PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

On the Production of Mannitol from Glucose by Species of Aspergillus

John Howard Birkinshaw, John Henry Victor Charles, Arthur Clement Hetherington and Harold Raistrick

Phil. Trans. R. Soc. Lond. B 1931 **220**, 153-171
doi: 10.1098/rstb.1931.0020

Studies in the Biochemistry of Micro-organisms.

Part IX.—*On the production of Mannitol from Glucose by Species of* Aspergillus.

By John Howard Birkinshaw, John Henry Victor Charles, Arthur Clement Hetherington *and* Harold Raistrick.

Mannitol ($C_6H_{14}O_6$) is the most common of the naturally occurring hexahydric alcohols. It is a curious fact that while glucose is the most commonly occurring hexose, the corresponding alcohol, sorbitol, is of relatively uncommon occurrence, while mannose and fructose, which are structurally related to mannitol, are not of such common occurrence as glucose.

Mannitol is a common constituent of many plants, but occurs principally in the curious plant exudation known as "manna," and is at present produced technically by two methods, both of which are open to obvious disadvantages.

(*a*) From "manna."

(*b*) By the bacterial fermentation of fructose.

(*a*) *Preparation from manna.*—Manna is a crystalline exudation occurring on various plants, but principally on the flowering or manna ash tree (*Fraxinus ornus*). The separation of mannitol from manna is a relatively easy process, only involving purification by fractional crystallisation. The product obtained is of good quality, but this method of supply is open to the serious objection that the quantity of manna available fluctuates very considerably during different seasons, and is largely dependent on weather conditions. Thus, in a wet season much of the manna produced is washed away on account of its ready solubility in water.

(*b*) *By the bacterial fermentation of fructose.*—For résumé and bibliography see Stiles, Peterson and Fred (1925).

It has long been known that mannitol is a metabolic product of certain bacteria grown on sugar solutions. The mannitic fermentation of wine has been intensively studied, and in recent years a process has been devised for the production of mannitol by bacterial fermentation. This method of preparation is open to two objections.

(1) The bacteria used convert only fructose into mannitol, and while they also act on glucose no mannitol is produced from this sugar, its place being taken by other decomposition products, chiefly lactic acid. Fructose is too expensive to use as a raw material for a fermentation process, and if sucrose, which gives rise to fructose

and glucose on hydrolysis, is used, the yield of mannitol is, of course, only half that produced from pure fructose. This, however, is not the most serious objection to the use of sucrose; its worst feature is the production of large quantities of soluble non-volatile by-products from the glucose part of the sucrose molecule. The main product arising from glucose, and equal in amount to the yield of mannitol from fructose, is lactic acid, which, either as the free acid or as the calcium salt, interferes very seriously with the recovery of mannitol from the fermentation solutions.

(2) Another objection to this method is the difficulty of recovering mannitol from the fermented liquors. This difficulty arises in part, as described in the last paragraph, from the production of calcium lactate, but is further intensified by the fact that the bacteria used for the fermentation require a source of organic nitrogen for growth, *e.g.*, proteins or peptones. These are not only expensive, and so contribute in part to the economic objections to this method, but they also add very considerably to the recovery difficulties.

During the course of investigations on the metabolic products of various common moulds it has been found here that mannitol is a fairly common product of the fermentation of glucose by numerous fungi. Since these micro-organisms grow on very simple media, requiring only inorganic and not organic nitrogen for their nutrition, and since they produce mannitol either from glucose or fructose, it was felt that the main objections to the above bacteriological method of producing mannitol might be overcome by using moulds as the active agents. Actual practice has indeed shown that the mannitol produced is very easily isolated from mould fermentation solutions.

The work carried out on the production of mannitol by various selected species of fungi is described in this paper.

It was pointed out as early as 1813 by VAUQUELIN and by BRACONNOT that mannitol is found in the *tissues* of the higher fungi. This has since been confirmed by a number of workers, who have shown that in a large variety of the agarics mannitol forms the chief part of the non-nitrogenous reserve material. More recently various observers have reported its occurrence in the mycelium of some of the lower fungi, particularly in different species of *Aspergillus* and *Penicillium*. BOURQUELOT (1889-90), working with *Aspergillus niger*, showed that mannitol and trehalose are present in the mycelium of this mould, and that the relative proportions of these two bodies vary in an interesting manner with different stages of growth.

All these observations, however, relate to the isolation and identification of mannitol in the *tissues* of the fungus investigated. It appears to be regarded as a reserve food material for the growing organism, comparable with the glycogen of yeast, rather than as a definite fermentation product, and no attempt seems to have been made to study it from the latter standpoint. In the course of the work about to be described it was shown that the metabolism solutions of certain species of *Aspergillus* contained large amounts of mannitol; yields up to 50 per cent. of the original glucose were obtained,

thus putting beyond doubt the view that mannitol is a genuine product of the fermentation of this sugar by moulds.

As a result of the systematic examination of various species of fungi, involving the preparation of carbon balance sheets for each species examined, as described in Parts II and III, it was found that certain species of *Aspergillus* showed in the metabolism solution a comparatively large amount of carbon which was not accounted for in any of the estimated products. This portion of the balance sheet, which was called "carbon unaccounted for," was in some cases very considerable, and with some species has been shown to consist almost entirely of mannitol. The history of these species on which work is described in this paper will now be given. They fall into three groups :—

(1) White species of *Aspergillus*.

(2) *Aspergillus elegans*.

(3) Species of *Aspergillus nidulans*.

Group 1. White species of *Aspergillus*.

(1) *Aspergillus* sp. Thom 4640.490, Catalogue No. Ac. 56. Obtained from Dr. Charles Thom, of the U.S.A. Bureau of Agriculture, Washington.

(2) *Aspergillus* sp. Thom 4640.489, Catalogue No. Ac. 55. Obtained from Dr. Thom.

