

The Products of Glucose Metabolism Formed by Various Species of Fungi (Helminthosporium, Clasterosporium, etc.)

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*Studies in the Biochemistry of Micro-organisms.*PART XVII.—*The Products of Glucose Metabolism formed by various species of Fungi*
(*Helminthosporium*, *Clasterosporium*, *etc.*).

By JOHN HOWARD BIRKINSHAW and HAROLD RAISTRICK.

In the following paper an account is given of the investigation of the metabolic products formed from glucose by various species of fungi belonging to different genera. These species were chosen for investigation since the carbon balance sheets prepared for them, when grown on CZAPEK-DOX glucose solution, indicated that each of them gives rise to considerable quantities of some metabolic product. The isolation, identification and approximate estimation of these products are described in detail.

The species of fungi chosen for examination were the following :—

- (a) *Helminthosporium geniculatum* TRACY et EARLE. Catalogue No. Ag. 93. This species was purchased in 1925 from the Centraalbureau voor Schimmelcultures at Baarn. For carbon balance sheet, see Part VI, Table VIII, p. 111.
- (b) *Clasterosporium* species, Catalogue No. Ag. 64. This species was isolated at Ardeer in 1924 from decaying cotton pulp, and its diagnosis was confirmed by Mr. F. T. BROOKS, of the School of Botany, Cambridge. For carbon balance sheet, see Part VI, Table XII, p. 118.
- (c) *Aspergillus Wentii* WEHMER. Catalogue No. Ac. 81. This species was received from Miss CHURCH, of the United States Bureau of Agriculture, at Washington, and bears the THOM and CHURCH Catalogue No. 4202·16C. For carbon balance sheet, see Part III, Table XI, p. 45.
- (d) *Fumago vagans* PERS. Catalogue No. Ag. 92. This species was purchased from Baarn in 1925. For carbon balance sheet, see Part VI, Table XI, p. 116.
- (e) *Penicillium* species. Catalogue No. Ad. 11. This species was isolated at Ardeer in 1922 from mouldy tobacco and was diagnosed by Dr. CHARLES THOM as “Ad. 11—one of the *P. chrysogenum* lot.” For carbon balance sheet, see Part IV, Table V, p. 63.

The method adopted for the preparation of sufficient material for investigation with each of the above species was to make use of the combined sterilizer-incubator described in Part VII. A quantity of the normal CZAPEK-DOX glucose medium having the composition given in Part I, p. 7, was prepared, and the moulds were grown in the sterilizer-incubator in the usual way.

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(a) *Helminthosporium geniculatum*.

Thirty litres of medium, distributed between six trays, were used in this experiment which commenced on 5th November, 1928.

Aeration was commenced on 13th November, 1928, 0·2 cu. ft. of air per tray per day being given for the first six days. From 20th November, 1928, to 9th January, 1929, 0·3 cu. ft. of air per tray per day was passed, and from 10th January, 1929, to the completion of the experiment on 31st January, 1929, 0·2 cu. ft. of air per tray per day was passed. Aeration was restricted throughout to six days per week. The incubation period thus lasted 87 days.

The six trays when taken off on 31st January, 1929, showed the results given in Table I.

TABLE I.

Number of Tray.	Percentage Glucose by Polarimeter.	Percentage Glucose by WOOD-OST Method.	Titration in c.c. N/1 NaOH per 250 c.c. Medium.
1	1·09	0·92	0·75
2	0·56	0·41	0·30
3	0·86	0·73	0·20
4	0·80	0·68	1·15
5	1·59	1·38	0·70
6	0·30	0·24	2·25

All the six trays were found to be free from contamination with the exception of Tray 5, which was found to be slightly contaminated, and was therefore discarded. The contents of the other five trays were filtered from the mycelium, and the mycelium (A), and filtrate (B), dealt with separately.

A. *Treatment of mycelium*.—The mycelium was thoroughly extracted with boiling water, the extract evaporated to small bulk *in vacuo* and precipitated with 2 vols. of 96 per cent. alcohol. A small amount of a substance resembling a polysaccharide was precipitated, but the quantity was too small for further examination. No other product of interest was found in the mycelium.

B. *Treatment of Filtrate*.—Examination of the carbon balance sheet for this species indicates that a comparatively large yield (16 per cent.) of some volatile neutral body is to be expected, together with almost as large an amount (12 per cent.) of some product or products included in the balance sheet as “carbon unaccounted for.” Hence a portion of the filtrate was distilled through a fractionating column and the distillate was refractionated. Two fractions of interest were obtained, the smaller one boiling below 77° C. and the main fraction boiling at 77°–80° C.

The fraction boiling below 77° C. gave a heavy yellow precipitate with 2:4-dinitrophenylhydrazine hydrochloride. This, on recrystallisation from absolute alcohol, had a melting-point of 158°–160° C., and corresponded with the 2:4-dinitrophenyl-

hydrazone of acetaldehyde, which according to BRADY and ELSMIE (1926), melts at 162°C .

A portion of the main fraction boiling at 77° – 80°C . was treated with somewhat less than the theoretical amount of *p*-nitrobenzoyl chloride according to the method described by BUCHNER and MEISENHEIMER (1905). The mixture was warmed until complete solution was obtained and then cooled, when yellowish crystals separated. These were filtered, shaken with sodium carbonate solution to remove any free acid, filtered and dried. The material melted at 55° – 56°C . and there was no depression of the melting point when mixed with an authentic specimen of ethyl *p*-nitrobenzoate. The main volatile neutral product is therefore ethyl alcohol, with small amounts of acetaldehyde.

The whole of the filtrate B was now evaporated *in vacuo* to low bulk and, on standing, became semi-solid and filled with crystals resembling mannitol. These crystals were not easily separated, however, so the whole residue was dissolved in water and precipitated with basic lead acetate, the lead precipitate (C) filtered off, excess lead removed from the filtrate by H_2S , lead sulphide filtered off and the filtrate evaporated *in vacuo* to a volume of 500 c.c.

One-half of this was diluted to one litre with water, 1 gm. of KH_2PO_4 added and the glucose present, amounting to about 60 gm., was removed by fermentation with a pure culture of yeast. At the end of the fermentation the yeast was filtered off, the filtrate treated with lead acetate, filtered from the lead precipitate which was rejected, and the lead removed from the filtrate with H_2S . The filtrate from the lead sulphide was evaporated *in vacuo* to remove H_2S , and made up to a volume of 500 c.c. Portions of this solution were now used for examination. 300 c.c. were evaporated to a syrup and treated while hot with sufficient absolute alcohol to produce incipient turbidity. On cooling, a mass of white needle crystals having a weight of 9.7 gm. = 3.23 per cent., separated. This was recrystallised and shown to consist of mannitol by the following means: A portion melted at 167° – 168°C ., the melting point being unchanged on admixture with pure mannitol. The optical rotation of another portion was $[\alpha]_{\text{Hg. yellow}}^{20} = -0.48^{\circ}$ in water ($c = 2.07$) changing to $[\alpha]_{\text{Hg. yellow}}^{20} = +35.2^{\circ}$ in 6 per cent. borax solution ($c = 0.829$). The value found in Part X for pure mannitol in 6 per cent. borax at a concentration of 0.829 per cent. was $+35.47^{\circ}$.

