosaccharide with authentic 6^G-β-fructosylsucrose in the four solvent systems listed revealed identical R_F values and colour shades for the two substances.

Transferase activity of banana invertase in vitro

Paper chromatography of the products of the banana invertase–sucrose reaction showed the presence of three spots with R_F values less than that of sucrose.

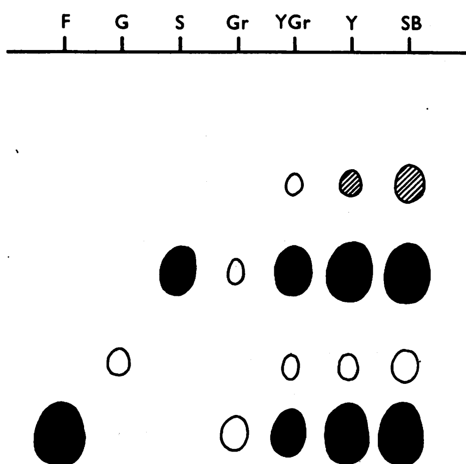

Fig. 1. Chromatogram of equal volumes (about 5 µl.) of extracts of 30 g. of banana pulp (fresh weight) in 80% (v/v) ethanol (final volume 250 ml.), at the stages of ripeness corresponding to green (Gr), yellow–green (YGr), yellow (Y) and speckled brown (SB). Reference sugars consisted of equal volumes (about 5 µl.) of 2·5% (w/v) fructose (F), 2·5% (w/v) sucrose (S) and 5% (w/v) glucose (G). The chromatograms were run for 18 hr. in butanol–acetic acid–water (5:1:4, by vol.) at 25°, dried and sprayed with 0·2% naphtharesorcinol solution (Bryson & Mitchell, 1951). The degree of shading indicates approximately the intensity of coloration of the spots.

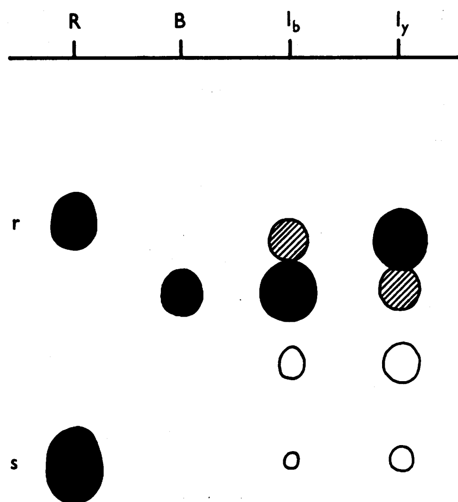

Fig. 2. Chromatogram of the oligosaccharides obtained from ripe bananas (B), the banana invertase–sucrose reaction (I_b) *in vitro* and the yeast invertase–sucrose reaction (I_y) *in vitro*. These products were separated chromatographically in butanol–acetic acid–water from glucose, fructose and sucrose (see Fig. 1), eluted, concentrated and run again in the same solvent system. B, Crystalline oligosaccharide component from ethanolic extract of banana. I_b, Partially purified banana invertase (see text) incubated with 20% (w/v) sucrose in 0·02M-sodium phosphate–0·01M-sodium citrate buffer, pH 4·0, for 120 min. at 25°; action was stopped by boiling. I_y, Yeast invertase incubated with 10% (w/v) sucrose in 0·01M-sodium acetate–acetic acid buffer, pH 5·0, for 30 min. at 25°; action was stopped by boiling; spots I, II and III are labelled after Bacon & Edelman (1950). R, Reference mixture of 2·5% (w/v) raffinose (r) and 2·5% (w/v) sucrose (s). The degree of shading indicates approximately the intensity of coloration of the spots.

The oligosaccharides produced by this reaction and also those produced by the yeast invertase–sucrose reaction were separated from the mono- and di-saccharides by paper chromatography, eluted and concentrated as already described for the oligosaccharide from the ethanolic extract of banana. Chromatographic comparison of the oligosaccharides from these sources (see Fig. 2) showed that the three spots produced by the banana invertase–sucrose reaction *in vitro* coincided with the spots numbered I, II and III by Bacon & Edelman (1950) from the yeast invertase–sucrose reaction. The single oligosaccharide spot from the banana extract had an R_F value identical with spot II (banana), which was the main component synthesized by the banana invertase *in vitro*. The banana invertase–sucrose reaction always gave a predominant spot II under the varying conditions of pH, buffer, temperature and time used, as compared with a predominant spot III in yeast invertase–sucrose tested similarly.

K_m values for banana and yeast invertases acting on sucrose

Preliminary experiments have shown that the pH optimum of banana invertase is at about pH 4. As with yeast invertase, the pH–activity curve has a rather broad peak but falls away steeply on each side. K_m values at various pH values so far determined reveal a large variation between banana and yeast invertases, e.g. at pH 5·1 the values were 0·5 and 20·0 mM respectively.

DISCUSSION

The results shown in Table 1 are in agreement with the findings of earlier workers (see Loesecke, 1949) that starch is converted into glucose, fructose and sucrose during ripening of the banana. In addition to these mono- and di-saccharides it is clear from the preceding section that at least one oligosaccharide is also formed during ripening. All these sugars appear at a very early stage when the fruit is quite firm and the cells intact, and they increase throughout ripening.

The oligosaccharide found in the banana appears to be identical with one formed by the action of banana invertase on sucrose *in vitro* and also with a component oligosaccharide of spot II from the yeast invertase–sucrose reaction (see Fig. 2). Spot II from the latter reaction has been separated chromatographically by Bacon (1954) using a charcoal–Celite column, and shown to consist of two trisaccharides, each of two fructose and one glucose residue. These two oligosaccharides, designated by Bacon as II_1 and II_2, have been investigated by several workers (see Edelman, 1956; Gottschalk, 1958) and the structures shown to be: II_1, 1-β-fructosylsucrose; II_2, 6^G-β-fructosylsucrose. The evidence obtained shows that the ripening banana contains 6^G-β-fructosylsucrose, which increases in amount along with sucrose, glucose and fructose during ripening. Banana invertase was shown to catalyse the formation of oligosaccharides *in vitro* and it is concluded that the banana oligosaccharide also is formed *in vivo* by transfructosylation from sucrose to other sucrose molecules (acting as acceptors) and catalysed by the same enzyme.

Although there have been numerous examples of such transfructosylation (and several of transglucosylation) by invertases since the discovery of the transferring action of these hydrolases *in vitro* (see Gottschalk, 1958), to our knowledge there has been no previous demonstration that this action can occur *in vivo* in higher plants. It might be argued that this reaction occurs only in cells which have undergone some type of degeneration. However, the anatomical evidence of early investigators (see Loesecke, 1949) strongly suggests that most cells of the ripening banana remain quite intact, with little or no degeneration of the cytoplasm, until the very last stages of ripening, and it is quite clear that the oligosaccharide component makes its appearance long before the banana reaches a 'mushy' stage.

The banana invertase–sucrose reaction produces, *in vitro*, in addition to the spot II (banana) already discussed, spots which are coincident with spots I and III from the yeast invertase–sucrose reaction. These latter have been shown (see Gottschalk, 1958) to be fructosylglucose and 6^F-β-fructosylsucrose respectively and it is likely that the sugars produced by the banana invertase are the same. It is not clear why only trisaccharide fraction II is found in the ethanolic extract of the banana whereas a number of different oligosaccharides are formed by banana invertase action on sucrose *in vitro*. It is possible that the other products found *in vitro* are also formed *in vivo* but are preferentially metabolized.

SUMMARY

1. The starch, sucrose and monosaccharide content of bananas of the variety *Musa cavendishii* has been investigated during ripening. In agreement with earlier workers using similar varieties it has been observed that starch is converted into glucose, fructose and sucrose during this process.

2. A trisaccharide also formed during the ripening process has been crystallized from banana extracts and provisionally identified as 6^G-β-fructosylsucrose.

3. The hydrolytic and transferase activities of banana invertase have been demonstrated *in vitro*.

The oligosaccharides formed have been compared chromatographically with the above trisaccharide and those components of the yeast invertase–sucrose reaction having similar R_F values. Conclusions are drawn about the transferase activity of banana invertase *in vivo*.

We are indebted to Dr A. Gottschalk of the Walter and Eliza Hall Institute of Research for his helpful discussions.

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Fungal detoxication

4. METABOLISM OF 2-METHOXYNAPHTHALENE BY *ASPERGILLUS NIGER**

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(*Received* 2 *July* 1958)

The metabolism of naphthalene by a variety of animals has been extensively studied by Young and by Boyland and their respective co-workers. The initial product in rats was shown by Young (1947) to be D-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene, both the diol and 1-naphthol and their conjugates having been isolated from urine. There appears to be little breakdown of the naphthalene ring, however (Boyland & Wiltshire, 1953), which is in sharp contrast with the degradation of naphthalene compounds by soil micro-organisms (Walker & Wiltshire, 1953, 1955; Treccani, Walker & Wiltshire, 1954; Canonica, Fiecchi & Treccani, 1957).

In previous work by Byrde, Harris & Woodcock (1956) the metabolism of ω-(2-naphthyloxy)-*n*-alkylcarboxylic acids by *Aspergillus niger* van Tiegh. was studied, the main features being nuclear hydroxylation, together with β-oxidation of all side chains except those of 2-naphthyloxyacetic acid and β-(2-naphthyloxy)propionic acid. In a later paper (Byrde & Woodcock, 1958) a similar investigation, in which *Sclerotinia laxa* Aderh. & Ruhl. was used with the same series of acids, showed β-oxidation to be the main process involved, nuclear hydroxylation being virtually absent. There was little evidence of ring breakdown of the naphthyloxy acid with either fungus, and the present investigations with 2-methoxynaphthalene as a non-polar sole-carbon source were made in order to examine the effect of this change on ring-fission.

* Part 3 Byrde & Woodcock (1958).

EXPERIMENTAL

All m.p.'s are uncorrected. Ultraviolet-absorption spectra were determined in methanol solution with a Unicam SP. 500 spectrophotometer. Infrared-absorption spectra were determined on mulls prepared by intimately grinding the samples with Nujol, by using a Hilger 800 spectrophotometer.

MATERIALS

2-*Methoxynaphthalene* (nerolin). This was used as obtained from British Drug Houses Ltd., m.p. 73–74°.

2-*Ethoxynaphthalene*. This was prepared from 2-naphthol with diethyl sulphate and NaOH. Crystallized from ethanol, it had m.p. 35–36°.

4-*Methoxysalicylic acid*. This was prepared from 2:4-dihydroxybenzoic acid by the method of Gomberg & Johnson (1917). It crystallized from water in long silky needles, m.p. 157–158° (Found: C, 57·1; H, 4·8. Calc. for $C_8H_8O_4$: C, 57·1; H, 4·7%). Gomberg & Johnson (1917) give m.p. 157°. The ultraviolet-absorption spectrum showed