(3) *Aspergillus* sp. Catalogue No. Ac. 10. Isolated in the laboratory at Ardeer, from a bench contamination of Czapek-Dox agar.

Group 2. *Aspergillus elegans*.

(4) *Aspergillus elegans* Gasparini, Catalogue No. Ac. 40. Purchased from the Centraalbureau voor Schimmelcultures at Baarn.

Group 3. Species of *Aspergillus nidulans*.

(5) *Aspergillus nidulans* Eidam, Catalogue No. Ac. 67. Purchased from the Centraalbureau voor Schimmelcultures at Baarn.

(6) *Aspergillus nidulans*, Catalogue No. Ac. 78. Purchased from Pribram, Vienna, and as received bore the label, *A. nidulans* (Hann).

(7) *Aspergillus nidulans* var. *Nicollei* Pinoy, Catalogue No. Ac. 85. Purchased from the Centraalbureau voor Schimmelcultures at Baarn.

(8) *Aspergillus nidulans* 110 (ascosporic), Catalogue No. Ac. 79. Purchased from the British National Collection of Type Cultures via Miss Church, of the U.S.A. Department of Agriculture, Washington.

(9) *Aspergillus nidulans*, Catalogue No. Ac. 98. Obtained from Mr. F. T. Brooks, of Cambridge, and identified by Miss Church.

The abridged balance sheets for these different species, as originally prepared in the metabolism experiments, are collected together in Table I for convenience of comparison.

Examination of these carbon balance sheets shows that, with the exception of the item " carbon as volatile neutral compounds," no other products are formed in appreciable amounts except those included under the heading " carbon unaccounted for." This has considerable practical importance, since, as the volatile neutral compounds can easily be removed by distillation, it follows that the only residual products in solution will be those under the heading of " carbon unaccounted for," so that it also follows that, if this item is shown to consist entirely or principally of mannitol, the recovery of this material must of necessity be a relatively simple matter.

Quantitative Estimation of Mannitol produced by Different Species of Aspergillus.

A number of the above species have been examined *quantitatively* from the point of view of mannitol production. The method adopted was the following :—

A Czapek-Dox solution containing 5 per cent. of glucose was distributed in 750 c.c. flasks, each flask containing 250 c.c. of medium. A number of these were then sown with spores of the particular species under investigation, and the necks of the flasks fitted with rubber connections, as described in Part II, p. 15. The flasks were incubated at 24° C., and each day about 300 c.c. of sterile air were passed through each flask during the course of half an hour. The flasks were then shut off from the air supply and no further air passed through until the following day. At the end of the incubation period the contents of the flask were filtered, and filtrate and washings were neutralised with N/1 sodium hydroxide solution to p_{H} 7, made up to a known volume, and a carbon balance sheet prepared on a portion of this solution exactly as described in Part II. The remainder of the metabolism solution was evaporated *in vacuo* and the distillate examined for alcohol. In all those cases where an appreciable amount of " carbon as volatile neutral compounds " occurs in the balance sheet, this was shown to consist almost entirely of ethyl alcohol. In those cases where it was considered advisable to do so, the residual glucose was then removed by fermentation with a pure culture of yeast. Whether this procedure was followed or not, the next step was to remove all precipitable material by treatment with normal and basic lead acetate. The filtrate was freed from lead by means of hydrogen sulphide, the solution filtered, and evaporated *in vacuo* to low volume, and then made up to a known volume for analysis.

In a portion of this solution the mannitol was estimated by treatment with borax and polarisation, as described in Part X. In another portion the mannitol was estimated by determining the acetyl value by treatment with acetic anhydride and anhydrous sodium acetate, as described in Part X. The results of these estimations are given in Table II.

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TABLE I.—Carbon Balance Sheets for Various Species of *Aspergillus*.

Experimental details.	*A.* sp. THOM 4640.490	*A.* sp. THOM 4640.489 (p_H of medium 4·2).	*A.* sp. THOM 4640.489 (p_H of medium 7·7).	*A.* sp.	*A. elegans.*	*A. elegans.*	*A. nidulans* EIDAM.	*A. nidulans.*	*A. nidulans* var. *Nicollei* PINOY.	*A. nidulans* (ascosporic).	*A. nidulans.*
Catalogue number	Ac. 56	Ac. 55	Ac. 55	Ac. 10	Ac. 40	Ac. 40	Ac. 67	Ac. 78	Ac. 85	Ac. 79	Ac. 98
Experiment number	96	92	118	139	52	68	95	111	120	112	130
Incubation period in days ...	48	46	71	51	25	38	35	44	61	40	41
Total carbon in solution gm.	3·061	2·609	2·576	2·380	2·969	2·591	2·663	2·064	2·207	2·143	2·273
Carbon as residual glucose ,,	0·760	0·309	0·288	0·802	1·174	0·336	1·179	0·245	0·131	1·158	1·240
Carbon as CO_2 in solution ,,	0·017	0·014	0·018	0·015	0·008	0·001	0·013	0·010	0·013	0·026	0·007
Carbon as volatile acids... ,,	0·071	0·094	0·092	0·022	0·046	0·047	0·025	0·020	0·027	0·043	0·011
Carbon as non-volatile acids ,,	0·069	0·070	0·095	0·141	0·145	0·135	0·076	0·080	0·090	0·091	0·104
Carbon as volatile neutral compounds ... ... gm.	0·130	0·299	0·231	0·012	0·710	0·758	0·673	0·831	1·026	0·030	0·006
Carbon as synthetic compounds gm.	0·048	0·052	0·132	0·207	0·067	0·066	0·057	0·082	0·122	0·142	0·108
Carbon unaccounted for (by difference) ... ... gm.	1·966	1·771	1·720	1·181	0·819	1·248	0·640	0·796	0·798	0·653	0·797

TABLE II.—Analyses of Metabolism Solutions.