Experiments were now carried out to estimate the amount of mannitol present in the solution, with the following results:—

- (a) True mannitol by polarimeter in borax solution = 3.56 per cent.
- (b) "Mannitol" by acetylation with acetic anhydride and sodium acetate = 6.16 per cent.
- (c) "Mannitol" by estimation of total carbon in solution, after removal of the acetic acid arising from the lead acetate used in precipitation = 6.83 per cent.

Consideration of these figures makes it obvious that only about half of the organic material in solution is mannitol, and since the figures obtained in (b) and (c) are approximately equal, it is obvious that the other metabolic products must be fully hydroxylated. The alcoholic mother-liquors from the 300 c.c. portion from which 9.7 gm. of mannitol had been isolated were therefore evaporated to a syrup, taken up in absolute alcohol and 2 vols. of dry ether added. The ether-alcohol solution was filtered from a certain amount of sticky material which was precipitated and evaporated to a syrup which weighed 5.28 gm.

2.2 gm. of this syrup were now treated with benzoyl chloride according to the method of LIPP and MILLER (1913), for the preparation of glycerol tribenzoate. This method has proved the most useful of any described in this paper for the isolation and identification of glycerol.

3.39 gm. of glycerol tribenzoate were obtained, which on recrystallisation from absolute alcohol melted at 72° – 72.5° C., the melting point being unchanged on admixture with a sample of pure glycerol tribenzoate.

From the weight of glycerol tribenzoate isolated it is obvious that glycerol must be a metabolic product of this strain of *Helminthosporium*, since, while a little glycerol may have arisen during the fermentation of the residual sugar by yeast, as described on p. 333, the glycerol thus formed would be only a small proportion of the amount actually isolated.

The main metabolic products of this strain of *Helminthosporium geniculatum* when grown on glucose are thus ethyl alcohol and mannitol, together with smaller amounts of glycerol and acetaldehyde.

(b) *Clasterosporium species*.

Thirty litres of medium distributed between 6 trays were used in this experiment which commenced on 16th February, 1928. Aeration was commenced on 25th February, 1928. Each tray received 0.3 cu. ft. of air per day, 6 days per week, during the rest of the incubation period which, in all, lasted 32 days. Samples were taken from each tray on the 29th day and gave the following figures for glucose by the polarimeter :— 0.53 per cent., 0.16 per cent., 1.14 per cent., 0.46 per cent., 0.45 per cent., 0.63 per cent., while an average sample gave 0.63 per cent. The experiment was stopped after 32 days, when all the six trays were found to be free from contamination. The metabolism solution was filtered from the mycelium, which was drained but not further examined, the volume of the solution measured and a well-mixed sample taken for analysis. The following results were obtained :—

Residual glucose :—(a) by polarimeter, 0.230 per cent.

(b) „ WOOD-OST, 0.244 „

(c) „ alkaline iodine, 0.544 per cent.

A partial carbon balance sheet prepared on the usual basis gave the results recorded in Table II.

TABLE II.

	Gm. Carbon per 250 c.c. Medium.
Carbon in residual glucose	Gm. 0.244
„ in CO ₂ in solution	—
„ in volatile acids	0.025
„ in non-volatile acids	0.086
„ in volatile neutral compounds... ..	0.824
„ in synthetic carbon	Nil
Total carbon accounted for	1.179
„ „ in solution	2.181
Carbon unaccounted for	1.002

Treatment of Metabolism Solution.—The results given in Table II are in substantial agreement with those previously recorded for the same species. They indicate that about 16 per cent. of some volatile neutral compound is to be expected, together with 20 per cent. of some product or products included in the balance sheet as “carbon unaccounted for.” Hence the metabolism solution which, after the removal of the portion for analysis, measured 22.2 litres, was evaporated *in vacuo*. The first portions of the vacuum distillate were then fractionated at ordinary pressure through a YOUNG’S column. There was thus obtained 45 c.c. of a liquid boiling at 77° C. to 79° C., which was shown to consist of ethyl alcohol, containing small amounts of acetaldehyde, by the method described on p. 332. Thus 2:4-dinitrophenylhydrazine gave rise to a hydrazone which on recrystallisation sintered at 160° C. and melted at 162°–162.5° C., while *p*-nitrobenzoyl chloride gave a nitrobenzoate which melted at 54.7°–56° C.

The whole of the metabolism solution which had now been evaporated to about 5 litres was not fermented by yeast, but was treated direct with basic lead acetate solution until no further precipitate was obtained and the solution (A) filtered from the lead precipitate (B), treatment of which will be given later.

Treatment of solution A.—Lead was removed from the solution (A) by H₂S and half of this lead-free solution was evaporated to a syrup which was then treated with hot absolute alcohol. On cooling, a considerable quantity of crystalline material separated, having a weight of 68.3 gm. (Fraction 1). This was filtered off, washed with absolute alcohol and shown to be mannitol by the method given on page 333.

The alcoholic mother-liquors were shown by analysis to contain a further 16.0 gm. of mannitol, which with Fraction 2, 1.7 gm. (p. 336) gives a total of 86 gm. equivalent to 172 gm. in the original volume. By treatment of the alcoholic mother-liquors as described on p. 334, 5.66 gm. of glycerol tribenzoate (M.Pt. 71°–72° C.) were isolated.

Treatment of lead precipitate (B).—The lead precipitate referred to on p. 335 was ground up with excess of dilute sulphuric acid. The lead sulphate formed was filtered off and the filtrate, freed from sulphuric acid by means of barium hydroxide, and from lead by means of sulphuretted hydrogen, was evaporated *in vacuo* to a thick syrup. The acids in this syrup were esterified by boiling with three separate portions of 250 c.c. of absolute ethyl alcohol containing 2·5 per cent. HCl. There separated from the acid esterification mixture a small amount of crystalline material which weighed 1·7 gm., was shown to be mannitol, and constituted Fraction 2. The filtrate from this fraction, after removal of alcohol, was extracted with ether, the ether extract washed with sodium carbonate and water, dried over anhydrous sodium sulphate and the esters distilled *in vacuo*. Two main fractions were obtained. Fraction 1 distilled at 106° C. to 130° C. at 18 mm. and weighed 7·8 gm. Fraction 2 distilled at 135° C. to 161° C. at 18 mm. and weighed 3·3 gm. 1 c.c. of the first fraction, on treatment with 2 c.c. of absolute alcohol and 2 c.c. of 50 per cent. hydrazine hydrate, gave a copious amount of a crystalline hydrazide which on recrystallisation separated in plates melting at 164°–165° C. This was shown to be succinic acid dihydrazide which has the same melting point. A mixed melting point gave 165° C. Fraction 2 on similar treatment with hydrazine hydrate gave rise to a small amount of a crystalline hydrazide melting at 176°–177° C. which was probably malic acid dihydrazide.

