523. *Studies of* Aspergillus Niger. *Part X.** *Polyol and Disaccharide Production from Acetate.*

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Growth of *A. niger* " 152 " on acetate as the sole carbon source is shown to produce mannitol, arabitol, erythritol, glycerol, maltose, and αα-trehalose intracellularly. Conditions for the incorporation of [^{14}C]acetate into the polyols and disaccharides are described.

Previous work [1] has shown that the major carbohydrate component produced intracellularly by *A. niger* " 152," grown on sucrose as the sole carbon source, is the linear polyglucosan nigeran. Early attempts [2] to grow this organism on sodium acetate as the sole carbon source were unsuccessful. Recently, however, we have been able to achieve this by heavy inoculation of a 1% sodium acetate medium with mycelia grown on media where the sole source of carbon was D-glucose. Two subsequent subcultures in 4% sodium acetate gave an organism which grew rapidly on 4% sodium acetate and survived in concentrations up to 10% sodium acetate but which was unable to withstand continuous subculturing in 4% sodium acetate. The present communication describes the identification of several polyols and disaccharides produced intracellularly when *A. niger* " 152 " is grown on 4% sodium acetate.

In all the work described the mineral constituents of the medium were similar to those used by Currie.[3] The mycelia obtained from 20 l. of medium containing sodium acetate (4%) were harvested, washed, and extracted successively with water, 80% ethanol, and 20% ethanol. Cooling the aqueous extract gave no precipitate,[1] indicating that the organism was unable to synthesise nigeran from acetate. A precipitate (A) in the cooled 80% ethanol extract showed none of the properties of nigeran and appeared to be a complex mixture of polysaccharide (galactose, glucose, and probably fructose detected in its hydrolysate) and protein (amino-acids detected on hydrolysis).

The combined extracts were fractionated on a charcoal–Celite column by Whistler and Durso's method.[4] Elution with water gave three fractions (I, II, and III) which, on

* Part IX, *J.*, 1957, 4865.

[1] Barker, Bourne, and Stacey, *J.*, 1953, 3084.
[2] Barker and Carrington, *J.*, 1953, 3588.
[3] Currie, *J. Biol. Chem.*, 1917, **31**, 15.
[4] Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677.

paper chromatography, were found to contain varying proportions of the acyclic polyhydric alcohols, mannitol, arabitol, and erythritol. A further fraction (IV), eluted with aqueous ethanol, appeared to contain maltose and trehalose. Crystalline D-mannitol was obtained from fraction II and was further characterised as its hexa-acetate. Fractionation of the mother-liquors on a cellulose column afforded crystalline erythritol which was further characterised as its di-*O*-benzylidene acetal. Further purification of some of the fractions from the cellulose column by separation on paper chromatograms gave crystalline D-arabitol. Fraction I afforded more di-*O*-benzylidene-erythritol, and fraction III yielded a crop of crystalline mannitol.

Fraction IV was fractionated on a cellulose column and afforded crystalline αα-trehalose dihydrate (further characterised as its octa-acetate) and a fraction which (*a*) showed an infrared spectrum identical with that of maltose and (*b*) gave glucose on treatment with the specific enzyme α-glucamylase.[5]

Two small cultures containing sodium acetate (4%) together with either $CH_3{\cdot}^{14}CO_2Na$ or $^{14}CH_3{\cdot}CO_2Na$ were incubated for 10 days at 30°. Activity determination showed that the highest proportion of labelled carbon was stored in the mycelia grown on $^{14}CH_3{\cdot}CO_2Na$. Radioautography of an extract of each mycelium showed little activity was present in the polyols or disaccharides. A much more efficient method of incorporating the activity was to delay addition of the labelled acetate until 7 days after incubation and then incubate the mixture for a further 3 days. Under these conditions highly active components corresponding to erythritol, arabitol, mannitol, maltose, trehalose, etc., were detected.

One sequence whereby acetate could be incorporated into the polyols would be initiated by its reaction as acetyl coenzyme A in the "glyoxylate cycle." This is the means by which it it believed [6] many moulds can meet all their carbon requirements from acetate. Subsequent conversion of malate, a component of this cycle, into phosphopyruvate, followed by the reversal of the reactions of glycolysis, could then yield hexoses. Hexoses in turn would supply pentoses *via* the pentose phosphate cycle. Present knowledge indicates that the acyclic polyhydric alcohols are synthesised by reduction either of the corresponding ketose phosphate [7] or of the ketose sugars [8] themselves.