Experimental details.	*A.* Sp. THOM 4640.490	*A.* Sp. THOM 4640.489 (p_H of medium 4·2).	*A. nidulans.* EIDAM.	*A. nidulans.*	*A. nidulans* var. *Nicollei* PINOY.	*A. nidulans* (ascosporic).	*A. nidulans.*
Catalogue number	Ac. 56	Ac. 55	Ac. 67	Ac. 78	Ac. 85	Ac. 79	Ac. 98
Experiment number	P 31	P 18	P 10	P 9	P 17	P 8	P 25
Incubation period in days ...	62	77	39	40	34	59	46
Total carbon in solution	2·819	2·325	1·633	1·505	1·880	2·509	2·349
Carbon as residual glucose ...	0·728	0·067	0·128	0·102	0·356	1·505	1·335
Carbon as CO_2 in solution... ...	0·021	0·004	0·033	0·022	0·015	0·008	0·021
Carbon as volatile acids	0·046	0·054	0·014	0·021	0·016	0·005	0·015
Carbon as non-volatile acids ...	0·129	0·144	0·131	0·109	0·109	0·094	0·113
Carbon as volatile neutral compounds	0·079	0·216	0·777	0·707	0·679	0·026	0·012
Carbon as synthetic compounds ...	0·034	0·047	0·035	0·053	0·053	0·078	0·069
Carbon unaccounted for (by difference)	1·782	1·793	0·515	0·491	0·652	0·793	0·784
Carbon as mannitol by polarimeter	1·131	1·669	0·388	0·397	0·494	—	0·644
Carbon as mannitol from acetyl value	1·124	1·768	0·449	0·451	0·580	—	0·729

N.B.—In the above table the figures given for different classes of carbon represent gm. carbon per 250 c.c. original medium. Since the medium contained 5 per cent. of glucose, *i.e.*, a total of 12·5 gm. glucose, which is equivalent to 5 gm. of carbon as glucose, multiplication of any of the above figures by 20 gives an approximate percentage yield of the particular product.

Isolation of Mannitol from the Metabolism Solutions of different Fungi.

In the results given in Table II mannitol was estimated but was not isolated. In the experiments described below, mannitol was isolated and identified, and a rough quantitative idea formed as to the yields obtained. It is interesting to note that in those cases where both a quantitative estimation and an isolation experiment have been carried out, the results agree quite reasonably well.

Before passing on to a detailed description of these experiments, note will be made of a difficulty which arose at the beginning of this work and which was not overcome for some time. It was found that while the quantitative results obtained in the metabolism experiments could be reproduced consistently, the yield of product was disappointingly small when an attempt was made to prepare larger quantities of fermentation solutions for investigation. The only difference between the conditions of experiment was that, whereas in the metabolism experiments each flask had a limited and known volume of air passed through it, in the earlier "bulk" experiments this method of aeration was altered, and replaced by plugging each of the flasks with an

ordinary cotton-wool plug. Hence, while in the metabolism experiments the aeration was limited, in the larger scale experiments it was unrestricted. The difference in results obtained will be best appreciated by comparing the salient features of two carbon balance sheets prepared for *Aspergillus elegans* (Ac. 40) grown under these two conditions.

It is obvious that unrestricted aeration prevents the accumulation either of alcohol (" volatile neutral compounds ") or of the " carbon unaccounted for."

The production of mannitol by different species will now be dealt with under each species investigated.

(1) *Aspergillus elegans* (Ac. 40).

(*a*) *With unrestricted aeration.*—Five litres of a CZAPEK-DOX glucose solution were made up and distributed in 350 c.c. lots in 1 litre flasks plugged with cotton wool. These were sterilized, sown with *Aspergillus elegans*, and incubated for 37 days at 23° C. At the end of this period the metabolism solution was filtered, and a carbon balance sheet prepared on a portion of it. This is given in Table III, column 3. The remainder

TABLE III.—Comparison of Carbon Balance-Sheets, Limited and Unrestricted Aeration.

	Ac. 40 grown under conditions of aeration as in metabolism experiments, *i.e.*, limited aeration.	Ac. 40 grown in flasks plugged with cotton wool, *i.e.*, unrestricted aeration.
Incubation period in days	48	37
Total carbon in solution	2·420	1·536
Carbon as residual glucose	0·658	0·969
Carbon as volatile neutral compounds	0·569	0·021
Carbon unaccounted for (by difference)	0·922	0·297

Carbon as different compounds all expressed as gm. carbon per 250 c.c. medium

of the solution was evaporated *in vacuo* to a thick syrup, extracted with hot alcohol, and allowed to stand overnight. The clear liquid was poured off, evaporated to a syrup, and re-extracted with absolute alcohol. Evaporation of the alcoholic extract gave rise to well-defined rhombic prisms which proved to be a compound of glucose with sodium chloride, $2C_6H_{12}O_6 . NaCl . H_2O$. As it was found impossible to isolate anything other than glucose, the residues were freed from alcohol, dissolved in water, and the glucose removed by fermentation with yeast. The glucose-free residue was evaporated to a syrup, and the syrup extracted with boiling alcohol. On standing, the alcoholic solution deposited clusters of needles, which were filtered off and dried.

On heating, these crystals showed signs of softening at 152° C., and melted completely

at 160°–162° C. On mixing with pure mannitol (melting point 163°–164° C.) they melted at 163° C. This indicated that the crystals consisted of impure mannitol. They were further characterized by conversion into a benzal derivative, using 0·1 gm. of the crude crystals, according to the method of FISCHER and FAY for *l*-iditol (1895). On shaking for a few minutes, after the addition of 0·2 c.c. concentrated hydrochloric acid and 0·2 c.c. benzaldehyde, the contents of the tube solidified. The mixture was allowed to stand overnight, then water was added and the solid filtered off and washed two or three times with alcohol, and finally with ether. After this treatment, perfectly colourless crystals were obtained melting at 215°–217° C. FISCHER gives the melting point of mannitol tribenzoylacetal as 215°–217° C.