The main metabolic products of this species of *Clasterosporium*, when grown on glucose, are thus mannitol and ethyl alcohol, together with smaller amounts of glycerol, acetaldehyde, succinic acid and probably malic acid.

(c) *Aspergillus Wentii*.

Sixty litres of medium distributed between twelve trays were used in this experiment which commenced on 10th June, 1927. Aeration was commenced on 15th June, 1927, each tray receiving 0·3 cu. ft. daily, six days per week, during the rest of the incubation period, which terminated on 22nd July, 1927. Samples were taken periodically in order to follow the course of the fermentation, and the results are given in Table III, since they illustrate very effectively a point which is evident from the original carbon balance sheet, *i.e.*, the production of some optically dextro-rotatory material which does not reduce alkaline copper solution. This is indicated by the large difference found between the glucose as estimated by the polarimeter and by the copper reduction method of Wood-Ost. (Table III.)

The experiment was terminated after 42 days' incubation. There was no contamination in any of the twelve trays, so that the whole of the metabolism solution from all the trays was mixed and filtered from the mycelium, which was pressed out, but not investigated further. The filtered metabolism solution had a total volume of 45·10 litres, of which 450 c.c. were taken for analysis and the remaining 44·65 litres were evaporated to low

Table III.—Percentage of glucose in different samples as estimated (a) Polarimetrically ;
(b) by the WOOD-OST Method.

Number of Tray.	After 17 Days' Incubation.		After 24 Days' Incubation.		After 35 Days' Incubation.		After 42 Days' Incubation.	
	By Polari-meter.	By WOOD-OST.	By Polari-meter.	By WOOD-OST.	By Polari-meter.	By WOOD-OST.	By Polari-meter.	By WOOD-OST.
2	2.77	2.26	2.19	1.67	1.21	0.61	—	—
4	3.26	2.70	2.49	1.97	1.52	0.95	—	—
9	2.98	2.50	2.27	1.74	1.02	0.53	—	—
11	2.90	2.48	2.09	1.61	0.90	0.38	—	—
Average sample	—	—	—	—	—	—	0.64	0.25

bulk *in vacuo*. A partial balance sheet prepared on the sample taken for analysis gave the following results :—

Titration value, 14.3 c.c. of N/1 NaOH per 250 c.c. medium.

Glucose by polarimeter, 0.641 per cent.

Glucose by WOOD-OST, 0.250 per cent.

Treatment of Metabolism Solution.

The results given in Table IV indicate that *Aspergillus Wentii* produces no alcohol, but, on the other hand, gives rise to about 20 per cent. of some non-volatile acid, the calcium salt of which is precipitated by 80 per cent. alcohol, together with about 16 per cent. of some product included as “carbon unaccounted for.” The whole of the

Table IV.

						Gm. Carbon per 250 c.c. Medium.
						Gm.
Carbon in residual glucose	0.250
„ in CO ₂ in solution	0.006
„ in volatile acids	0.015
„ in non-volatile acids	0.991
„ in volatile neutral compounds...	Nil
„ in synthetic carbon	0.067
Total carbon accounted for	1.329
„ „ in solution	2.141
Carbon unaccounted for...	0.812

metabolism solution was therefore evaporated *in vacuo* to about 9 litres. Of this, two-thirds was stored in the cold room under toluene, while *one-third* was completely precipitated with basic lead acetate. There was a very large precipitate of lead salts (A), which was filtered off and washed well with water. The treatment of the filtrate and washings (B) will be described later.

A. Treatment of Lead Precipitate.—The acids were regenerated from the lead precipitate by treatment with dilute sulphuric acid in the usual manner, excess of sulphuric acid being removed from the filtrate quantitatively by means of barium hydroxide. The solution of regenerated acids was now maintained at 90°–95° C. in presence of an excess of calcium carbonate. When the solution was apparently neutral to litmus, calcium hydroxide was added until a permanently neutral solution was obtained, in order to convert any lactone present into the free acid. The solution of the calcium salts was filtered from the excess of calcium carbonate, which was carefully washed, and the filtrate and washings evaporated *in vacuo* to incipient crystallisation. On standing in the cold, large quantities of a crystalline calcium salt were deposited, which were removed from time to time. In all, four crops were obtained having a total weight of 191·6 gm. This crystalline calcium salt consisted of practically pure calcium gluconate, which it resembles in crystalline form and in its characteristic method of crystallisation. For identification, a portion of it was converted into the phenylhydrazide by heating on a water bath with phenylhydrazine and acetic acid. The phenylhydrazide was recrystallised from water and decolorised with a little animal charcoal. It separated in shining white plates having a melting point of 204°–205° C., which was unchanged on admixture with synthetic gluconic acid phenylhydrazide. Another portion of the calcium salt was recrystallised from water and the calcium gluconate content determined by :—

- | | |
|---|------------------|
| (a) determination of optical activity using the figure given by MAY, HERRICK, THOM and CHURCH (1927), of $[\alpha]_D^{20} = +9\cdot8^\circ$ | = 95·8 per cent. |
| (b) ashing in presence of sulphuric acid | = 95·1 „ |

It may be noted here that 191·6 gm. of calcium gluconate were actually isolated from *one-third* of the metabolism solution, so that it seems reasonable to suppose that the amount of calcium gluconate actually present is considerably higher than this. On the other hand, if it is assumed that *all* the carbon present in the balance sheet in Table IV as non-volatile acids is present as gluconic acid, only 176·2 gm. of calcium gluconate should be present in this volume of metabolism solution. The explanation of this discrepancy is almost certainly the fact that gluconic acid is present in the original metabolism solution, partly as the free acid, which is then precipitated as the calcium salt in 80 per cent. alcohol, and partly as the lactone, which is not precipitated as the calcium salt, but which is shown as “carbon unaccounted for.” Further support for this view will be given later, when it is shown that, in spite of the high

figure for "carbon unaccounted for," only relatively small amounts of products other than gluconic acid could be isolated.

B. Treatment of Filtrate and Washings.—The filtrate and washings were freed from lead by means of H_2S and then evaporated *in vacuo* to a syrup which did not crystallise even after long standing. It was, therefore, re-dissolved in 1 litre of water and the residual glucose, amounting to about 37 gm., fermented with 40 gm. of pure yeast. After three days the yeast was removed by filtration, the filtrate cleaned with basic lead acetate, and the filtrate from the lead precipitate freed from lead by means of H_2S and then evaporated *in vacuo*. Even now the resultant syrup refused to crystallise, and the explanation was found in the fact that the syrup contained considerable quantities of sodium acetate. This was removed by estimating the acetic acid in a small portion of the syrup and then distilling the remainder *in vacuo* with the calculated amount of sulphuric acid necessary to decompose the sodium acetate present. After removal of the acetic acid, an estimation of the carbon present in solution gave an amount only half (24.8 gm.) of what was expected from the balance sheet, *i.e.*, 48.6 gm. of carbon should be present as "carbon unaccounted for" in one-third of the metabolism solution. This supports the view already expressed that part of the "carbon unaccounted for" is gluconic acid lactone, which by the method adopted will have been isolated as calcium gluconate.

