CLXXI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXIV. THE METABOLIC PRODUCTS OF THE *PENICILLIUM BREVI-COMPACTUM* SERIES.

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Thom, in his book "The Penicillia" [1930], groups together a number of species in what he calls the *P. brevi-compactum* series, corresponding with Biourge's [1923] Hemizonata. This series, of which the outstanding species may be taken to be *P. brevi-compactum* Dierckx and *P. stoloniferum* Thom, includes a number of other species or strains which are very widely distributed. Thom regards *P. brevi-compactum* and *P. stoloniferum* as synonyms.

The series is particularly interesting from a biochemical point of view since there is a certain amount of scattered information leading to the belief that the metabolic products of some of the species are definitely toxic. In 1896 Gosio was engaged in Italy on a study of mouldy maize in the belief that pellagra is caused by the toxic substances present in such maize. Although this belief is no longer held, Gosio's work [1896] led to the introduction of a test, which has been largely used in Italy and in America, for the detection of spoilage of maize by mould infection. This test was amplified by Black and Alsberg [1910] and consists in extracting the ground maize with chloroform. To the chloroform solution is then added ferric chloride, and in the presence of marked infections various colours such as blue, green, violet and red are obtained. Gosio isolated from mouldy maize a strain of *Penicillium* to which he gave the name *P. glaucum*, although it is evident now that his culture was inadequately named and was, in fact, one of the *brevi-compacta*. When this species was grown, either on maize itself or on Raulin's medium, a metabolic product was isolated which melted at 145°, and gave an intense blue colour with $FeCl_3$ when pure, but a violet shade when slightly impure. Unfortunately, very little of the substance was available for analysis, and hence, on results of a single combustion and without a molecular weight determination, Gosio assigned the formula $C_9H_{10}O_3$ to this product.

Alsberg and Black [1913], in a study of maize deterioration by mould infections, found that a fungus isolated from a sample of spoiled Italian maize, and identified as *Penicillium stoloniferum* Thom, is, at any rate in part, responsible for this deterioration. This organism, when grown on Raulin's medium, gave the very strong and characteristically violet ferric chloride reaction of Gosio [1896]. They isolated the metabolic product responsible for the ferric chloride reaction, and called it mycophenolic acid. This acid was obtained in the form of white needles, having a melting-point of 140°, and the formula $C_{17}H_{20}O_6$, deduced from analysis, titration value and molecular weight estimations. Alsberg and Black described its general properties, including the fact that it gives a violet colour with $FeCl_3$ in aqueous solution, but they did not work out its constitutional formula. There is, in our opinion, no doubt that Gosio's acid and Alsberg and Black's mycophenolic acid are one and the same substance. Neither of these substances was found to be toxic to mice.

Turesson [1917] found that some members of the *P. brevi-compactum* series were pathogenic to honey bees, and his experiments indicate that these species produce a phenolic substance in quantities sufficient to produce this toxic effect.

We have collected from various sources a number of species or strains of *Penicillium* belonging to the *P. brevi-compactum* series, and found that when these cultures are grown on Raulin's medium, or to a lesser degree when they are grown on a Czapek-Dox medium containing glucose as the sole source of carbon, the metabolism solution gives with ferric chloride a range of colours varying with the length of incubation and the particular species used. In the earlier stages after, say, 7 days' incubation, the colour is brown or brownish-red, and then changes with continued incubation to a port-wine red or purple, or bluish-red, or even, in some cases, bluish-violet.

Other characteristic reactions are obtained by addition to the metabolism solution of bromine water or alkaline hypochlorite solution. The cautious addition of bromine water gives first a reddish-brown colour, discharged completely on further addition of the reagent. Excess of bromine gives in most cases a voluminous precipitate. Bleaching powder or sodium hypochlorite solutions give a coloration varying from pale brown to rich brownish-red, according to the length of the period of incubation. The colour fades very slowly if the optimum amount of hypochlorite is used but is rapidly discharged by excess of the reagent.

