CLXV. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XX. ON THE PRODUCTION OF MANNITOL FROM HEXOSES AND PENTOSES BY A WHITE SPECIES OF *ASPERGILLUS*.

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THE production of mannitol by bacterial fermentation has been extensively studied, since the organisms concerned are a source of trouble in the wine industry. Gayon and Dubourg [1901] showed that although glucose, sorbose, galactose, mannose, sucrose, maltose, lactose, raffinose and xylose are all fermented, laevulose alone yields mannitol. They found that arabinose and trehalose are not attacked. Peterson and Fred [1920, 1] reported that certain pentose-fermenting bacteria isolated from silage and manure also possess the power of producing mannitol. These organisms ferment glucose, galactose, mannose [Peterson and Fred, 1920, 2], xylose and arabinose [Fred, Peterson and Anderson, 1921], but again mannitol is obtained from laevulose alone, and not from any of the other sugars. With sucrose and raffinose some strains give traces of mannitol [Stiles, Peterson and Fred, 1925]. This fact was also observed by Gayon and Dubourg [1901], and it appears probable that fermentation of these sugars is not preceded by hydrolysis to monosaccharides, since invert sugar yields relatively large amounts of mannitol. It is most probable that mannitol arises by direct hydrogenation of one molecule of laevulose, coincidently with the oxidation of another molecule to lactic acid, acetic acid and carbon dioxide [Peterson and Fred, 1920, 1].

The presence of mannitol in the tissues of higher fungi was first discovered by Braconnot [1811], whose observation has been confirmed repeatedly and extended to the lower fungi. Obaton [1929], in a review on the evolution of mannitol in plants, studied in detail the production of mannitol and of trehalose in the mycelium of *Aspergillus niger*, but found no correlation between these two products, since factors affecting the one were without any effect on the other.

Recently Birkinshaw, Charles, Hetherington and Raistrick [1931] have shown that mannitol may be regarded as a product of mould fermentation. In the case of one white species of *Aspergillus* (Ac. 55), the yield of mannitol

approached 50 % of the glucose utilised. To obtain this result the rate of aeration must be carefully controlled. Since the source of mannitol is glucose, which on chemical reduction gives sorbitol, an intensive search was made for this alcohol, but no trace was discovered. The other metabolic products found in addition to mannitol were ethyl alcohol and carbon dioxide with traces of volatile and non-volatile acids.

In view of these results it was decided to extend this investigation, and the results of metabolism experiments on mannose, galactose, laevulose, xylose and arabinose are now reported. This work was restricted to one organism, *i.e.* that which gave the optimum yields of mannitol from glucose. In all experiments the medium consisted of 250 cc. of a 5 % solution of the sugar under investigation together with the usual Czapek-Dox salts, the medium being contained in a 750 cc. conical flask. It may be noted that this organism grows better in a medium where K_2HPO_4 is substituted for KH_2PO_4. The latter was used throughout, however, since the chance of a purely chemical decomposition of the sugar is thus decreased.

EXPERIMENTAL.

Carbohydrates.

A list of the sugars used in this work is given below, together with the observed optical rotations as a criterion of purity. The experiments with glucose, referred to in the introduction, were not repeated, but the results previously obtained [Birkinshaw, Charles, Hetherington and Raistrick, 1931] have been incorporated in Table II for comparison.

d-*Mannose* (British Drug Houses), as obtained gave $[\alpha]_{5461}^{22} + 18\cdot1°$ and $[\alpha]_{5790}^{22} + 16\cdot1°$ in water (c, 0·9948).

d-*Xylose* (Swann Chemical Co., Birmingham, Alabama), was recrystallised from aqueous alcohol. The final product gave $[\alpha]_{5461}^{18} + 22\cdot05°$ and $[\alpha]_{5790}^{18} + 19\cdot12°$ in water (c, 0·2900).

d-*Galactose* (Pfanstiehl), as obtained gave $[\alpha]_{5461}^{19} + 93\cdot5°$ and $[\alpha]_{5790}^{19} + 83\cdot0°$ in water (c, 1·000).

l-*Arabinose* (British Drug Houses), as obtained gave $[\alpha]_{5461}^{20} + 120\cdot4°$ and $[\alpha]_{5790}^{20} + 106\cdot9°$ in water (c, 1·1341).

d-*Laevulose* (Merck), as obtained gave $[\alpha]_{5461}^{22} - 107°$ and $[\alpha]_{5790}^{22} - 94\cdot7°$ in water (c, 2·009).