The acetic acid-free solution was now evaporated, when considerable quantities of pure sodium sulphate were separated. The resulting syrup was then fractionally precipitated with alcohol and finally with alcohol and ether, giving rise to

- (a) a series of alcohol and ether-alcohol precipitates,
- (b) an ether-alcohol solution.

(a) *Treatment of Alcohol and Ether-Alcohol Precipitates.*—The following fractions were obtained after treatment with alcohol and ether-alcohol :—

TABLE V.

Fraction.	Treatment.	Weight in Gm.	Percentage of Sulphated Ash.
1	Crystalline magma from 70 per cent. alcohol ...	9.73	32.4
2	Immediate precipitate from 95 per cent. alcohol ...	12.6	9.07
3	Precipitate from 95 per cent. alcohol on standing ...	2.51	0.85
4	Precipitate from 1 : 1 ether-alcohol	6.84	5.43
5	Precipitate from 2 : 1 ether-alcohol	1.32	4.04
6	Syrupy residue	16.0	0.13

Apart from a little mineral matter, as indicated by the figures for sulphated ash, fractions 2–5 consisted almost entirely of mannitol. Thus, a portion of fraction 3

crystallised from water and alcohol in the manner characteristic of mannitol, in needles having a melting point of 164.5° – 166° C., which was not altered on admixture with a sample of pure mannitol. Actual estimations of the amount of mannitol present were carried out by the method described in Part X, and indicated the presence of a total of 28.0 gm. of mannitol in one-third of the total metabolism solution.

(b) *Treatment of Ether-alcohol Solution.*—The ether-alcohol solution on evaporation left 16 gm. of a syrup referred to as fraction 6 in Table V. A portion of this syrup was very carefully dried *in vacuo*, when it lost 1–2 per cent. of its weight. 6.3 gm. of this dried syrup were treated with 26 gm. of phenyl isocyanate and the mixture warmed on a sand bath under a reflux condenser until the reaction commenced, when the source of heat was immediately removed until the initial violent reaction had moderated. Heating was then resumed for a further 20 minutes. The product, which solidified on cooling, was broken up, washed with cold benzene and then with water, and recrystallised from alcohol. The solid residue was then fractionally recrystallised from alcohol and benzene, and after 28 separate recrystallisations the purified products were found to group themselves around two melting points, *i.e.*, 183° – 185° C. and 235° C., the latter corresponding to diphenyl urea.

The fraction melting at 183° – 185° C. was shown to be the triphenylurethane of glycerol. Its melting point was not depressed on admixture with a synthetic sample of the triphenylurethane of glycerol, with which it also agrees in appearance and crystalline form. The melting point which was found for this material differs from the only melting point given in the literature, *i.e.*, 160° C. to 180° C. of TESSMER (1885). A combustion on the material melting at 183° C. to 185° C. gave the following results:—

0.1295 gm. of urethane gave 0.0590 gm. H_2O and 0.3055 gm. CO_2 , corresponding to 5.10 per cent. hydrogen and 64.33 per cent. carbon.

0.1161 gm. gave 0.0561 gm. H_2O and 0.2755 gm. CO_2 , corresponding to 5.41 per cent. hydrogen and 64.70 per cent. carbon.

0.1428 gm. gave 11.02 c.c. nitrogen at 13.5° C. and 774.1 mm., corresponding to 9.27 per cent. nitrogen.

The calculated results for glycerol triphenylurethane ($C_{24}H_{23}O_6N_3$) are: carbon = 64.15 per cent., hydrogen = 5.16 per cent., nitrogen = 9.35 per cent.

The total yield of glycerol triphenylurethane isolated was found to correspond to over 50 per cent. of the theoretical, assuming that the original syrup consisted entirely of glycerol. Since no trace of any other phenylurethane could be isolated, and making due allowance for the known losses arising during recrystallisation, the syrup must, obviously, consist mainly of glycerol.

The objection might be raised that part, at any rate, of this glycerol had arisen during the removal of residual sugar by fermentation with yeast, as described on p. 339. For this reason a second portion, consisting of one-third of the original evaporated metabolism solution, was worked up in the same manner as has already been described, with the exception that the residual glucose was not removed by fermentation, but was

precipitated with alcohol and ether in the mannitol fraction. By this means a syrup weighing 20.2 gm. was obtained which on analysis gave 75.3 per cent. and 75.6 per cent. of glycerol by the pyridine acetylation method and 76.2 per cent. by the ZEISEL-FANTO method. It is thus evident that glycerol is really a metabolic product of *Aspergillus Wentii*.

The main product of the metabolism of *Aspergillus Wentii* when grown on glucose is thus gluconic acid (about 16 per cent. isolated) which is present in the metabolism solution partly as the free acid, the calcium salt of which is precipitated by 80 per cent. alcohol, and partly as the lactone which in the metabolism experiments appears in the fraction "carbon unaccounted for." Mannitol (2.9 per cent.) and glycerol (1.6 per cent.) are the other main metabolic products, while no alcohol is formed by this species.

(d) *Fumago vagans*.

Sixty litres of medium distributed between 12 trays were used in this experiment which commenced on 6th October, 1928. Aeration was commenced on 13th October, 1928, each tray receiving 0.2 cu. ft. per day, six days per week. Eight trays were taken off on 13th November, 1928, while the remaining four, which were somewhat slower, were taken off on 19th November, 1928.

Samples were taken and analysed periodically and a selection of the results is given in Table VI. These results indicate the production of some optically dextro-rotatory material which does not reduce alkaline copper solution. The production of a large amount of titratable acid is also a noteworthy feature. During the estimation of the titratable acid it was always found that the initial end-point was very transitory and that it was necessary to add considerable volumes of standard sodium hydroxide after the apparent end-point has been reached before a really permanent and final pink colour was obtained. The same feature was noted with *Aspergillus Wentii* and is probably due to the presence of gluconic acid lactone.

Eight trays were taken off after 38 days' incubation and the remaining four after 44 days. All the trays were entirely free from contamination so that the whole of the metabolism solution (A) from all the trays was mixed and filtered from the mycelium (B). The filtered metabolism solution had a total volume of 44.49 litres. 100 c.c. were taken for analysis and gave the following analytical results :—

Titration value = 26.2 c.c. N/1 NaOH per 250 c.c. medium.

Glucose by polarimeter = 0.874 per cent.

Glucose by WOOD-OST = 0.21 per cent.

A carbon balance sheet was not prepared for this particular sample, but a balance sheet prepared on a repeat experiment, which followed almost identical lines, is appended. (Table VII.)