It is thus evident that mannitol is one of the products of the growth of *Aspergillus elegans* on glucose, even under conditions of unrestricted aeration, although under these conditions the yield of mannitol is very small.

(*b*) *With restricted aeration.*—Three litres of the usual 5 per cent. glucose CZAPEK-DOX medium were distributed between twelve 750 c.c. flasks. After sterilising and sowing with Ac. 40, each flask was fitted with a rubber bung and aeration tubes, as in the metabolism experiments. The flasks were aerated for half an hour each day, and the rate at which the air was supplied was, as far as possible, uniform and comparable with the rate in the quantitative experiments. The flasks were incubated at 23° C. for 48 days, and at the end of this time a carbon balance sheet was prepared on a portion of the mixed, neutralised, and filtered metabolism solution. This is given in Table III, column 2. The remainder of the filtrate was evaporated *in vacuo* to low bulk and immediately crystallised on cooling. Treatment of the distillate will be dealt with later. The crystalline residue was diluted somewhat with water, treated with a considerable quantity of hot, absolute alcohol, and set aside to crystallise. 12 gm. of almost white crystals, having the typical appearance of mannitol, separated, and further quantities were obtained from the mother liquors. Assuming that all the "carbon unaccounted for" is due to mannitol, the yield should be 24·7 gm., and the amount obtained indicates that if allowance is made for the solubility of mannitol, this compound must constitute the greater part of the "carbon unaccounted for."

A sample of the recrystallised product melted at 163°–164° C. and there was no lowering of the melting point when it was mixed with a sample of pure mannitol. It gave the following results on combustion :—

	Observed.	Calculated.
Carbon	39·73	39·54
Hydrogen	7·57	7·75

The distillate obtained on evaporation of the metabolism solution was fractionated three times. The final fractions consisted of 1–2 c.c. collected below 78° C., and 10–12 c.c. collected from 78°–78·5° C. Both fractions gave the usual qualitative

tests for ethyl alcohol, and neither fraction gave any reaction for aldehydes or ketones when tested with *p*-nitrophenylhydrazine.

(2) *Aspergillus* species (Ac. 10).

(*a*) *With unrestricted aeration.*—The effect of unrestricted aeration in preventing the accumulation of the "carbon unaccounted for," as observed with Ac. 40, was also seen in experiments carried out with Ac.10.

Two flasks of Czapek-Dox 5 per cent. glucose were sown with Ac. 10, and incubated at 23° C., with cotton wool plugs in the necks of the flasks. The salient features of the carbon balance sheet on the mixed metabolism solutions from these two flasks are given in Table IV, column 3. In column 2 of the same table, corresponding values are given for comparison for the same mould grown under conditions of restricted aeration.

Table IV.—Results of metabolism experiments with Ac. 10.

	Ac. 10 grown under conditions of aeration as in metabolism experiment, *i.e.*, restricted aeration.	Ac. 10 grown in flasks plugged with cotton wool, *i.e.*, unrestricted aeration.
Incubation period in days	51	35
Total carbon in solution	2·380	2·836
Carbon as residual glucose	0·802	2·238
Carbon as volatile neutral compounds	0·012	0·013
Carbon unaccounted for (by difference)	1·181	0·251

Carbon as different compounds all expressed as gm. carbon per 250 c.c. medium.

A small amount of mannitol was isolated from the evaporated metabolism solution from these flasks.

(3) *Aspergillus* species (Ac. 55).

(*a*) *With varying degrees of aeration.*—The effect of varying degrees of aeration on the growth of Ac. 55 and on the yield of water-soluble metabolic products was investigated in some detail. Since experience showed that the chief water-soluble metabolic product of Ac. 55 is mannitol, complete carbon balance sheets were not prepared in this series of experiments, but a measure of the amount of mannitol produced was obtained by estimating the *total* carbon present in solution and subtracting from this the carbon as residual glucose (Table V, column 6).

The experiment was carried out as follows: A number of 1 litre conical flasks, each containing 350 c.c. of the usual Czapek-Dox 5 per cent. glucose solution, were sown

with Ac. 55. Some of the flasks were fitted with sterile rubber bungs and side tubes, as in the metabolism experiments and measured amounts of sterile air, varying from 50 c.c. to 1,000 c.c. per day, were passed through the appropriate flasks daily during the incubation period. The remainder of the flasks were loosely plugged with cotton wool and were left undisturbed until taken off for analysis.

The results obtained are given in Table V, in which all weights are expressed as gm. per 250 c.c. of medium.

TABLE V.—Effect of aeration on yield of metabolic products of Ac. 55.

Degree of aeration.	Incubation period in days.	Weight of mycelium.	Carbon in glucose.	Total carbon in solution.	Difference, *i.e.*, carbon as metabolic products in solution.	Percentage yield of metabolic products calculated on glucose consumed.
		gm.	gm.	gm.	gm.	
50 c.c.	46	0·28	3·874	4·527	0·653	58·0
50 c.c.	66	0·31	3·092	4·274	1·182	62·0
100 c.c.	46	0·49	3·492	4·285	0·793	52·6
100 c.c.	65	0·69	2·426	3·838	1·412	54·9
200 c.c.	46	0·76	2·830	3·893	1·063	49·0
200 c.c.	65	1·08	1·460	3·281	1·821	51·4
300 c.c.	47	0·96	2·276	3·631	1·355	49·7
300 c.c.	64	1·24	0·910	2·936	2·026	49·5
400 c.c.	47	1·14	1·778	3·290	1·512	46·9
400 c.c.	64	1·46	0·264	2·470	2·206	46·6
600 c.c.	48	1·37	1·731	3·082	1·351	41·3
600 c.c.	63	1·76	0·142	2·075	1·933	39·8
800 c.c.	48	1·53	1·656	3·035	1·378	41·2
800 c.c.	63	1·93	0·112	1·835	1·723	35·3
1,000 c.c.	49	1·88	0·727	2·411	1·683	39·4
1,000 c.c.	62	1·97	0·090	1·585	1·495	30·4
Unrestricted aeration. Cotton wool plugs	16	0·56	4·032	4·364	0·332	34·3
	19	0·64	3·896	4·295	0·399	36·2
	23	1·00	3·074	3·833	0·759	39·4
	26	0·90	3·178	3·930	0·752	41·3
	34	1·90	1·385	2·728	1·343	37·2
	36	1·62	1·761	2·928	1·167	36·0
	50	2·85	0·127	0·846	0·719	14·8
	68	2·73	0·027	0·466	0·438	8·8