In working up the metabolic products from a number of these species, it soon became evident that a complex mixture of metabolic products is formed. In almost all cases mycophenolic acid, in greater or smaller amount, was isolated, but in addition to this compound, other metabolic products, having the empirical formulae $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ and $C_8H_6O_6$ were isolated.

The colours given by the metabolism solution with ferric chloride or with bleaching powder solution and the reactions with bromine water are obviously the sums of the separate reactions of the various pure products with these reagents. The reactions of the pure materials are described in detail later.

It is the purpose of this paper to describe the culture methods employed for the production of these substances, and the chemical methods used for their isolation. The question of their constitution will be dealt with in subsequent papers.

EXPERIMENTAL.

Cultures.

The following list indicates the organisms used and their source.

Table I.

Catalogue No.	Species	Source
P 75	*P. brevi-compactum* Dierckx	Received from Centraalbureau voor Schimmelcultures, Baarn, 14. x. 30. Biourge's culture
M 3 (1)	Unnamed	Isolated at London School of Hygiene and Tropical Medicine by J. H. V. Charles from Italian mouldy maize
M 3 (3)	Unnamed	,, ,, ,,
M 3 (4)	Unnamed	,, ,, ,,
P 151	*P. scabrum* Biourge	From Biourge, 23. xii. 31
A 11	*P. brevi-compactum* Dierckx	Isolated from cotton and identified by G. Smith
S 30	Unnamed	Isolated at London School of Hygiene and Tropical Medicine by J. H. V. Charles from soil
P 84	*P. Hagemi* Zaleski	Type strain from Baarn, 14. x. 30
P 99	*P. Szaferi* Zaleski	Type strain from Baarn, 14. x. 30
D 8	Unnamed	Plate contaminant isolated by Dr Duncan, London School of Hygiene and Tropical Medicine
P 90	*P. patris-mei* Zaleski	Type strain from Baarn, 14. x. 30
P 37	*P. griseo-brunneum* Dierckx	From Thom, 29. iv. 30, No. 4733.68
Ad 89	*P. stoloniferum* Thom	Ardeer Collection, Thom Type 27
Ad 87	*P. Biourgeianum* Zaleski	Ardeer Collection, contaminant of culture of Alga. Identified by Thom
P 69	*P. aurantio-griseum* Dierckx var. *Poznaniensis* Zaleski	Type strain, from Baarn, 14. x. 30

All the species were examined as to purity by plating out, and, where necessary, were purified by the isolation of single-spore colonies.

Detailed morphological examination showed that all the species used were true "brevi-compacta" in Thom's sense, the penicillus typical of this group being, when once recognised, easily distinguishable from all others. The named species corresponded sufficiently closely with the published diagnoses to allow of no doubt as to correct naming, but a number of them showed slight divergencies from type, particularly as regards the presence or absence of fine markings on the conidiophores and conidia. The unnamed species all corresponded very closely with the descriptions of *P. brevi-compactum* given by Biourge and of *P. stoloniferum* given by Thom—*P. brevi-compactum* is regarded by Thom as a synonym for *P. stoloniferum*—and may be regarded as strains of that species.

Media.

Three media were tried. The first (A) which we describe as "Raulin-Thom" is identical with that quoted by Thom [1930, p. 36] as due to Raulin, except for the omission of potassium silicate. This medium, however, differs from that originally described by Raulin [1869], the ammonium nitrate of Raulin's medium being replaced by ammonium tartrate. Experiments were also tried on true Raulin's solution (B), again with omission of potassium silicate. Finally, Czapek-Dox medium (C) was used in test-tube experiments. Of the three media, which have the following composition, Raulin-Thom proved the best and was used throughout the routine experiments.