TABLE VI.—Showing (a) Per cent. Glucose estimated polarimetrically; (b) Per cent. Glucose estimated by Wood-Ost Method, and (c) Titration in c.c. N/1 NaOH per 250 c.c. solution.

Tray No.	After 20 Days' Incubation.			After 27 Days' Incubation.			After 33 Days' Incubation.			After 37 Days' Incubation.			Glucose by Wood-Ost.	Glucose by Polari-meter.	Titra-tion in c.c.
	Glucose by Polari-meter.	Glucose by Wood-Ost.	Titra-tion in c.c.	Glucose by Polari-meter.	Glucose by Wood-Ost.	Titra-tion in c.c.	Glucose by Polari-meter.	Glucose by Wood-Ost.	Titra-tion in c.c.	Glucose by Polari-meter.	Glucose by Wood-Ost.	Titra-tion in c.c.			
2	2.11	1.77	23.6	1.47	1.00	27.9	1.05	0.51	30.7	0.86	—	30.5	—	—	—
10	2.33	1.66	26.0	1.71	0.91	30.5	1.34	0.50	32.7	1.18	—	32.7	—	—	—
Average Sample	—	—	—	—	—	—	—	—	—	—	—	—	0.874	0.210	26.2

TABLE VII.

						Gm. Carbon per 250 c.c. Medium.
						Gm.
Carbon in residual glucose	0·345
„ in CO ₂ in solution	Nil
„ in volatile acids	—
„ in non-volatile acids	1·198
„ in volatile neutral compounds	Nil
„ in synthetic carbon	0·017
„ in compounds precipitated by alcohol	0·211
Total carbon accounted for	1·771
„ „ in solution	2·521
Carbon unaccounted for	0·750

It will be noticed that one of the items in the carbon balance sheet, *i.e.*, “carbon in compounds precipitated by alcohol” = 0·211 gm., has not hitherto appeared in the carbon balance sheets. It was found, however, in dealing with *Fumago vagans* that a heavy precipitate was formed on the addition of four volumes of alcohol to the metabolism solution on which the estimation of the carbon in non-volatile acids was being carried out. This precipitate was therefore filtered off, and its carbon content estimated. The calcium salts of the non-volatile acids were then precipitated in the filtrate. The nature of this alcohol insoluble precipitate (C) will be dealt with later.

A. *Treatment of metabolism solution.*—The results given in Table VII indicate that *Fumago vagans* produces no alcohol, but, on the other hand, gives rise to large amounts of non-volatile acids (24 per cent.) together with about 15 per cent. of “carbon unaccounted for” and about 4 per cent. of some product which is precipitated from aqueous solution by the addition of four volumes of alcohol. Hence, the whole of the metabolism solution was evaporated *in vacuo* to about 3 litres and of this *one-half*, 1560 c.c., was taken for further treatment. An equal volume of 66 O.P. alcohol was added very slowly with continuous stirring, causing the formation of a voluminous precipitate which was allowed to settle overnight. This precipitate, which was neutral in reaction and which constitutes the alcohol insoluble precipitate (C), was filtered off, and washed with 50 per cent. alcohol.

The filtrate and washings were evaporated *in vacuo* to about 700 c.c. and, since a test carried out on a small portion of this solution indicated the presence of considerable amounts of gluconic acid, an attempt was made to fractionate whatever acids were present. This was carried out as follows: To the evaporated and strongly acid filtrate were added two volumes of alcohol with constant stirring. A strongly acid syrup weighing 157 gm. was obtained and this was dissolved in water, neutralised with sodium hydroxide

and treated with a solution of calcium acetate in water. The calcium salts were then fractionally precipitated with alcohol giving rise to Fractions B1 to B5.

The filtrate from the above syrup was now neutralised, enough alcohol added to give 80 per cent. by volume, and calcium acetate added gradually and with constant stirring. A very voluminous precipitate was obtained which was filtered off, washed and fractionally crystallised from water. This gave rise to Fractions C1 to C8. The alcoholic filtrate from the above calcium salts insoluble in 80 per cent. alcohol was evaporated to remove all the alcohol and then set aside to crystallise. This gave rise to Fractions D1 to D3. Treatment of the mother-liquors from D1 and D3 will be described later.

Each of the different fractions B1 to B5, C1 to C8 and D1 to D3 was now recrystallised from water and the mother-liquors from the recrystallisations used for the preparation of lead salts, as described later. The different fractions were then analysed by the following method which will indicate the presence of any material other than calcium gluconate. The calcium gluconate content of the fraction under examination was first determined by the polarimeter using $[\alpha]_D^{20} = +9.8^\circ$ (MAY, HERRICK, THOM and CHURCH, 1927) as the accepted figure for pure calcium gluconate. The calcium gluconate in the same sample was then estimated by determining the sulphated ash.

The quotient $R \equiv \frac{\text{calcium gluconate by polarimeter}}{\text{calcium gluconate by sulphated ash}}$ is, of course, unity in the case of pure calcium gluconate. The value obtained for each of the different fractions, together with their weights, is given in Table VIII.

The figures given for R in Table VIII show a remarkable approximation to unity considering the range of fractions, and indicate that almost all the fractions consist of fairly pure calcium gluconate. Two of the fractions were examined for the presence of other acids, *i.e.*, fraction C4, having a value for $R = 0.944$ and fraction D3, having a value for $R = 0.482$. 10 gm. of fraction C4 were dissolved in water, the calcium removed with oxalic acid, filtered, the filtrate evaporated and extracted with ether. A small amount of crystals of succinic acid was obtained. Fraction D3, on similar treatment, gave a very strong smell of acetic acid, and it is evident that this fraction contained a considerable amount of calcium acetate, which would explain the low value for R.

An attempt was also made to detect other acids in the mother-liquors from the recrystallisation of the calcium salts referred to above. The mother-liquors from all the calcium salt recrystallisations were combined and treated with the theoretical amount of oxalic acid to precipitate the amount of calcium present, filtered from the calcium oxalate and the filtrate precipitated with basic lead acetate. The lead salts were then filtered, washed and dried. 202 gm. of dry lead salts were obtained, and these were converted into esters by FOREMAN'S method (1912), involving esterification with absolute ethyl alcohol and dry HCl, filtration from precipitated lead chloride and removal of excess of HCl as NH_4Cl by treatment with a solution of dry NH_3 in absolute alcohol. The alcoholic solution of the esters when

TABLE VIII.