It is evident that even under conditions of unrestricted aeration considerable quantities of a water-soluble metabolic product, presumably mannitol, are formed, and are subsequently destroyed by the mould as the available glucose disappears. The yield of product is not so large, however, as is obtained when the air supply is controlled, and this yield, calculated on the glucose metabolized, decreases *pari passu* with the amount of air supplied.

(*b*) *Restricted aeration.*—The balance sheet given for Ac. 55 in column 2, Table II, and labelled experiment No. P 18 was prepared from a portion of solution arising as follows :—12 flasks, each containing 250 c.c. of CZAPEK-DOX 5 per cent. glucose medium, were incubated for 77 days and about 300 c.c. of sterile air was passed through each flask each day. At the end of the incubation period the filtered metabolism solution and washings were made up to 4 litres, and of this 250 c.c. were used for preparing the carbon balance sheet, 250 c.c. were used for the mannitol estimations, and the remaining 3,500 c.c. were evaporated *in vacuo* to a syrup which immediately set to a mass of crystals on cooling. This residue was redissolved in boiling water, treated with 100 c.c. of 20 per cent. normal lead acetate solution, and allowed to stand for 3 hours. It was then filtered and the filtrate treated with 100 c.c. of basic lead acetate solution and left overnight. (Treatment of lead precipitates is given later.) In the morning the solution was filtered, excess lead removed from the filtrate with hydrogen sulphide, and the filtrate from the lead sulphide evaporated *in vacuo* to a syrup. The syrup was dissolved in the minimum quantity of boiling 70 per cent. alcohol and, while hot, boiling absolute alcohol was added until a slight permanent turbidity was obtained. The boiling solution was filtered and set aside to crystallise. Mannitol crystallised in beautiful white crystals which were filtered off and dried.

Weight of mannitol, 34·4 gm.
Melting point, 162–163° C.

These crystals gave no reduction of BENEDICT'S solution.

Mother-liquors and washings were evaporated, and a second crop of mannitol weighing 1·95 gm. was obtained. The total yield of mannitol isolated is thus 36·35 gm., representing 77·5 per cent. of the theoretical amount, assuming that all the "carbon unaccounted for" is mannitol, *i.e.*, 47·05 gm. No further mannitol could be isolated from the mother-liquors. It is evident from the above, together with the quantitative figures given in Table II, that mannitol must constitute practically the whole of the "carbon unaccounted for" with Ac. 55. This conclusion is further supported by results given in Part X, p. 201.

The lead precipitates referred to above were too small in amount from this quantity of material for successful investigation, but, as considerable quantities had been accumulated in working up a larger scale experiment with Ac. 55, referred to on p. 170, these were investigated and the treatment of these lead precipitates may with advantage be dealt with here.

The acids were set free from the lead precipitate by treatment with hydrogen sulphide, and a portion of these acids (corresponding to about half of the total, *i.e.*, 30 litres of metabolism solution) was taken for esterification. The aqueous solution of the regenerated acids was evaporated *in vacuo* to a thick syrup, and this was esterified by heating for 5 hours with 250 c.c. of absolute alcohol containing 2·5 per cent. of hydrochloric acid

on three successive occasions—the alcohol being removed by distillation at the end of each period of heating and replaced by fresh alcoholic hydrogen chloride at the start of the next period. The esters were then extracted with ether, the ether solution washed with dilute potassium hydroxide solution and then with water, and dried over anhydrous sodium sulphate. After removal of the ether the esters were fractionated *in vacuo*.

The following fractions were obtained :—

	Boiling Point.	Weight.
		Gm.
Fraction I	70°–82° C. at 45 mm.	3·18
Fraction II	82°–110° C. at 20 mm.	1·24
Fraction III	115° C. at 20 mm. 111° C. at 13 mm.	6·56
Fraction IV	125° C. at 14 mm.	1·50
Fraction V	132° C. at 14 mm.	1·77
Fraction VI	173° C. at 17 mm.	A few drops

The lower boiling fractions of the esters (Fractions I, II and III) were then refractionated at atmospheric pressure, giving

	Boiling Point.		
Fraction A	75°–85° C.	2·58 gm.	mainly alcohol.
,, B	85°–98° C.	1·52 ,,	
,, C	100°–209° C.	0·58 ,,	
,, D	209°–218° C. (mostly ca. 214°)	3·66 ,,	

The fractionation was continued *in vacuo*.

Lower fractions (continued).

	E	75°–100° C. (97° C.)	at 15 mm. 1·30 gm.
4th fraction added			
	F	90°–115° C.	at 16 mm. 1·07 gm.
5th fraction added			
	G	117°–130° C.	at 17 mm. 1·68 gm.
6th fraction added			
	H	114°–132° C.	at 14 mm. 1·33 gm.
	I	131°–170° C.	at 15 mm. 1·25 gm.

From each of these fractions 1 c.c. was removed and placed in a test tube along with 2 c.c. hydrazine hydrate (50 per cent.) and 2 c.c. absolute alcohol and left overnight. By this treatment the esters of many polybasic acids give rise to the corresponding hydrazides.