Experimental

Growth of Aspergillus niger "152".—Medium (1 l.) was prepared containing NH_4NO_3 (5·0 g.), KH_2PO_4 (2·0 g.), $MgSO_4,7H_2O$ (0·5 g.), $FeSO_4,7H_2O$ (0·05 g.), anhydrous $ZnCl_2$ (0·05 g.), N-HCl (2 c.c.), and glucose (100 g.). After sterilisation by autoclaving at 15 lb./sq. in. for 20 min., the medium was inoculated with *Aspergillus niger* "152" and incubated for 5 days at 30°. Three further batches (each 1 l.) of medium were prepared which contained the mineral constituents mentioned above but in which glucose was replaced by sodium acetate trihydrate (1%; 4%; 4% respectively). Mycelia obtained from the glucose culture were then subcultured successively into these acetate media. Incubation times were 1%, 17 days; 4% (I), 18 days; 4% (II), 8 days and the final pH values of the media 1%, 6·61; 4% (I), 6·0; 4% (II), 7·15. Mycelia from 4% (II) were finally subcultured into twenty 1 l. batches of medium containing sodium acetate trihydrate (4%) and incubated for 7 days at 30°. The mycelia were harvested and then extracted twice with boiling water (1 l.) for 10—15 min. On cooling, no nigeran was deposited. The residual mycelia were extracted successively with boiling 80% ethanol (1 l.) and with boiling 20% ethanol (1 l.). On cooling, the 80% ethanol extract deposited a solid A (0·6 g.). All the extracts were combined, concentrated *in vacuo*, and freeze-dried to a powder B (15·3 g.). Paper chromatography of B in butanol–ethanol–water–ammonia (40 : 10 : 49 : 1) and spraying the chromatogram with a silver nitrate–sodium hydroxide [9] spray revealed

[5] Barker, Bourne, and Fleetwood, *J.*, 1957, 4865.
[6] Kornberg and Krebs, *Nature*, 1957, **179**, 988.
[7] Wolff and Kaplan, *J. Biol. Chem.*, 1956, **218**, 849.
[8] Moore and Rainbow, *J. Gen. Microbiol.*, 1955, **13**, 190.
[9] Trevelyan, Proctor, and Harrison, *Nature*, 1950, **166**, 444.

components with the same mobilities as maltose, mannitol (or glucose), arabitol, erythritol, and glycerol together with another component of R_G 0·75.

Fractionation of B. Fraction B was dissolved in water (100 c.c.) and fractionated on a charcoal–Celite column (l 65 cm., diam. 4·5 cm.) by Whistler and Durso's method.[4] Elution with water gave fraction I, 5·01 g.; fraction II, 3·15 g.; and fraction III, 1·06 g. Further elution with increasing concentrations of aqueous ethanol gave fraction IV, 0·248 g.

Fraction I was extracted with boiling methanol (3 × 100 c.c.; 1 × 50 c.c.), and the extracts were concentrated to a syrup (1·3 g.). A portion (0·1 g.) crystallised from aqueous ethanol, to give an unknown product, m. p. 326—328°, which on combustion left a residue (21·6%) and was not further investigated. The mother-liquors were concentrated to a syrup and treated with benzaldehyde (3·5 c.c.) and concentrated hydrochloric acid (4 c.c.) at room temperature for 3 days. The mixture was then poured into water and extracted with ether (3 × 10 c.c.), and the extract washed with aqueous sodium hydrogen carbonate (2 × 5 c.c.), then sodium hydrogen sulphite (3 × 10 c.c.), dried (Na_2SO_4), and evaporated. The residue crystallised from ethanol and after recrystallisation gave needles, m. p. 194—196° (Jayme and Maris [10] give 197° for dibenzylidene-erythritol).

Fraction II left a small residue (0·12 g.) on extraction with boiling methanol (100 c.c.). On cooling, the methanol extract deposited crystals (0·79 g.) which after several recrystallisations from methanol–water gave D-mannitol (0·45 g.), m. p. and mixed m. p. 164·5—166° (Found: C, 39·7; H, 7·7. Calc. for $C_6H_{14}O_6$: C, 39·6; H, 7·7%). Acetylation with sodium acetate–acetic anhydride gave mannitol hexa-acetate, m. p. 123—124° (Found: C, 49·8; H, 6·0. Calc. for $C_{18}H_{26}O_{12}$: C, 49·8; H, 6·0%).

The mother-liquors from the crystallisation of mannitol were evaporated to a syrup (2 g.), and a part (1 g.) was fractionated on a cellulose column washed with the organic phase of butanol–ethanol–water–ammonia (40 : 10 : 49 : 1). After analysis of the fractions collected, all those containing erythritol were concentrated to a syrup (0·37 g.). Crystallisation from ethanol afforded *meso*-erythritol, m. p. and mixed m. p. 118—119° (Found: C, 39·6; H, 8·5. Calc. for $C_4H_{10}O_4$: C, 39·35; H, 8·3%). Treatment of a portion (0·05 g.) with benzaldehyde (0·2 c.c.) and concentrated hydrochloric acid (0·25 c.c.) (as above) gave dibenzylidene-erythritol, m. p. 200—201°. The remaining fractions (0·61 g.) were refractionated twice on a cellulose column without affording pure arabitol. A part (38 mg.) was therefore separated on sheets of Whatman No. 1 paper, the bands containing the arabitol being eluted and freeze-dried (32 mg.). Crystallisation from aqueous ethanol gave D-arabitol, m. p. and mixed m. p. 104—105°. Its infrared spectrum (700—3000 $cm.^{-1}$) was also identical with that of D-arabitol.