Number of Fraction.	Weight of Fraction in Gm.	Value of R
		$= \frac{\text{Calcium Gluconate by Polarimeter.}}{\text{Calcium Gluconate by Sulphated Ash.}}$
B 1	6.76	0.986
B 2	15.15	0.966
B 3	1.33	1.060
B 4	36.69	1.049
B 5	5.17	0.925
C 1	10.23	0.988
C 2	17.68	0.962
C 3	87.50	0.998
C 4	32.09	0.944
C 5	6.87	0.985
C 6	17.95	0.920
C 7	29.60	0.938
C 8	41.90	0.913
D 1	13.12	0.957
D 2	3.73	1.16
D 3	12.87	0.482
Theoretical for calcium gluconate	—	1.000
—	Total weight of different fractions = 338.64	—

freed from HCl was evaporated to remove all alcohol, and then shaken with dry ether. The ether solution was dried over Na_2SO_4 , the ether removed and the residue, which was very small, distilled *in vacuo*. Only 2 to 3 drops of an oily liquid distilled, having a boiling point about 130°C . at 4 to 5 mm., nor could any distillable esters be obtained from the ether insoluble part, even though an attempt was made to distil the esters in a mercury vapour vacuum. It is thus apparent that the mother-liquors contain practically no acids giving esters which can be distilled, and it appears probable that no acid other than gluconic acid is present in any appreciable amount.

The mother-liquors from fractions D1 to D3 of the calcium salts contain the whole of the metabolic products in solution, except such as were precipitated with 50 per cent. alcohol, *i.e.*, the alcohol insoluble precipitate C, together with those acids precipitated as calcium salts in 80 per cent. alcohol. For further investigation, these mother-liquors were therefore evaporated to small bulk, and sufficient 5N. H_2SO_4 added (220 c.c.) to give an acid reaction to congo red. The precipitated calcium sulphate was filtered off and the filtrate extracted with ether in a continuous extraction apparatus. 11.6 gm. of a crystalline product were obtained on evaporation of the ether, and this was found to consist of succinic acid. On recrystallisation from alcohol and sublimation in a

high vacuum, this product melted at 192° – 193° C., and this melting point was not altered on admixture with an authentic sample of succinic acid. 0.2426 gm. required 40.96 c.c. of N/10 NaOH for neutralisation corresponding to an equivalent of 59.24 (theoretical for succinic acid = 59.02).

The ether extracted solution was neutralised and evaporated to small bulk. It was now mixed with wood meal in order to convert it into a crumbly mass suitable for extraction, and was then dried down *in vacuo* to a friable mass, which was extracted with acetone in a continuous extraction apparatus. The acetone solution was evaporated, the residue taken up in absolute alcohol and the alcoholic solution treated with 2 volumes of dry ether. The ether-alcohol solution was filtered and evaporated, leaving 10.73 gm. of syrup, which was shown to consist principally of glycerol. 1 gm. of this syrup on treatment with benzoyl chloride, according to the method of LIPP and MILLER (1913) gave 2.65 gm. of crude glycerol tribenzoate, which on recrystallising twice from absolute alcohol, melted at 71.5° – 72° C., the melting point being unchanged on admixture with a sample of pure glycerol tribenzoate. This weight of glycerol tribenzoate corresponds to about 60 per cent. of pure glycerol in the syrup. An estimation of the glycerol content by oxidation with periodic acid according to the method of MALAPRADE (1928) gave the following results: glycerol by the iodine absorbed = 69.9 per cent., and glycerol by the acidity formed = 68.9 per cent. Since the agreement between these two methods is good, it may be assumed that no product susceptible to oxidation by periodic acid is present in the syrup other than glycerol.

At no time was there any evidence of the presence of mannitol in the metabolism solution.

B. *Treatment of Mycelium*.—The mycelium (see p. 341) was treated with about 2 litres of distilled water, steamed, and pressed out while still hot, giving rise to a sticky solution. This extraction was repeated four times in all, and to the combined filtered extracts was now added an equal volume of alcohol. This gave rise to a voluminous precipitate, which weighed 8.2 gm., and which was identical with the “alcohol precipitate C,” referred to on p. 343, and isolated from the metabolism solution. The two fractions were therefore combined for further treatment.

C. *Treatment of Alcohol Insoluble Precipitate*.—The alcohol insoluble precipitate C obtained from the metabolism solution weighed 24.5 gm., and was in the form of a greyish powder. This was combined with the same product obtained from the mycelium (wt. = 8.2 gm.), and a portion of it was purified by fractional precipitation from aqueous solution with alcohol. By this means the dark colouring matter present was precipitated in the first fractions, and the material arising in the middle fractions consisted of a white flocculent precipitate, which when washed with alcohol and dry ether, and then quickly dried *in vacuo* gave rise to a light white powder. This purified material was examined, and was shown to be of the nature of a neutral polysaccharide, which is apparently different from any previously described.

General Properties of the Alcohol Insoluble Precipitate.—This material is readily soluble in hot water, from which it slowly separates, on cooling, as a white amorphous precipitate. The aqueous solution is neutral to litmus, and gives no colour with iodine.

A portion of it gave the following results on combustion, corresponding to the empirical formula $(C_6H_{10}O_5)_n$: 0.2483 gm. of material dried *in vacuo* to constant weight gave 0.4031 gm. CO_2 and 0.1456 gm. H_2O corresponding to 44.27 per cent. of carbon and 6.56 per cent. of hydrogen (theoretical for $C_6H_{10}O_5$, carbon = 44.42 per cent., hydrogen = 6.22 per cent.).

It is very strongly dextro-rotatory. A 1.099 per cent. solution had a rotation corresponding to $[\alpha]_{Hg, yellow}^{18} = +217^\circ$.

It is readily hydrolysed by boiling with dilute acids and on hydrolysis gives rise to no product other than glucose. This was shown as follows:—1.0985 gm. of purified and dried material containing 0.0025 gm. of ash, and equivalent, therefore, to 1.096 gm. of dry ash-free material, were dissolved in water and made up to 100 c.c. 50.00 c.c. of this solution were now taken, 10 c.c. of N/1 HCl added, boiled under reflux and the rotation determined at intervals with the following results:—The readings were taken in the mercury yellow light in a 10 cm. tube. Initial reading, $+1.98^\circ$; after $2\frac{1}{4}$ hours' hydrolysis, $+0.61^\circ$; after $3\frac{3}{4}$ hours', $+0.49^\circ$; after $5\frac{3}{4}$ hours' $+0.48^\circ$.

The hydrolysis was stopped after $5\frac{3}{4}$ hours and 50.02 c.c. of the hydrolysis solution were removed and titrated with N/1 NaOH. 8.41 c.c. were required, and since the calculated amount of acid present equals $\frac{5}{6} \times 10.00 = 8.33$ c.c., it is evident that no acid has been formed during the hydrolysis.

The neutralised solution was now made up to 100 c.c., and the glucose content estimated with the following results:—

	Per cent.
(a) By polarimeter	= 0.474
(b) By WOOD-OST	= 0.475
(c) By alkaline iodine	= 0.489

Accepting the polarimeter and WOOD-OST figures as being correct, the actual weight of glucose produced from 1.096 gm. of dry ash-free material is thus 1.140 gm., from which it is obvious that no product other than glucose is formed during the hydrolysis.