D showed crystals (0·14 gm.) which, when filtered and washed, melted at 160°–162° C. The mother-liquor deposited further crystals which melted at 155°–158° C. (0·16 gm.)

E also gave crystals melting at 158°–162° C. which, when recrystallised, melted at 162°–163·5° C.

F gave crystals melting at 155°–158° C.

All these three fractions appeared to give the same hydrazide in different states of purity. It was suspected that this was the dihydrazide of succinic acid, although the melting point was somewhat low. Accordingly 2 gm. of fraction D were hydrolysed with baryta, the acid liberated with sulphuric acid, and extracted with ether. On attempting to sublime the residue from the ether extract a liquid was obtained which distilled, but on cooling crystallised on the sides of the tube. A portion of the crystals was removed and resublimed, and, at about 140° C. a small amount of white sublimate formed which melted at 184° C. (corr.) and was probably succinic acid. A sample of pure succinic acid melted at 185° C. (corr.) M.P. of mixture = 185° C., thus confirming succinic acid. The liquid which distilled was shown to be succinic anhydride after recrystallising a small portion from alcohol; it melted at 117°–119° C. (uncorr.). (M.P. of succinic anhydride = 118°–120° C.). A titration on 0·028 gm. gave an equivalent of 52. Calculated value for succinic anhydride = 50.

Some of the recrystallised hydrazine derivative from Fraction E gave the following combustion figures:—0·1120 gm. hydrazide gave 0·0704 gm. H_2O and 0·1355 gm. CO_2, equivalent to 7·03 per cent. hydrogen and 32·99 per cent. carbon (theoretical for succinic acid dihydrazide is hydrogen 6·90 per cent., carbon 32·87 per cent.).

Fractions G and H were so similar that they were united and treated with hydrazine hydrate. This ester gave a crystalline hydrazide almost immediately on the addition of the hydrazine hydrate. When recrystallised it melted at 180°–181° C. The hydrazide from pure *l*-malic ester melted at 181·5° C. The mixture melted at 181°–182° C., *i.e.*, no lowering of melting point.

The benzylidene compound which was prepared by the method described by Rundshagen (1926), by dissolving the hydrazide in dilute hydrochloric acid and shaking with benzaldehyde, yielded anomalous results. That prepared from fractions G and H melted at 174° C. after recrystallisation, whereas the derivative from *l*-malic acid melted at 183°–184° C. Rundshagen gives the melting point as 164°–166° C. Possibly the differences are due to the presence of optical isomers in varying proportions. To confirm malic acid a combustion on the hydrazide was carried out. 0·1027 gm. hydrazide gave 0·0583 gm. H_2O and 0·1116 gm. CO_2.

	Observed.	Calc. for the dihydrazide of malic acid.
H	6·37 per cent.	6·22 per cent.
C	29·64 ,,	29·63 ,,

The last fraction, I, did not give a hydrazine derivative except for a very small amount of the dihydrazide of malic acid.

The acids which were thus identified as arising from the growth of Ac. 55 on glucose under conditions of restricted aeration were succinic and malic acids. This constitutes in the carbon balance sheet for Ac. 55 a part of the "carbon as non-volatile acids," but it should be borne in mind that they are relatively so small in amount as to have no appreciable effect on the subsequent recovery of mannitol.

(4) *Aspergillus* species (Ac. 56).

(*a*) *With varying degrees of aeration.*—The effect of varying degrees of aeration on the growth of Ac. 56 and on the yield of water-soluble metabolic products was investigated in a similar manner to that described for Ac. 55 (see p. 161). The results obtained are given in Table VI, and in this case also all weights are expressed as gm. per 250 c.c. medium. The figures given in the last column represent the amount of mannitol, calculated as gm. carbon per 250 c.c. estimated by the polarimetric method given in Part X.

TABLE VI.—Effect of aeration on yield of Metabolic Products of Ac. 56.

Degree of aeration.	Incubation period in days.	Weight of mycelium.	Carbon in glucose.	Total carbon in solution.	Difference, *i.e.*, carbon as metabolic products in solution.	Percentage yield of metabolic products calculated on glucose consumed.	Carbon as mannitol estimated polarimetrically.
		gm.	gm.	gm.	gm.		gm.
100 c.c.... ...	40	0·34	3·812	4·485	0·673	56·6	0·231
100 c.c.... ...	69	0·47	3·258	4·138	0·880	50·5	—
200 c.c.... ...	40	0·49	3·018	4·165	1·147	57·9	—
200 c.c.... ...	69	0·74	2·052	3·607	1·555	52·8	0·647
300 c.c.... ...	40	0·59	3·039	3·948	0·909	46·4	—
300 c.c.... ...	69	0·94	1·393	3·212	1·819	50·4	0·725
400 c.c.... ...	40	0·67	2·572	3·851	1·279	52·7	0·493
400 c.c.... ...	68	0·99	1·155	3·036	1·881	48·9	—
600 c.c.... ...	40	0·78	2·373	3·692	1·318	50·2	—
600 c.c.... ...	68	1·26	0·596	2·643	2·047	46·5	0·631
800 c.c.... ...	40	0·96	2·119	3·436	1·317	45·7	—
800 c.c.... ...	68	1·59	0·139	2·086	1·947	40·1	0·558
Unrestricted aeration ...	32	2·31	0·257	1·981	1·724	36·3	—

The results given in Table VI indicate that, as was the case with Ac. 55, the yield of water-soluble metabolic products obtained is very greatly influenced by the degree of aeration. The figures given in the last column show that, unlike Ac. 55, metabolic products other than mannitol are formed by Ac. 56, since mannitol only forms 30-40 per cent. of the total metabolic products of Ac. 56 over a wide range of air supply.