Medium.

Sugar	25·00 g.
$NaNO_3$	1·00 g.
KH_2PO_4	0·50 g.
KCl	0·25 g.
$MgSO_4.7H_2O$	0·25 g.
$FeSO_4.7H_2O$	0·005 g.
Water	... to 500 cc.

The salts were dissolved separately and added to the solution of the sugar. 250 cc. of the medium were measured into each of two 750 cc. conical flasks and these were then sterilised by steaming for 30 minutes on each of three successive days.

Organism.

The organism used was an unnamed white species of *Aspergillus*, which was sent originally to Ardeer in 1923 by Dr Charles Thom, of the U.S. Department of Agriculture. It bore the Thom and Church Catalogue No. 4640·489, and the Ardeer Catalogue No. Ac. 55. Its carbon balance sheet was worked out [Birkinshaw, Charles, Raistrick and Stoyle, 1931] and it was then used for the preparation of mannitol from glucose [Birkinshaw, Charles, Hetherington and Raistrick, 1931]. All inoculations were made from beer-wort agar slopes grown at 25° for 5 days. A suspension in 5 cc. of distilled water of the heavily sporing mycelium from one test-tube culture was used for the inoculation of each flask of medium.

Incubation.

After inoculation, the two flasks were each fitted with a sterile rubber bung carrying an inlet glass tube which reached practically to the surface of the culture medium, and an outlet tube, the end of which only just passed through the bung. Each end of each tube was fitted with a cottonwool plug and the whole fitting—bung and glass tubes—was sterilised in the autoclave immediately before use. The inlet tube was connected to a controlled air supply and the outlet tube to a water seal, ensuring a closed system. The whole apparatus was maintained at a temperature of 25° ($\pm$ 1°). Aeration was not commenced until a continuous felt of mycelium had been established (7–17 days). Thereafter 300 cc. of sterile air were passed into each flask daily, except Sundays. It had been shown previously that the omission of one day in seven only prolongs the period of incubation without affecting the ultimate result. After a few days of aeration, the thin film grew considerably and formed a thick white felt with a dry surface of sterile, woolly, aerial hyphae. This then remained unaltered, except for a slight wrinkling, until the supply of easily assimilable carbon became low, when the culture showed distinct signs of autolysis, shrinking and becoming water-logged. The flasks were then removed for examination.

With mannose, galactose and xylose the growth appeared normal and similar in character to that obtained on glucose. With arabinose, however, even after 3 months no sign of autolysis had appeared. One flask (*a*) was therefore removed after 93 days and examined. The other flask (*b*) was incubated for 11 more days and then, as autolysis became evident, it was also removed for examination. The residual sugar in both was high and, as will be seen later, the yield of mannitol was very much less than with glucose, mannose, galactose and xylose.

With laevulose the results were surprising, as after 3 weeks of incubation only rudimentary growth had occurred. A few tiny islets of mycelium formed very slowly, but even after 5 weeks these showed no signs of further development. It was thought possible that the laevulose might contain a trace of some toxic substance. That this was not so was shown by the abundant growth of other moulds on the same medium. Samples from one of the flasks were transferred to test-tubes, sterilised by one steaming for 30 minutes, and inoculated with six different species of *Aspergillus* and *Penicillium*. All of these showed normal growth in a few days.

A second attempt was made to grow the white *Aspergillus* on a fresh batch of the same medium. In spite of very heavy inoculations the same result was obtained—traces of growth which showed no appreciable development after the first fortnight. Since this organism prefers an alkaline medium, 2·0 cc. of sterile N NaOH were added to each flask after 3 weeks. This is equivalent to converting the acid phosphate into the dibasic salt. The flasks were shaken thoroughly to ensure mixing, but were not inoculated again since definite traces of growth were present. Ten days later a distinct improvement was apparent. In both flasks a ring of growth could be observed round the edge of the surface, together with a few discrete colonies. Aeration was now commenced, but the growth remained almost stationary, and 9 weeks later the flasks were removed for analysis.