It was definitely shown that the hydrolysis product is glucose, and not some other carbohydrate, by hydrolysing 2 gm. of material with 50 c.c. of N/10 HCl for 5 hours. 5 c.c. of N/1 NaOH were added to neutralise the acid, then 6 c.c. of phenylhydrazine and 3 c.c. of glacial acetic acid. The mixture was heated on the boiling water bath for half an hour, and the osazone which separated in yellow needles was filtered off, washed and dried. The dry osazone melted at $206^\circ C$. when quickly heated and 0.2 gm. dissolved in 4 c.c. of pyridine and 6 c.c. of alcohol and polarised in a 10 cm. tube gave a

rotation of -1.35° and -1.45° on different fractions. NEUBERG (1899) gives the value for glucose under these conditions as -1.50° .

The polysaccharide is not hydrolysed by either of the enzymes, invertase or diastase, since solutions of the polysaccharide to which solutions of the enzymes had been added showed no alteration in rotation even after 3 days' incubation at 37°C .

The main product of the metabolism of *Fumago vagans* when grown on glucose is thus gluconic acid. A new dextro-rotatory polysaccharide, composed entirely of glucose units, is also formed in considerable amounts. Glycerol and succinic acid are also formed in smaller amounts, but no mannitol was isolated.

(e) *Penicillium species Ad.* 11 (*P. chrysogenum*).

Sixty litres of medium distributed between 12 trays were used in this experiment, which commenced on 19th May, 1927. Aeration was commenced on 23rd May, 1927, each tray receiving 0.33 cu. ft. per day on 6 days per week. A sample of gas taken on 27th May, 1927, before aeration, showed an entire absence of oxygen and the presence of 21 per cent. of CO_2 . Four trays were taken off on 22nd June, 1927, and the remaining 8 trays were taken off 2 days later. All the trays were free from infection, so that the metabolism solution from all the trays was mixed and filtered, and had a total volume of 45 litres. Analyses are given in Table IX on all the trays on the day they were taken off.

TABLE IX.

Number of Tray.	Titration in c.c. N/1 NaOH per 250 c.c. Medium.	Percentage Glucose by Polarimeter.	Percentage Glucose by WOOD-OST.
1	17.2	0.56	0.07
2	19.2	0.67	0.18
3	16.5	0.75	0.29
4	16.1	0.71	0.25
5	17.1	0.66	0.20
6	16.6	0.59	0.17
7	19.6	0.61	0.12
8	22.0	0.32	0.10
9	19.7	0.66	0.15
10	17.6	0.74	0.20
11	19.9	0.70	0.16
12	20.1	0.67	0.14

It was noted on carrying out the estimation of the titratable acidity that the initial end-point was very transitory, and that a considerable quantity of standard alkali was required to effect a permanent end-point. This is almost certainly due to the presence of an acid lactone.

A carbon balance sheet was prepared on a mixed sample obtained by taking 20 c.c. from each tray. The results are given in Table X.

Titration value	20·3 c.c. N/1 NaOH per 250 c.c. medium.
Glucose by polarimeter	0·552 per cent.
Glucose by WOOD-OST	0·122 per cent.

TABLE X.

						Gm. Carbon per 250 c.c. Medium.
						Gm.
Carbon in residual glucose	0·122
„ in CO ₂ in solution	—
„ in volatile acids	—
„ in non-volatile acids	0·833
„ in volatile neutral compounds	Nil
„ in synthetic carbon	0·106
Total carbon accounted for	1·061
„ „ in solution	2·472
Carbon unaccounted for	1·411

Treatment of Metabolism Solution.—The results given in Table X indicate that this strain of *P. chrysogenum* gives rise to about 16 per cent. of some non-volatile acid, the calcium salt of which is precipitated by 80 per cent. alcohol. There is also about 28 per cent. of some compound or compounds included as “carbon unaccounted for,” and in view of previous experience it seems probable that part, at any rate, of the “carbon unaccounted for” may be present as the lactone of a hydroxy-acid.

The whole of the metabolism solution, measuring 45 litres, was evaporated *in vacuo* to a thin syrup measuring 1,770 c.c. (solution A). This was preserved with toluene, and portions of it were examined.

500 c.c. of the original solution A (measuring 1,770 c.c.) were diluted to 3½ to 4 litres, and incubated at 30° C. for 24 hours with 22 gm. of pure yeast in order to remove all fermentable sugar. The solution was now filtered from the yeast, and the filtrate and washings precipitated with basic lead acetate solution until no further precipitate was obtained and the reaction of the liquid was definitely alkaline. The large lead precipitate was filtered off and washed by grinding three times with water. The filtrate and washings were combined, evaporated to small bulk and reprecipitated with basic lead acetate. The second lead precipitate, which was small in bulk, was united with the first lead precipitate and worked up as lead precipitate (B). The filtrate and washings (C) are dealt with later.

(a) *Treatment of Lead Precipitate (B).*—The washed lead precipitate from the first and second lead precipitations was ground up with a slight excess of dilute sulphuric acid, filtered from lead sulphate, and the excess of sulphuric acid removed from the filtrate and washings with barium hydroxide. The lead and sulphuric acid-free solution was now boiled with an excess of pure calcium carbonate for about 12 hours. Towards the end of this period a small quantity of calcium hydroxide was added, and when permanent neutrality was obtained the solution was filtered from excess calcium carbonate. The aqueous solution was evaporated and crystallised, giving rise to the following crops of crystals :—

	gm.
Crop 1	158·4
„ 2	14·89
„ 3	1·82
„ 4	12·24

The sulphated ash on these fractions was determined with the following results :—

	Per cent.
Crop 1	31·33
„ 2	32·99
„ 2 recrystallised, 1st fraction	31·30
„ 2 recrystallised, 2nd fraction	31·21
„ 3	33·14
„ 3 recrystallised	31·33
„ 4	{ 29·26
	{ 30·59

From these figures it appears that all of these crops consist almost entirely of calcium gluconate, which has a sulphated ash content of 31·62 per cent. This was confirmed by determining the quotient :

$R \equiv \frac{\text{Calcium gluconate by polarimeter}}{\text{Calcium gluconate by sulphated ash}}$ as described on p. 344. The results obtained are given in Table XI.

TABLE XI.

Number of Fraction.	Weight of Fraction in Gm.	Value of R
		$\equiv \frac{\text{Calcium Gluconate by Polarimeter.}}{\text{Calcium Gluconate by Sulphated Ash.}}$
Crop 1	158·4	0·982
„ 2	14·89	1·042 (recrystallised, first fraction).
„ 2	14·89	1·025 (recrystallised, second fraction).
„ 3	1·82	1·073 (once recrystallised).
„ 4	12·24	1·028

Crop 1 was further shown qualitatively to consist of calcium gluconate by the following means :—

- (a) A portion was converted into the phenylhydrazide by heating with acetic acid and phenylhydrazine. The phenylhydrazide crystallised from water in white plates melting at 198° – 199° C., this melting point being unchanged on admixture with an authentic sample of gluconic acid phenylhydrazide.
- (b) An attempt was made to esterify a portion by boiling with absolute alcohol containing 2·5 per cent. dry HCl. No ester was obtained, but a small quantity of *d*-gluconic acid lactone (see FISCHER, 1890) was isolated.