Fraction III, on paper chromatography, was shown to contain more arabitol and mannitol.

Fraction IV on paper chromatographic and ionophoretic examination showed two components with the mobilities of maltose and trehalose. Separation on a cellulose column (as above) gave one fraction (14 mg.) containing maltose and another (120 mg.) containing trehalose. The maltose fraction was characterised by (*a*) its infrared spectrum which was identical with that of an authentic specimen and (*b*) incubation of a portion (5 mg.) with α-glucamylase (10 mg.) at 30°, whereafter glucose could be detected (30 min.) and all the maltose was hydrolysed (12 hr.). The trehalose fraction crystallised from aqueous ethanol, to give αα-trehalose dihydrate (0·09 g.), m. p. and mixed m. p. 96—98°. Treatment of a portion (50 mg.) with sodium acetate (50 mg.) and acetic anhydride (0·5 c.c.) gave trehalose octa-acetate, m. p. 97—98° (Hudson and Johnson [11] give 96—98°) (Found: C, 49·3; H, 5·8. Calc. for $C_{28}H_{38}O_{19}$: C, 49·6; H, 5·6%).

Examination of A. Material A was insoluble in boiling water and in cold N-sodium hydroxide. Hydrolysis of a portion (20 mg.) with 2N-sulphuric acid at 100° for 3—4 hr. gave, *inter alia*, glucose, fructose, galactose, and amino-acids when examined paper chromatographically.

Incorporation of [^{14}C]*Acetate into the Polyols and Disaccharides.*—(*a*) Mineral medium (5 c.c.; composed as above) containing sodium acetate trihydrate (4%) and $CH_3{\cdot}^{14}CO_2Na$ (0·8 mg.; 50 μc) was sterilised and then inoculated with *A. niger* " 152 " from the 4% acetate (I) culture described above. After incubation for 10 days, the mycelia produced were filtered off, washed with water, and freeze-dried (1·6 mg.). When spread on an aluminium disc, these mycelia showed an activity of 1647 counts per min. per mg. in position 3 of a counter. The mycelia were

[10] Jayme and Maris, *Ber.*, 1944, **77**, 383.
[11] Hudson and Johnson, *J. Amer. Chem. Soc.*, 1915, **37**, 2748.

extracted with boiling water (2 × 2 c.c.), 80% ethanol (2 × 2 c.c.), and 20% ethanol (2 × 2 c.c.), and the material deposited from the 80% ethanol extract removed by centrifugation. The mixed extracts were concentrated to 0·5 c.c. Portions (50 μl.; 125 μl.) with activities 800 c/min. and 2000 c/min. respectively were separated on Whatman No. 1 paper irrigated with the organic phase of butanol–ethanol–water–ammonia (40 : 10 : 49 : 1) for 3 days. After exposure to *X*-ray film for 15 days no mobile components could be detected in the smaller portion, and only maltose and trehalose could be detected weakly in the larger. In both portions most of the radioactivity remained on the base line.

The above procedure was repeated with $^{14}CH_3 \cdot CO_2Na$ (1·0 mg.; 25 μc). The mycelia obtained (2·5 mg.) had 2611 counts per min. per mg. in position 3 of the counter. A portion (50 μl.; 1000 c/min.), when analysed on a chromatogram as above, showed weakly active components corresponding to maltose and trehalose.

(*b*) Mineral medium (5 c.c.) containing sodium acetate trihydrate (4%) was sterilised and inoculated in the usual manner. After incubation for 7 days, $CH_3 \cdot {}^{14}CO_2Na$ (1·0 mg.; 63 μc) in sterile water (1 c.c.) was added and the culture incubated for a further 3 days. The mycelia obtained (6 mg.) showed 1320 counts per min. per mg. in position 4 of the counter and were extracted as above. A portion (150 μl.; 21,000 c/min.) of the extract was analysed by paper chromatography and radioautography (exposure time, 7 days). Strongly active components corresponding to maltose, trehalose, mannitol, arabitol, and two unknown components were detected together with a weakly active component corresponding to erythritol.

A similar experiment was carried out involving addition of $^{14}CH_3 \cdot CO_2Na$ (0·975 mg.; 25 μc) in sterile water (0·5 c.c.) to a 4% sodium acetate culture (5·25 c.c.) after incubation for 7 days. After further incubation for 3 days, radioautography of an extract of the mycelia (3 mg.) again showed strongly active polyols and disaccharides.

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