(*b*) *With restricted aeration.*—Nine litres of 5 per cent. Czapek-Dox glucose medium were made up and 250 c.c. of this distributed in each of 36 flasks. 300 c.c. of sterile air were passed through each flask daily, and at the end of the incubation period the metabolism solutions were mixed, filtered, and filtrate and mycelium washings neutralised with sodium hydroxide to *p*H 7·0, and made up to 12 litres. Of this, 250 c.c. were used for the preparation of the balance sheet given in Table II, column 2, and 250 c.c. for the mannitol estimations given in the same column. The remaining 11·5 litres were treated with normal and basic lead acetate, filtered, the lead removed from the filtrate by hydrogen sulphide, and the lead-free filtrate evaporated *in vacuo* to a syrup which crystallised on standing. The crystalline mass was then thoroughly extracted with 400 c.c. of absolute alcohol. The mixture was filtered and the crystals washed and dried. They consisted of fairly pure mannitol, and weighed 67 gm., corresponding to 43·1 per cent. of the total "carbon unaccounted for," and 67·9 per cent. of the mannitol estimated to be present by polarimeter. It is evident that with Ac. 56 a considerable proportion of the "carbon unaccounted for" is some product other than mannitol, a conclusion which is supported by the results given in Table VI.

The syrupy filtrate from the mannitol crystals was diluted to rather more than a litre with absolute alcohol, when a certain amount of sticky material was precipitated. The clear alcoholic solution was poured off, evaporated *in vacuo* and made up again to about a litre with fresh absolute alcohol. A further precipitate was produced, and the clear alcoholic solution was again separated and to it were added 2 litres of ether. A third precipitate was thus obtained and the ether-alcohol solution was poured off and evaporated *in vacuo*. The residue from this was further purified by subjecting it to a second precipitation, by redissolving in absolute alcohol and reprecipitating with two volumes of dry ether. A thick sticky residue was deposited, and the clear ether-alcohol solution was poured off and evaporated, giving rise to a clear syrup having a weight of 10 gm. Treatment of this dry syrup will be given later.

The three sticky precipitates contained considerable amounts of glucose. They were collected together and fermented for three days with a pure culture of yeast. The fermented solution was filtered, cleared with lead acetate, and the lead-free filtrate evaporated to a thick syrup. This was then subjected to alcohol-ether treatment as described above, giving rise to a second clear syrup, the weight of which was 3·5 gm.

The two syrups described above, weighing respectively 10 gm. and 3·5 gm., were shown to consist almost entirely of glycerol by the following means.

(*a*) The mean molecular weight of a portion of the syrups was determined by estimating the depression of the freezing point of water. They gave a mean value of about 80 (molecular weight of pure glycerol = 92).

(*b*) A portion of each syrup was benzoylated by shaking an aqueous solution of it with benzoyl chloride and sodium hydroxide solution. An almost quantitative yield of glycerol tribenzoate was obtained having the following

characteristics :—Melting point, 71·5°–72° C. A synthetic sample of pure glycerol tribenzoate gave a melting point of 71°–72° C., and a mixture of the benzoate isolated from the syrup and of synthetic glycerol tribenzoate had a melting point of 70°–71° C.

Combustions on the benzoate isolated from the syrup gave the following results.

	Combustion (1)	Combustion (2)	Theoretical for glycerol tribenzoate.
C	71·6 per cent.	71·2 per cent.	71·2 per cent.
H	5·06 ,,	5·12 ,,	4·99 ,,

Further proof of the presence of glycerol was obtained as follows :—

5·6 gm. of this syrup (first crop) were used for a phenyl *iso*cyanate reaction. The only products which could be identified after repeated recrystallisation of the mixture were glycerol triphenylurethane and diphenyl urea.

In a further attempt to gain some information as to whether any product other than glycerol was present in the syrup, a portion of it was subjected to the action of periodic acid by the method of MALAPRADE (1928).

Two figures were obtained for the amount of glycerol present, one from the amount of periodic acid reduced to iodic acid and the other from the titratable acidity produced by the reaction. This method, whilst admittedly not very accurate, is at any rate more specific than the rest of the glycerol methods and enables a distinction to be made between glycerol and ethylene glycol, since the latter produces no acidity.

Glycerol by reduction of HIO_4 = 92·7 per cent.
Glycerol by acidity produced = 89·7 ,,

Making due allowance for the error of the method, it may be said that the agreement is good, and that the syrup remaining after removal of mannitol consists of glycerol. Any other substances, except water, are present only in negligible amounts.

Hence, unlike Ac. 55, for which mould the "carbon unaccounted for" is almost entirely mannitol, with Ac. 56 rather more than half of the "carbon unaccounted for" is mannitol and a considerable proportion of the remainder, if not all of it, is glycerol.

The acids precipitated as lead salts, as described on p. 163, were worked up in a similar manner to that adopted for the lead precipitate of Ac. 55 described on p. 164. The only hydrazide isolated from the acids from Ac. 56 was one having a melting point of 178°–179° C. The hydrazide of pure *l*-malic acid melts at 181·5° C. and a mixture of the hydrazide isolated and of synthetic *l*-malic acid hydrazide melted at 179° C. The benzylidene derivative prepared from the above hydrazide melted at 166°–168° C. This agrees substantially with the melting point given by RUNDSHAGEN for the *l*-malic acid derivative (164°–166° C.) though it disagrees with the melting points observed here and given on p. 165. The explanation is probably that offered on p. 165.

The only acid identified as arising from the growth of Ac. 56 on glucose under conditions of restricted aeration was thus *l*-malic acid.

Production of Glycogen by Aspergillus *species Ac.* 56.