The total yield of calcium gluconate is thus, $158\cdot4 + 14\cdot89 + 1\cdot82 + 12\cdot24 = 187\cdot35$ gm. 187·4 gm. of calcium gluconate corresponds to 62·75 gm. of carbon. This amount of carbon is therefore present as gluconic acid or as lactone in 500 c.c. of the evaporated metabolism solution (solution A measuring 1,770 c.c.), which in its turn corresponds to 12·71 litres of the original metabolism solution. The amount of carbon actually isolated as calcium gluconate thus corresponds to 1·234 gm. carbon per 250 c.c. of the original metabolism solution before evaporation. If this figure is compared with the figure for carbon in non-volatile acids given in the carbon balance sheet in Table X, *i.e.*, 0·833 gm., it is obvious that only a portion of the gluconic acid present is precipitated as a calcium salt insoluble in 80 per cent. alcohol, the remainder being presumably present as a lactone.

(b) *Treatment of Filtrate and Washings (C).*—The combined filtrate and washings (C) were freed from lead by means of H_2S and evaporated to small bulk. A considerable quantity of mannitol separated, which on recrystallisation weighed 21·26 gm. and melted at 162° C. The filtrate and mother-liquors did not give rise to any further mannitol on concentration and this was shown to be due to the presence of considerable quantities of sodium acetate. In order to remove this, the theoretical amount of N/1 H_2SO_4 , necessary to decompose all the sodium acetate present, was added. The acetic acid was then distilled off *in vacuo* and a considerable quantity of sodium sulphate crystallised out and was filtered off.

An actual estimation of the total mannitol present in solution A was carried out and is described in detail below. It was thus found that 57·4 gm. of mannitol are present in 500 c.c. of solution A. Therefore, since 21·26 gm. have already been isolated, there will now be remaining in the acetic acid-free solution about 36 gm. of mannitol. Also, a total carbon estimation on this solution showed that no appreciable amount of organic material other than mannitol could be present in the solution, since its total carbon content corresponded to 50 gm. of mannitol. Hence, only about 14 gm. of any material other than mannitol were present. For this reason it was not considered worth while to investigate this fraction further.

Estimation of Mannitol.—5 c.c. of the evaporated metabolism solution (solution A measuring 1,770 c.c.) were treated by the method described in Part X for solutions

containing both glucose and mannitol. The total amount of mannitol found was 0.574 gm., corresponding to 57.4 gm. in 500 c.c. of the solution A. A companion analysis carried out by the estimation of the acetyl value of solution A gave a figure of 53.6 gm. of mannitol per 500 c.c. of solution A. Accepting as correct the figure 57.4 gm. of mannitol in 500 c.c. of the evaporated metabolism solution A, this is equivalent to 22.71 gm. of carbon or to 0.447 gm. of carbon in 250 c.c. of the original metabolism solution.

Estimation of Gluconic Acid.—An estimation was carried out of the gluconic acid present in the evaporated metabolism solution (solution A measuring 1,770 c.c.) by polarising this solution after complete neutralisation with sodium hydroxide, and calculating the gluconic acid present from an experimentally determined rotation for sodium gluconate in water.

0.2510 gm. of pure gluconic acid lactone was neutralised completely with N/1 NaOH (found, 1.43 c.c. N/1; theory=1.41 c.c.) and made up to 25 c.c. with water. This was then polarised in a 20 cm. tube using the mercury yellow light. The mean of 20 closely agreeing readings taken at intervals over 24 hours, gave $+0.335^\circ$, corresponding to $[\alpha]_{\text{Hg. yellow}} = +16.68^\circ$ for sodium gluconate weighed as lactone.

5.05 c.c. of the evaporated metabolism solution were neutralised with N/1 NaOH (0.78 c.c. needed) and made up to 50.06 c.c., filtered and polarised in a 10 cm. tube with the mercury yellow light. The mean of 10 closely agreeing readings was $+0.874^\circ$. An estimation of the glucose present, by the WOOD-OST method, indicated the presence of 0.379 per cent. glucose corresponding to a rotation of $+0.199^\circ$. Therefore, the net rotation due to sodium gluconate = $+0.675^\circ$, which corresponds to 4.406 per cent. of gluconic acid lactone, and is equivalent to 1.597 gm. of carbon present as gluconic acid or the lactone in 250 c.c. of the original unevaporated medium. This is equivalent to 64.6 per cent. of the total carbon in solution, while the “carbon as non-volatile acids” figure from the balance sheet, *i.e.*, 0.833 gm., is only 33.6 per cent. of the total carbon in solution. On the other hand, almost complete precipitation of the gluconic acid is effected by two precipitations with basic lead acetate, as is shown by the following figures:—When a portion of the original evaporated metabolism solution (A) was diluted and precipitated twice with basic lead acetate 50.0 per cent. of the total carbon in solution was precipitated in the first precipitation and 13.0 per cent. in the second precipitation, making a total of 63.0 per cent., which agrees quite well with the figure of 64.6 per cent. given above for the total gluconic acid content.

The carbon present in the original medium, either as mannitol (0.447 gm.) or as gluconic acid or gluconic acid lactone (1.597 gm.) is, therefore, 2.044 gm. of carbon per 250 c.c. medium.

From the balance sheet 0.833 gm. of carbon is present in the original metabolism solution as “non-volatile acids” and 1.411 gm. of carbon as “carbon unaccounted for,” making a total of 2.244 gm. Hence, it is obvious that, at the most, only very small amounts of any products other than mannitol and gluconic acid are formed from glucose by *P. chrysogenum*. These products are present to the extent of approximately

9 per cent. of mannitol, 16 per cent. of gluconic acid as the free acid, and 16 per cent. of gluconic acid as the lactone.

Summary.

An investigation has been carried out of the metabolism products of five different species of fungi belonging to five different genera. These species were all grown on the usual CZAPEK-DOX 5 per cent. glucose solution, and had been chosen for investigation because of some interesting point in their carbon balance sheets.

(a) *Helminthosporium geniculatum*.—The main metabolic products of this species are ethyl alcohol and mannitol. Smaller amounts of glycerol and acetaldehyde were also found.

(b) *Clasterosporium species*.—The main metabolic products of this species are mannitol and ethyl alcohol. Smaller amounts of glycerol, acetaldehyde, succinic acid and probably malic acid were also found.

(c) *Aspergillus Wentii*.—The main metabolic product of this species is gluconic acid, present in solution partly as the free acid, and partly as the lactone. Smaller amounts of mannitol and glycerol were found, but no alcohol.

(d) *Fumago vagans*.—The main metabolic product of this species is gluconic acid, together with smaller amounts of glycerol and succinic acid, but no mannitol. An interesting feature of this species is the production by it, in considerable quantities, of a new polysaccharide composed entirely of glucose units. The preparation and properties of this polysaccharide are described.

(e) *Penicillium species* Ad. 11 (*P. chrysogenum*).—The main metabolic products of this species are gluconic acid and mannitol.