Examination of metabolism solution.

The following procedure was adopted in all the experiments. The metabolism solution was filtered, without sterilisation, from the mycelium, which was washed 5 times with boiling water. Filtrate and combined washings from two flasks, corresponding to 500 cc. of original medium, were made up to 1 litre, constituting solution A. Aliquot portions of this were used for the various analyses and for the isolation of mannitol.

The collected results are given in Table II in which the results for glucose previously obtained [Birkinshaw, Charles, Hetherington and Raistrick, 1931] are incorporated.

The following estimations were carried out.

I. *Residual sugar*, (a) by polarimeter and (b) by Shaffer and Hartmann method.

(a) By polarimeter. The solution was polarised in a 4 dm. tube using both the mercury green and yellow lines, λ5461 and λ5790.

(b) By the Shaffer and Hartmann method. The normal procedure was followed without modification, the factors for the different sugars being determined by control estimations carried out on standard solutions of each sugar.

The results obtained by the two methods are given in item 4, Table II, and are in good agreement throughout.

II. *Acidity produced*. This was determined by titration with standard sodium hydroxide to phenolphthalein. The figures given in item 5, Table II,

Table I.

		Mannose	Galactose	Laevulose	Xylose	Arabinose (a)	Arabinose (b)
1	Concentration of sugar in standard metabolism solution A, calculated from mean polarimeter results (g. per 100 cc.)	Nil	0·516	1·46	0·290	0·735	0·750
2	Volume in cc. of metabolism solution A made up to 50 cc. with 3 g. of borax (solution B)	40·0	40·0	40·0	25·0	40·0	40·0
3	Concentration of sugar in solution B (g. per 100 cc)	Nil	0·413	1·168	0·145	0·588	0·600
4	Rotation of solution B in 4 dm. tube λ 5461	+1·17°	+0·38°	−1·39°	+0·50°	+0·67°	+0·42°
	λ 5790		+0·36°	−1·27°	+0·45°	+0·57°	+0·39°
5	Rotation in 6·0 % borax of synthetic sugar solution of same concentration as in solution B λ 5461	Nil	−0·59°	−1·39°	+0·04°	+0·29°	+0·29°
	λ 5790		−0·52°	−1·27°	+0·04°	+0·26°	+0·26°
6	Rotation due to mannitol (Item 4 − Item 5) λ 5461	+1·17°	+0·97°	Nil	+0·46°	+0·38°	+0·13°
	λ 5790		+0·88°	Nil	+0·41°	+0·31°	+0·13°
7	Percentage mannitol in solution B. Calculated from Item 6 λ 5461	0·730	0·60	Nil	0·27	0·22	0·084
	λ 5790		0·61	Nil	0·27	0·20	0·074
8	Total mannitol in metabolism solution A (g.) λ 5461	9·13	7·5	Nil	5·4	1·4	0·53
	λ 5790		7·6	Nil	5·4	1·3	0·46

Table II.

		Glucose	Mannose	Galactose	Laevulose	Xylose	Arabinose (a)	Arabinose (b)
1	Incubation period (days)	77	65	72	94*	70	93	104
2	Aeration period (days)	70	49	55	62	60	83	96
3	Initial sugar (g.)	25·0	25·0	25·0	25·0	25·0	12·5	12·5
4	Residual sugar (g.) (a) by pol. λ 5461	—	Nil	5·13	14·6	2·90	3·66	3·75
	λ 5790			5·18	14·6	2·90	3·70	3·75
	(b) by Shaffer-Hartmann	0·34	Nil	4·55	14·6	—	3·57	4·15
	Decrease of			*Decrease of*	*Decrease of*		*Decrease of*	*Decrease of*
5	Acidity produced (cc. N/NaOH)	1·0	0·2	0·08	0·3	0·4	0·8	0·9
6	Mannitol estimated by polarimeter (g.)	8·4	9·1	7·5	Nil	5·4	1·35	0·50
7	Mannitol isolated (g.)	6·9	9·3	7·4	Nil	4·9	0·2†	0·1†
8	Percentage estimated yield of mannitol on sugar utilised	34·2	36·4	37·8	Nil	24·4	15·3	5·7
9	Percentage yield of mannitol isolated on sugar utilised	28·0	37·2	37·3	Nil	22·2	—	—

* NaOH (2 cc. N) added after 22 days' incubation.   † Isolated as tribenzylidene compound

have been corrected for the original acidity of the medium. The acidity produced was practically negligible in all cases.