In an experiment with *Aspergillus* species Ac. 56, which was carried out in the combined sterilizer incubator described in Part VII, the production of a polysaccharide was observed, which on investigation proved to be glycogen. The experimental details follow :—

One tray containing five litres of Czapek-Dox 5 per cent. glucose medium, with 2 gm. per litre of ammonium nitrate in place of the usual 2 gm. of sodium nitrate, was used for this experiment. The actual nitrogen present was thus about twice the standard amount. After sterilization the medium was sown with Ac. 56 and aerated at the rate of 0·25 cu. ft. per day at room temperature. The incubation period lasted 47 days. The contents of the tray were shown to be free from contamination, and the filtered metabolism solution then showed a strong opalescence and gave a characteristic red-brown reaction with iodine. It was evaporated *in vacuo* and an equal volume of alcohol was added. An amorphous white powder was precipitated which was very difficult to filter. It was washed by centrifuging with alcohol and ether and dried. Yield of crude material containing 9·5 per cent. of ash = 7 gm.

The substance was purified as follows : It was dissolved in hot water, filtered from the insoluble portion, acidified with 5 c.c. concentrated HCl and precipitated by the addition of 1·5 volumes of 96 per cent. alcohol. It was filtered off, drained, and reprecipitated twice from *neutral* solution with an equal volume of alcohol, washed with alcohol and ether and dried.

The final product is a pure white amorphous powder containing no ash and neutral in reaction. Its aqueous solution is slightly opalescent in the cold and gives a characteristic brownish-red colour with iodine.

0·2280 gm. was dissolved in hot water, made up to 50 c.c., and polarised in a 20-cm. tube. The readings obtained were + 1·753° with the mercury yellow light corresponding to $[\alpha]^{20}_{\text{Hg. yellow}} = +192{\cdot}2°$ and + 1·932° with the mercury green light corresponding to $[\alpha]^{20}_{\text{Hg. green}} = +211{\cdot}9°$.

0·4028 gm. was hydrolysed by heating with 10 c.c. of N/1 H_2SO_4 for four hours on a boiling water bath. The hydrolysis mixture was neutralised, made up to 50 c.c. and filtered. The glucose content as estimated by the polarimeter was 0·836 per cent. and by the Wood-Ost method 0·826 per cent.

As the sugar formed on hydrolysis was definitely proved to be glucose, it is evident from these figures that the material is a polyglucose, and from its optical rotation and its colour reaction with iodine it is either identical with, or closely allied to, glycogen.

Large Scale Experiments on the Production of Mannitol by Aspergillus *species Ac.* 55.

Experiments on a 60 litre scale were carried out in the combined sterilizer-incubator described in detail in Part VII of this series.

Five litres of CZAPEK-DOX 5 per cent. glucose solution (pH = 7·4) were placed in each of the twelve trays. Each tray was also fitted with a support for the mycelium, consisting of a grid of aluminium wire-netting of 1 inch mesh, the edges of which were turned over so that the grid was supported just below the surface of the liquid. This grid was found to be necessary in order to prevent the mycelium sinking into the liquid during the manipulations described later. After sterilizing and sowing the trays with Ac. 55, the whole apparatus was incubated at room temperature for a period of 59 days and during the whole incubation period sterile air at the rate of 0·25 cubic feet per tray was passed daily.

At the end of the incubation period the metabolism solution was removed through the inoculation openings by means of sterile syphons, care being taken to disturb the mycelium as little as possible.

The metabolism solution was treated with basic and neutral lead acetates in the usual way, the washed lead precipitates being examined by the method already described on page 163. The filtrate from the lead precipitates was freed from lead, evaporated, and a total yield of 608 gms. of mannitol was isolated corresponding to about 24 per cent. of the glucose fermented.

An experiment was now carried out to determine the effect of replacing the withdrawn metabolism solution by fresh sterile CZAPEK-DOX 5 per cent. glucose solution. The experimental details follow. After removal of the exhausted metabolism solution as described above, each tray was washed out with 4 litres of sterile distilled water, introduced beneath the mycelium, and removed by means of sterile syphons. Five litres of fresh sterile CZAPEK-DOX 5 per cent. glucose solution were now introduced into each tray beneath the mycelium. It was possible to carry out these manipulations without submerging the mycelium because of the support given to the mycelial felt by the aluminium grids described.

Incubation at room temperature and aeration as previously described were carried out for a period of 25 days. By this time almost all the glucose had disappeared in most of the trays and yields of mannitol varying between 39·9 per cent. and 50·2 per cent., calculated on the glucose metabolized, were obtained. As was inevitable, contaminations were found in a small proportion of the trays, but in the uncontaminated trays an average yield of 46·3 per cent. of mannitol was obtained.

It is thus evident that by the use of a fresh medium with a mycelial felt already established, not only is the incubation period necessary for the fermentation of a given quantity of glucose considerably curtailed, but the yield of mannitol obtained is considerably increased. It is also significant that, even under these optimum conditions, the yields of mannitol obtained definitely tend to a maximum of 50 per cent. of the

glucose fermented corresponding to the production of one molecule of mannitol from two molecules of glucose. This lends support to the view expressed on p. 9 of the introductory paper (Part I) of this series that the initial step in the metabolism of glucose by certain species of fungi is a CANNIZZARO reaction involving the production from two molecules of glucose of one molecule of mannitol and one molecule of gluconic acid.

Summary.

The formation of mannitol from glucose by three unnamed white species of *Aspergillus*, one strain of *A. elegans*, and five different strains of *A. nidulans*, has been investigated.

The fundamental effect of variations in the air supply on the yield of mannitol obtained is described.

It has been found that a well-developed mycelium of the white species Ac. 55 may be used to ferment to mannitol a fresh quantity of glucose solution, supplied in place of the exhausted medium. By this means, yields of mannitol approaching 50 per cent. of the glucose fermented were obtained.

Other metabolic products found in addition to mannitol were :

(*a*) Small quantities of succinic acid and malic acid produced by the white species Ac. 55.

(*b*) A considerable quantity of glycerol, a complex carbohydrate closely resembling, if not identical with, glycogen and small quantities of *l*-malic acid produced by the white species Ac. 56.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES