721. *Studies of* Aspergillus niger. *Part II.** *Transglycosidation by* Aspergillus niger.

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Aspergillus niger (strain 152) (Yuill, *Chem. and Ind.*, 1952, 755) synthesises nigeran from a wide variety of carbohydrate substrates. It contains a transglucosidase responsible for the synthesis of *iso*maltose and panose from maltose, and transfructosidase(s) which utilise sucrose in the formation of trisaccharides.

Nigeran (originally named mycodextran), which was first isolated by Dox and Niedig (*J. Biol. Chem.*, 1914, **18**, 167; 1915, **20**, 83), has been shown recently (Barker, Bourne, and Stacey, *Chem. and Ind.*, 1952, 756; Part I*) to be a polyglycosan in which most, if not all, of the α-1 : 4- and α-1 : 3-linkages are arranged alternately. Before attempting to isolate the enzyme(s) responsible for the synthesis of nigeran, from *Aspergillus niger* "152," it was decided to investigate the carbohydrate metabolism of this mould, and to determine the influence of trace ions present in the culture medium on the production of this novel glucosan.

Comparison of the growth of *A. niger* "152" in sucrose and Currie's medium (*J. Biol. Chem.*, 1917, **31**, 15), which contains Mg^{++}, Zn^{++}, and Fe^{++}, with that obtained in the absence of one of these ions, revealed that while the omission of Mg^{++} prevented growth, Fe^{++} deficiency produced a greater weight of mycelium and of nigeran than was obtained in the control medium. Although Zn^{++} deficiency did not prevent the formation of a mycelium, no nigeran was produced.

A study of the utilisation of a series of carbohydrate substrates and the storage of nigeran by *A. niger* "152" was followed by an examination of the residual culture medium (A), the soluble (C) and insoluble carbohydrates (B) present in a boiling-water extract of the mycelium after cooling, and the hydrolysable carbohydrates (D) retained by the extracted mycelium. Sodium acetate failed to support growth, and D-galactose, lactose, αα-trehalose, methyl α-D-glucopyranoside, and dipotassium α-D-glucose 1-phosphate gave only small mycelia in which nigeran could not be detected. D-Glucose, D-fructose, sorbitol, D-xylose, maltose, sucrose, starch, and inulin produced large mycelia which stored nigeran intracellularly, but only small amounts of the glucosan were obtained from mycelia grown on L-sorbose and D-mannitol (see Table 3, p. 3590).

Table 1. *Metabolism of certain substrates.*

Substrate	Other sugars produced having R_F values identical with those of:
Maltose	Glucose, *iso*maltose, panose, tetrasaccharide (trace : *iso*maltotriose, nigerose).
Starch	Glucose (trace : *iso*maltose).
Sucrose	Fructose, glucose, trisaccharides (trace : tetrasaccharide).
Inulin	Fructose (*inter al.*).
Fructose	Oligosaccharides? ($R_{Fructose}$ 0·63, 0·50, 0·41, 0·36).

No sugars other than the substrate were detected in (A) when the mould was grown on D-xylose, D-glucose, D-galactose, D-mannitol, sorbitol, L-sorbose, lactose, dipotassium α-D-glucose 1-phosphate, and methyl α-D-glucoside. After 10 days D-xylose and D-glucose had been completely utilised, but some sorbitol still remained. Substrates which gave rise to other sugars in the medium are listed in Table 1.

With all substrates which gave rise to nigeran, a component having an R_F value identical with that of glucose was found in (C). In all cases where growth was observed, the extracted mycelium, after hydrolysis, gave three components having mobilities identical with those of glucose, galactose, and fructose, suggesting the storage of these sugars as polysaccharides.

A closer investigation of the storage of nigeran by *A. niger* growing on glucose, sucrose, and maltose revealed that, whereas no nigeran could be detected after 3 days, it reached a maximum after about 5 days, further incubation only serving to increase the weight of

* Part I, *J.*, 1953, 3084.

mycelium obtained (Table 4, p. 3591). After the incubation of resting cells of *A. niger* (mycelium grown on sucrose, 3 days) with these same sugars, larger amounts of nigeran could be isolated than with a saline control. Similar but smaller amounts of nigeran were obtained by using the same resting cells after freeze-drying (Table 5, p. 3591).

With the exception of the production of the supposed oligosaccharides from fructose, the sugars formed from the substrates listed in Table 1 by propagating cells of *A. niger* were also produced by resting cells and by a cell-free extract of the mould. Since it was believed that the possible precursors of nigeran might be a trisaccharide or a disaccharide phosphate in which one of the glucosidic links (α-1 : 3 or α-1 : 4) was pre-formed, the synthesis of oligosaccharides from sucrose and maltose was investigated more closely.

The oligosaccharides produced from maltose by a cell-free extract of the mould were fractionated on a charcoal column (Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677) and characterised as crystalline sugars or their derivatives. The isolation of *iso*-maltose (6-*O*-α-D-glucopyranosyl-D-glucose), panose [*O*-α-D-glucopyranosyl-(1 ⟶ 6)-*O*-α-D-glucopyranosyl-(1 ⟶ 4)-α-D-glucopyranose], and glucose, and the detection of *iso*-maltotriose indicates the presence of an intracellular transglucosidase analogous to the extracellular enzymes produced by *A. niger* NRRL-337 (Pan, Andreasen, and Kolachov, *Science*, 1950, **112**, 115; *Arch. Biochem.*, 1951, **30**, 6; Pan, Nicholson, and Kolachov, *J. Amer. Chem. Soc.*, 1951, **73**, 2547) and by *A. oryzae* (Pazur and French, *J. Amer. Chem. Soc.*, 1951, **73**, 3536; *J. Biol. Chem.*, 1952, **196**, 265). The synthesis of a second disaccharide having a mobility equal to that of nigerose (3-*O*-α-D-glucosyl-D-glucose) is being further investigated.

Two trisaccharides, separated by the gradient elution method of Alm, Williams, and Tiselius (*Acta Chem. Scand.*, 1952, **6**, 826), and a tetrasaccharide were isolated from the products of the action of a cell-free extract of *A. niger* on sucrose. One trisaccharide, which constituted the major part of the trisaccharide fraction, was obtained crystalline. It was non-reducing, contained two D-fructose and one D-glucose residue, and had an R_F value identical with *O*-α-D-glucopyranosyl (1 ⟶ 2)-*O*-β-D-fructofuranosyl (1 ⟶ 2)-β-D-fructofuranoside, a trisaccharide produced from sucrose by using " Takadiastase " (Bacon and Bell, *J.*, in the press) and kindly supplied to us by Dr. J. S. D. Bacon. It had $[\alpha]_D^{16}$ +29·2° in H_2O, which was similar to the values between +30·5° and +32·6° reported by Bacon and Bell (*loc. cit.*) for their syrupy trisaccharide. The other trisaccharide was also non-reducing and contained two D-fructose and one D-glucose residue. Structural studies on these trisaccharides are in progress and will be reported later. Both of them, as well as the tetrasaccharide fraction, which contained three fructose and one glucose residue, presumably arose by transfer of fructosyl groups to sucrose, a phenomenon also found with extracts of other moulds (Wallenfels and Bernt, *Angew. Chem.*, 1952, **64**, 28; Bealing and Bacon, *Biochem. J.*, 1953, **53**, 277; Pazur, *J. Biol. Chem.*, 1952, **199**, 217).

Experimental

Effect of Trace Ions on the Synthesis of Nigeran.—A medium (250 c.c.) was prepared containing sucrose (37·5 g.), NH_4NO_3 (0·625 g.), KH_2PO_4 (0·25 g.), $MgSO_4,7H_2O$ (0·0625 g.), N-HCl (0·25 c.c.), $ZnSO_4,7H_2O$ (0·010 g.), and $FeSO_4,7H_2O$ (0·010 g.), together with three other similar media from which zinc sulphate, ferrous sulphate, and magnesium sulphate were severally omitted. The sulphate of the medium deficient in magnesium sulphate was supplied as ammonium sulphate (0·0625 g.). After inoculation with *Aspergillus niger* " 152," and incubation for 6 days at 30°, no growth was observed in the Mg^{++}-deficient medium. Nigeran (fraction 1) was isolated by washing each mycelium thoroughly with distilled water (6 × 100 c.c.) and extracting it with boiling water (2 × 100 c.c.) for 0·5 hr., successive extracts being filtered while hot, combined, and set aside at 0° for 18 hr. The nigeran precipitate was separated by centrifuging, washed with alcohol, and dried *in vacuo* over P_2O_5 at 60°. The extracted mycelium was dried at 100° and weighed (see Table 2). Further nigeran fractions were isolated by autoclaving the mycelium with water (100 c.c.) at 15 lb./sq. in. for 30 min. (fraction 2), and by extraction with cold N-sodium hydroxide (50 c.c.) for 24 hr. at room temperature, nigeran (fraction 3) separating on neutralisation of the filtered extract. Portions (20 mg.) of each fraction were submitted to partial hydrolysis by N-sulphuric acid at 90° for 2 hr. and the neutral-

ised hydrolysates analysed chromatographically as the free sugars and as their benzylamine derivatives (Bayly and Bourne, *Nature*, 1953, **171**, 385) with a *n*-butanol (40%), ethanol (10%), water (49%), ammonia (1%) solvent mixture. Components having R_F values identical with those of glucose, maltose, and 3-*O*-α-D-glucosyl-D-glucose were detected. The optical rotation of each nigeran fraction was determined in N-sodium hydroxide.

TABLE 2. *Effect of trace ions on the synthesis of nigeran.*

	Control		Fe^{++}-deficient		Zn^{++}-deficient
Medium :	g.	$[\alpha]_D$	g.	$[\alpha]_D$	g.
Fr. 1	0·137	+225°	0·216	+243°	None
Fr. 2	0·049	+254	0·136	+250	None
Fr. 3	0·264	+234	0·450	+218	None
Mycelium	3·354	—	6·033	—	1·122

On repetition, the above experiment gave similar results. Extension of the time of incubation of the Zn^{++}-deficient medium from 6 to 12 days resulted in an increased yield of the extracted mycelium but this was not accompanied by the production of nigeran. The aqueous extract had only a negligible rotation but was stained blue with iodine.

Metabolism of Various Substrates by Propagating Cells of Aspergillus niger.—The carbohydrate substrate was dissolved in the mineral component of Currie's medium (10 g. in 100 c.c.), steam-sterilised, inoculated with aliquot volumes (1 c.c.) of a suspension of *A. niger* " 152 " and incubated at 30°. During incubation, spots of the medium were submitted to paper chromatography as described above, the papers being sprayed with aniline hydrogen phthalate (Partridge, *Nature*, 1949, **164**, 443) for reducing sugars, naphtharesorcinol (Partridge, *Biochem. J.*, 1948, **42**, 238) for keto-sugars, and ammoniacal silver nitrate (Hough, *Nature*, 1950, **165**, 400) for non-reducing sugars and sugar-alcohols. After 10 days, the residual culture medium (A) was decanted from the mycelial felts, the mycelia were washed and extracted with boiling water (100 c.c. × 1; 50 c.c. × 3), and the nigeran (B) was obtained as described above. The supernatant extract (C) was concentrated to a small volume (10 c.c.) and analysed chromatographically. The residual mycelium (D) was washed with alcohol and ether and dried. No growth was observed with sodium acetate as the substrate.

TABLE 3. *Production of nigeran from various substrates.*

	Yield (as % of substrate)				
Substrate	*B* (Nigeran)	*D*	$[\alpha]_D$ of *B*	Sugars in hydrolysate of *B*	Infra-red analysis of *B*,* 700—960 cm.$^{-1}$
D-Xylose	0·39	13·67	+231°	Glucose	Nigeran
D-Glucose	2·77	23·50	+243	Glucose	Nigeran
D-Galactose	None	0·38	—	—	—
D-Fructose	1·37	21·80	+209	Glucose	Nigeran
L-Sorbose	0·01	17·70	—	Glucose	Nigeran
D-Mannitol	0·01	8·33	—	Glucose	Nigeran
D-Sorbitol	1·04	12·46	+226	Glucose	Nigeran
α-D-Glucose 1-phosphate	None	0·20	—	—	—
Methyl α-D-glucoside ...	None	0·20	—	—	—
Maltose	1·80	19·04	+253	Glucose	Nigeran
Sucrose	1·60	19·44	+247	Glucose	Nigeran
Lactose	None	0·89	—	—	—
αα-Trehalose	None	0·20	—	—	—
Melezitose	None	6·61	—	—	—
Starch	0·82	25·30	+201	Glucose (fructose)	Nigeran
Inulin	1·50	18·22	+202	Glucose (fructose)	Nigeran

* Barker, Bourne, Stacey, and Whiffen, *Chem. and Ind.*, 1953, 196.

Rates of Storage of Nigeran by Aspergillus niger.—Digests containing severally glucose, maltose, and sucrose (15 g.), together with mineral medium (150 c.c.), were each prepared in triplicate, sterilised, inoculated with aliquots (5 c.c.) of a suspension of *A. niger* in sterile distilled water, and incubated at 30°. After 3, 5, and 7 days a culture of each of the three substrates was removed for analysis. Spots of the medium were analysed chromatographically and nigeran was extracted as above from each mycelium with boiling water (5 × 50 c.c.). The extracted cell residues were dried in the usual manner.

Metabolism of Resting Cells of Aspergillus niger *with Various Substrates.*—Freeze-dried washed cells (1·009 g.) of *A. niger*, which had been previously grown on sucrose, were suspended

in water (20 c.c.) and portions (1 c.c.) added to each of a number of 2·5% carbohydrate solutions (2 c.c.) in centrifuge tubes; the mixtures were shaken and incubated at 30°. Spots of the supernatant liquid obtained after centrifuging were removed after 3 and 24 hr. and the

TABLE 4. *Storage of nigeran by* A. niger.

Substrate	Days of incubation	Yield of nigeran (as % of substrate)	$[\alpha]_D$ of nigeran	Yield of extracted cells (as % of substrate)	Sugars detected in medium: Main	Trace
Glucose ...	3	None	—	6·1	Glucose	—
,, ...	5	1·86	+254°	15·9	—	Glucose
,, ...	7	1·78	+248	23·4	—	—
Sucrose ...	3	None	—	6·0	Sucrose, glucose, fructose	Trisaccharide
,, ...	5	1·97	+249	15·9	Glucose, fructose	Sucrose
,, ...	7	1·62	+259	26·4	—	—
Maltose ...	3	None	—	4·7	Maltose, glucose	Panose, *iso*maltose
,, ...	5	1·44	+247	14·6	Glucose	Maltose, *iso*maltose, panose
,, ...	7	1·46	+252	18·2	—	*iso*Maltose, glucose

cells resuspended. The spots were analysed on paper chromatograms with the solvents and sprays enumerated above.

Effect of Freeze-drying on the Synthesis of Nigeran by Resting Cells.—A washed three-day mycelium of *A. niger*, grown on sucrose, was shredded in a Waring Blender and again washed free from cell debris with water. The moist cells were divided, one-half was freeze-dried and stored at 0°, and the other added in equal amounts (4·0 g.) to sterile solutions of glucose, maltose, and sucrose (7·5 g.) in water (50 c.c.) and to a control solution of N-sodium chloride (50 c.c.). A comparative experiment was carried out with freeze-dried cells in equivalent amounts (1·23 g.). After 7 days' incubation, the nigeran was obtained from each digest by four boiling-water extractions in the usual manner. The extracted cell residues were dried and weighed. A control extraction of fresh resting cells (4 g.) gave 0·834 g. of extracted cell residue and 0·026 g. of nigeran (see Table 5).

TABLE 5. *Effect of freeze-drying on resting cells.*

	Fresh resting cells			Freeze-dried resting cells		
Substrate	Nigeran (g.)	$[\alpha]_D$ of nigeran	Extracted cell residue (g.)	Nigeran (g.)	$[\alpha]_D$ of nigeran	Extracted cell residue (g.)
Glucose	0·058	+260°	1·283	0·048	+239°	0·918
Maltose	0·091	+279	1·186	0·067	+231	0·590
Sucrose	0·072	+278	1·070	0·038	+232	0·335
Saline	0·030	+238	0·464	0·027	+267	0·329

Preparation of a Cell-free Intracellular Enzyme Extract.—A suspension of freeze-dried cells of *A. niger* (1 g.) in water or buffer was shaken at 0° with glass beads for 20 min. in a Mickle Tissue Disintegrator, and the cell debris washed out with water (20 c.c.), shaken for 1 hr., and centrifuged at 5000 r.p.m. to eliminate cell debris. A spot of the extract was stained with carbofuchsin to confirm the absence of unbroken cells.

Effect of a Cell-free Extract of A. Niger *on Various Substrates.*—Digests containing the cell-free enzyme extract (1 c.c.) with each of a series of 2·5% carbohydrate substrate solutions (2 c.c.) were incubated at 37° and spots of the solutions analysed chromatographically in the manner previously described.

Isolation of the Oligosaccharides synthesised from Maltose by a Cell-free Extract.—A cell-free 0·2M-citrate extract (pH 6·2; 20 c.c.) of freeze-dried *A. niger* (0·786 g.) was added to a maltose solution (6·985 g. in 200 c.c. of water), layered with toluene, and incubated at 30—35°; the production of oligosaccharides was followed on a paper chromatogram. After 8 days, enzyme action was arrested by heating at 100° for 15 min., the absence of polysaccharide in the culture filtrate confirmed by addition of methanol (3 vol.), and the solution concentrated to dryness *in vacuo*. The residue was extracted with dry methanol (200 c.c.) under reflux; the extract was cooled, filtered from insoluble citrate, and concentrated *in vacuo*. The residue was dissolved in water (100 c.c.) and fractionated on a charcoal column with increasing concentrations of aqueous ethanol (Whistler and Durso, *loc. cit.*). After paper-chromatographic analysis, similar

fractions were combined, concentrated, purified by methanol extraction, and freeze-dried. The yields and contents of these fractions are shown in Table 6.

TABLE 6. *Oligosaccharide fractions.*

Fraction	Chromatographic analysis	Yield (g.)
I	Glucose	0·651
II	*iso*Maltose	0·789
III	Maltose	0·959
IV	Panose	0·923
V	Multicomponent mixtures	0·379

Characterisation of the Oligosaccharide Fractions.—Crystallised from absolute methanol, fraction I gave α-D-glucose (0·476 g.), m. p. and mixed m. p. 141·5—143·5°, $[\alpha]_D^{17}$ +52° (*c*, 1·039 in H_2O; equil.) (Found: C, 40·0; H, 6·8. Calc. for $C_6H_{12}O_6$: C, 40·0; H, 6·7%). The glucose was further characterised as its β-penta-acetate, m. p. and mixed m. p. 130—132° (Found: C, 49·1; H, 5·6. Calc. for $C_{16}H_{22}O_{11}$: C, 49·15; H, 5·6%).

Fraction II was chromatographically pure *iso*maltose, having $[\alpha]_D^{17}$ +122·9° (*c*, 0·154 in H_2O) when corrected for ash (4·88%), and giving only glucose on hydrolysis with 2N-sulphuric acid. The *iso*maltose (0·0684 g.) was acetylated at 30—35° for 48 hr. with freshly distilled acetic anhydride (0·7 c.c.) and pyridine (3·5 c.c.). The resulting solution was heated at 100° for 30 min., cooled, and poured into ice-water. The crude acetate was extracted with chloroform and recrystallised from absolute ethanol. The β-*iso*maltose octa-acetate (0·0286 g.) obtained had m. p. 142·5—144·5°, alone or in admixture with an authentic specimen kindly supplied by Prof. M. L. Wolfrom (Found: C, 49·9; H, 5·7. Calc. for $C_{28}H_{38}O_{19}$: C, 49·6; H, 5·65%).

Fraction IV was heated under reflux in hot aqueous methanol (H_2O 2 c.c.; methanol 4 c.c.) for 8 hr., cooled, seeded, and allowed to crystallize according to the method of Pan, Nicholson, and Kolachov (*loc. cit.*). The panose obtained (0·367 g.) had m. p. 224—225·5° (decomp.), undepressed on admixture with an authentic specimen kindly supplied by Dr. Pan, and showed $[\alpha]_D^{17}$ +154° ⟶ +152·3° (36 hr.; equil.) (*c*, 0·559 in H_2O) (Found: C, 42·8; H, 6·3. Calc. for $C_{18}H_{32}O_{16}$: C, 42·9; H, 6·4%). Its infra-red absorption spectrum in the region 11—14 μ was identical with that of the authentic specimen. Hydrolysis with 1·5N-sulphuric acid for 5 hr. at 100° resulted in a 97·3% conversion into glucose. Partial hydrolysis with 0·34N-H_2SO_4 at 90° for 1 hr. gave glucose, maltose, *iso*maltose, and unchanged panose (identified by paper chromatography). Partial hydrolysis of the aldonic acid formed by oxidation of the trisaccharide (0·005 g.) in water (5 c.c.) with 0·1N-iodine (1 c.c.) and 0·2M-bicarbonate buffer (2 c.c.; pH 10·6) at room temperature for 2·5 hr. gave glucose and *iso*maltose as the only unoxidised sugars.

Crystalline material (0·077 g.), obtained from the mother-liquors after the crystallization of panose, had m. p. 213—214° (decomp.). Chromatographic analysis (benzylamine technique: Bayly and Bourne, *loc. cit.*) showed the presence of panose together with small amounts of a second trisaccharide having R_F identical with that of *iso*maltotriose.

Among the mixtures constituting fraction V, some contained a second disaccharide having R_F identical with that of 3-*O*-α-D-glucosyl-D-glucose and a tetrasaccharide.

Isolation of the Oligosaccharides synthesised from Sucrose by a Cell-free Extract.—A freeze-dried cell-free extract of *A. niger* (1·2 g.) was incubated with a sucrose solution (65 g. in 400 c.c. of water) at 37° and the production of oligosaccharides followed on a paper chromatogram. After 52 hr. the enzyme action was arrested and a preliminary fractionation effected on a charcoal column as described above. Fractions containing mainly glucose, fructose, or sucrose were discarded, and the higher oligosaccharides eluted by aqueous ethanol were obtained in two fractions. One fraction (1·088 g.) contained chromatographically pure trisaccharide I, while the other (1·671 g.), after gradient elution with 0—12% aqueous ethanol on a charcoal column (Alm, Williams, and Tiselius, *loc. cit.*), gave trisaccharide II (0·040 g.), further trisaccharide I (0·359 g.), and a tetrasaccharide fraction (0·082 g.), together with traces of higher oligosaccharides.

Examination of Oligosaccharide Fractions.—Trisaccharide I (1·088 g.) was dissolved in dry methanol, filtered, concentrated, precipitated by addition of dry ethanol, redissolved, and set aside to crystallize. Several crops of crystalline trisaccharide I (in all 0·587 g.) were obtained, having m. p. 82—88° (indef. with decomp.), $[\alpha]_D^{16}$ +29·2° (*c*, 0·584 in H_2O) unchanged after 18 hr. Its R_F value on a paper chromatogram was identical with that of *O*-α-D-glucopyranosyl (1 ⟶ 2)-*O*-β-D-fructofuranosyl (1 ⟶ 2)-β-D-fructofuranoside, the syrupy trisaccharide isolated by Bacon and Bell (*loc. cit.*). It was non-reducing to Shaffer–Hartmann solution (*J. Biol. Chem.*,

1921, **45**, 349). On partial hydrolysis it gave components having R_F values identical with those of sucrose, glucose, and fructose, and after complete hydrolysis the ratio of fructose to glucose was 2·1 : 1 (Van der Plank, *Biochem. J.*, 1936, **30**, 460).

Trisaccharide II showed $[\alpha]_D^{18}$ +33·6° (*c*, 0·417 in H_2O), was non-reducing, and after complete hydrolysis the ratio of fructose to glucose present was 1·8 : 1.

The tetrasaccharide fraction showed $[\alpha]_D^{18}$ +16·7° (*c*, 0·042 in H_2O), was non-reducing, and after complete hydrolysis the ratio of fructose to glucose present was 2·7 : 1.

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