III. *Mannitol estimated by polarimeter.* The method used was that described by Raistrick and Young [1931] for the estimation of mannitol in fermentation solutions. This method is based on the fact that, although in aqueous solution mannitol gives a very small rotation, $[\alpha]_{5461}^{20} - 0.49°$, in presence of 6.00 % borax a high positive rotation is given, the exact values of which vary with the concentration of mannitol and are given by the above authors. Since, however, the optical rotations of all the sugars used are greatly affected by the borax, a correction must be made for this effect. The procedure was as follows. The concentration of sugar in the metabolism solution A having been determined as described in Section I, a measured volume of the metabolism solution, together with 3.00 g. of borax ($Na_2B_4O_7.10H_2O$) was made up to a volume of 50 cc. and polarised. A solution of the sugar in question, in the same concentration as above, was also polarised in presence of 6.00 % borax. The difference between these two readings gave the rotation due to mannitol. The results obtained are given in Table I.

Isolation of mannitol.

A measured volume of the metabolism solution having been retained for the above estimations the remainder was used for the isolation of the product. 10 cc. of basic lead acetate solution were added and the precipitate was allowed to settle overnight. Excess lead was removed from the filtrate by H_2S and the solution was then concentrated *in vacuo* to 20–50 cc., the temperature of the water-bath being kept below 50°. At this stage a certain amount of crystalline material had usually separated. It was dissolved by warming and then 2 volumes of boiling absolute alcohol were added. On cooling, a voluminous crystalline mass of fine white needles was obtained. This was filtered, washed, dried and weighed. The mother-liquors were concentrated again and the process repeated until no further crystalline product separated.

The residual sugar remained in solution and did not interfere with the crystallisation of the mannitol, except in the case of arabinose. As shown in Table II, the two flasks of arabinose were examined separately, each solution being made up to a volume of 500 cc. No mannitol was isolated from either by direct crystallisation. With the first one a yield of 2.5 g. of white crystalline material was obtained, and estimations of reducing power and rotation showed that this product contained about 80 % of arabinose. The small amount of mannitol present could not be separated by direct means. It was, therefore, converted into the tribenzylidene derivative [Meunier, 1891] which was readily isolated and purified, as the sugar does not yield a benzylidene compound with HCl and benzaldehyde. The method, however, is not quantitative, only about 60 % of the calculated yield being obtained from synthetic mixtures of arabinose and mannitol. With the second arabinose metabolism solution no attempt was made to isolate a crystalline product directly. The concentrated

solution was desiccated *in vacuo* over P_2O_5 and then treated with HCl and benzaldehyde, by which means tribenzylidene mannitol was again obtained.

Each fraction of crystalline material obtained by the above treatment from the mannose, galactose and xylose metabolism solutions had M.P. between 158° and 163°. On recrystallisation from aqueous alcohol all fractions had M.P. 165–166° which was not depressed on mixing with an authentic specimen of pure mannitol (M.P. 166°). The optical rotation was also measured in each case and found to be $[\alpha]_{5461}^{20} - 0.49°$ in water.

From each of the purified samples the tribenzylidene compound was prepared by the action of concentrated HCl and benzaldehyde [Meunier, 1891]. After recrystallisation from absolute alcohol the M.P. was 217–219°. The mixed M.P. with tribenzylidene mannitol was 217–219°. The optical rotation found was $[\alpha]_{5461}^{17} - 17.3°$ and $[\alpha]_{5790}^{17} - 15.6°$ in chloroform; Patterson and Todd [1929] give $[\alpha]_{5461}^{25} - 17.5°$ and $[\alpha]_{5790}^{25} - 15.5°$.
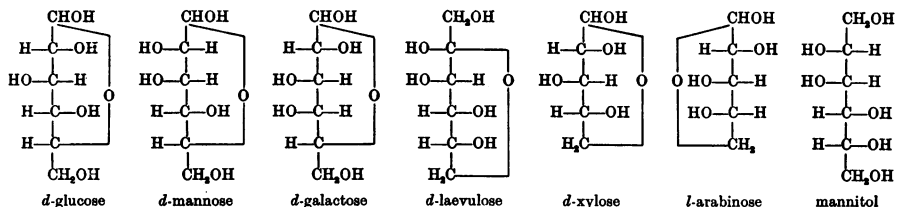
As a final confirmation of the identity of the mannitol a weighed amount of each specimen was polarised in 6·00 % borax solution when the calculated results agreed with the actual amounts used.

In the case of arabinose, as already stated, the mannitol was isolated as the tribenzylidene derivative. With laevulose no mannitol could be detected by the polarimetric method and none was isolated.

DISCUSSION.

As is shown in Table II, the yield of mannitol from glucose, mannose and galactose is approximately the same, about 35 %. Xylose produced 24 %, but it is doubtful whether one can place any significance on this ratio, since Birkinshaw, Charles, Hetherington and Raistrick [1931] have shown that with unrestricted aeration the organism slowly utilises the mannitol formed. With arabinose the growth is less satisfactory and the yield of mannitol, although still appreciable, is poorer. Laevulose is apparently not a satisfactory source of carbon for this organism and is metabolised very slowly. The residual metabolism solution, however, contained only 60 % of the original sugar, so that 10 g. of laevulose had been utilised. In spite of this, no mannitol could be isolated and the rotation of the solution in 6·00 % borax confirmed the absence of even traces of this product (Table I). It may be that, since laevulose is so resistant to attack, any mannitol formed is broken down preferentially, but even were this the case, one would expect to find a small amount in the solution.

These results are rather unexpected, running as they do exactly contrary to the results of bacterial fermentation. In the latter case mannitol is produced from laevulose and not from any other hexose or pentose, while with this species of *Aspergillus* mannitol is obtained from all the sugars so far tested with the exception of laevulose. A consideration of the structural formulae renders the problem even more complex.

CHOH	CHOH	CHOH	CH₂OH	CHOH	CHOH	CH₂OH

The structural Fischer projection formulae are labelled:

d-glucose   *d*-mannose   *d*-galactose   *d*-laevulose   *d*-xylose   *l*-arabinose   mannitol

Mannose would naturally yield mannitol by direct hydrogenation, and for glucose one could postulate a Lobry de Bruyn type of transformation. Acceptance of such a hypothesis is rendered difficult by the laevulose results—since this sugar also gives mannose by the Lobry de Bruyn transformation, especially in alkaline solution—and becomes untenable when the pentoses are considered.

It might be claimed that the theory advanced to account for the alcoholic fermentation of sugar by yeast can be applied. Both the yeasts and the bacteria, however, show a fundamental difference in their metabolism as compared with the fungi. In the first cases the process is almost exclusively "catabolic" while with the fungi syntheses of an "anabolic" type are a striking characteristic. We have no evidence at present as to whether a hypothetical intermediate product, of the type of methylglyoxal, *etc.*, is transformed directly into mannitol. It appears at least equally possible that a hypothetical intermediate product is first built up into a reserve polysaccharide which is broken down as required and forms the immediate precursor of mannitol, which latter must then be regarded as a product of indirect synthesis. It is proposed to investigate this point by examination of the mycelium at various stages of growth, the mannitol produced being estimated at the same time.

SUMMARY.

The metabolism of a species of white *Aspergillus* has been studied on synthetic media with controlled aeration. Mannitol is formed by this organism in considerable amounts (15–35 % of utilised sugar) from glucose, mannose, galactose, xylose and arabinose. The organism does not, however, produce mannitol from laevulose. These results are directly contrary to those reported for mannitol-producing bacteria.

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