Medium A (Raulin-Thom)		Medium B (Raulin)		Medium C (Czapek-Dox)	
Water	1500	Water	1500	Water	1000
*Glucose	75	*Glucose	75	Glucose	50
Tartaric acid	4	Tartaric acid	4	$NaNO_3$	2
Amm. tartrate	4	NH_4NO_3	4	KH_2PO_4	1
Amm. phosphate	0·6	Amm. phosphate	0·6	KCl	0·5
K_2CO_3	0·6	K_2CO_3	0·6	$MgSO_4, 7H_2O$	0·5
$MgCO_3$	0·4	$MgCO_3$	0·4	$FeSO_4, 7H_2O$	0·02
$(NH_4)_2SO_4$	0·25	$(NH_4)_2SO_4$	0·25		
$ZnSO_4, 7H_2O$	0·07	$ZnSO_4, 7H_2O$	0·07		
$FeSO_4, 7H_2O$	0·07	$FeSO_4, 7H_2O$	0·07		

* In each case 5 % glucose was used in place of the usual 4·6 % cane sugar (Thom) or sugar candy (Raulin).

Technique for cultivation of the micro-organisms in the routine metabolism experiments.

350 cc. quantities of the Raulin-Thom medium (A) were placed in 101 one litre conical flasks, which were plugged with cotton wool and sterilised by steaming for half an hour on each of three consecutive days. Each batch of 101 flasks was sown with a suspension in the above medium of spores prepared from two wort-agar test-tube cultures, 3 to 4 weeks old, of the desired strain. The flasks were thoroughly shaken and incubated at 24°.

Eleven of these flasks were separately removed at varying times during the incubation period, and the following analyses carried out, the contents of the remaining 90 flasks being worked up for metabolism products.

1. Determination of p_H.
2. Glucose by polarimeter.
3. Bromine absorption by Koppeschaar's method.
4. Determination of ferric chloride reaction.

The results of the first three estimations are summarised in Table II.

Change of p_H.

When the figures (Table II) for change of p_H are plotted, the curves for P 75, M 3 (1), M 3 (3), M 3 (4), P 151, A 11, S 30, P 84, P 99, D 8, P 90 and P 37 all follow approximately the same course (S-shaped) showing usually a small initial decrease in p_H from the initial value of 3·9 over the first 6 days and

Table II.

R.S.P. = % residual sugar determined by polarimeter. B.A. = bromine absorption in mg. Br per cc.

Organism	P 75			M 3 (1)			M 3 (3)			M 3 (4)			P 151		
Days	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H
0	—	—	3·9	5·27	0·11	3·9	5·48	0·14	3·9	—	—	—	4·97	0·11	3·9
1	—	—	—	—	—	—	—	—	—	5·32	0·14	3·9	—	—	—
2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	—	—	—	3·75	0·35	3·65	3·89	0·61	3·8	3·83	0·59	3·8	3·88	0·30	3·7
5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
7	2·87	1·03	3·8	2·38	1·55	3·65	2·33	1·09	3·8	2·27	0·82	3·9	2·67	0·70	3·6
8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9	—	—	—	1·94	1·87	4·1	—	—	—	1·43	0·99	4·0	—	—	—
10	1·59	1·82	3·9	—	—	—	1·27	1·54	4·0	—	—	—	1·27	1·10	4·0
11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
12	—	—	—	0·97	2·18	4·4	0·78	1·70	4·4	0·67	1·36	4·6	—	—	—
13	0·84	2·28	4·3	—	—	—	—	—	—	—	—	—	0·48	1·50	4·6
14	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
15	—	—	—	0·34	2·67	4·95	0·26	2·00	5·2	0·29	1·63	5·3	—	—	—
16	0·53	2·72	4·7	—	—	—	—	—	—	—	—	—	—	—	—
17	—	—	—	—	—	—	0·16	2·26	5·7	—	—	—	0·31	1·60	5·3
18	—	—	—	0·25	2·75	4·95	—	—	—	0·11	1·79	5·4	—	—	—
19	0·26	3·26	5·1	—	—	—	0·07	2·24	6·4	—	—	—	—	—	—
20	—	—	—	0·12	3·39	5·2	—	—	—	—	—	—	0·02	1·87	6·4
21	0·19	3·36	5·5	—	—	—	—	—	—	0·02	1·89	5·9	—	—	—
22	—	—	—	—	—	—	0·00	2·27	7·1	—	—	—	—	—	—
23	0·06	3·58	6·1	0·02	3·07	5·5	—	—	—	0·00	1·98	6·1	—	—	—
24	—	—	—	—	—	—	—	—	—	—	—	—	0·00	1·90	7·5
25	0·04	3·36	6·4	—	—	—	0·00	2·32	7·5	—	—	—	—	—	—
26	—	—	—	0·03	3·49	6·5	—	—	—	0·00	1·84	6·65	—	—	—
27	0·00	3·28	6·9	—	—	—	—	—	—	—	—	—	—	—	—
28	—	—	—	—	—	—	0·00	2·24	7·7	—	—	—	0·00	1·90	7·1
29	0·00	3·36	6·9	0·00	3·74	6·65	—	—	—	0·00	2·00	6·8	—	—	—
30	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
31	0·00	3·15	6·9	—	—	—	—	—	—	—	—	—	0·00	2·00	7·3
32	—	—	—	0·00	3·73	7·0	—	—	—	—	—	—	—	—	—
33	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
34	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Organism	A 11			S 30			P 84			P 99			D 8		
Days	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H
0	5·32	0·08	3·9	5·03	0·11	3·9	5·13	0·11	4·0	5·27	0·10	3·9	5·27	0·14	3·9
1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	4·74	0·14	3·8	—	—	—	—	—	—
4	4·00	0·24	3·7	—	—	—	—	—	—	3·80	0·48	3·3	4·46	0·26	3·7
5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	—	—	—	3·31	0·96	3·85	3·02	0·26	3·65	—	—	—	—	—	—
7	2·65	0·66	3·5	—	—	—	—	—	—	2·31	1·24	3·2	2·59	0·93	3·7
8	—	—	—	—	—	—	2·01	0·35	3·8	—	—	—	—	—	—
9	1·89	1·04	3·7	1·88	1·34	3·65	—	—	—	1·43	1·74	3·2	1·68	1·76	3·7
10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
12	1·02	1·52	4·1	—	—	—	—	—	—	0·82	2·16	3·9	0·65	2·54	4·1
13	—	—	—	0·94	3·17	4·3	0·69	0·61	4·8	—	—	—	—	—	—
14	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
15	0·48	1·86	4·6	—	—	—	—	—	—	0·26	2·59	4·6	0·41	2·94	4·5
16	—	—	—	0·50	3·34	4·6	0·22	0·86	5·5	—	—	—	—	—	—
17	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
18	0·15	2·10	5·0	—	—	—	—	—	—	0·09	2·80	5·1	0·12	2·66	5·3
19	—	—	—	0·24	3·72	5·1	—	—	—	—	—	—	—	—	—
20	—	—	—	—	—	—	0·08	1·09	6·1	—	—	—	—	—	—
21	0·11	2·24	5·4	—	—	—	—	—	—	0·02	2·86	5·9	0·03	2·51	6·6
22	—	—	—	0·09	3·74	5·6	—	—	—	—	—	—	—	—	—
23	0·03	2·32	5·6	—	—	—	0·00	1·09	6·4	0·00	3·04	6·9	0·00	2·99	6·85
24	—	—	—	0·05	3·66	6·25	—	—	—	—	—	—	—	—	—
25	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
26	0·00	2·50	6·3	—	—	—	0·00	1·02	6·3	0·00	3·03	7·25	0·00	2·48	7·4
27	—	—	—	0·00	3·87	6·8	—	—	—	—	—	—	—	—	—
28	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
29	0·00	2·50	6·7	—	—	—	0·00	1·15	6·5	0·00	2·96	7·5	0·00	2·88	7·4
30	—	—	—	0·00	4·43	7·2	—	—	—	—	—	—	—	—	—
31	—	—	—	—	—	—	0·00	1·17	6·4	—	—	—	—	—	—
32	0·00	2·46	7·0	—	—	—	—	—	—	0·00	2·88	7·6	0·00	2·11	7·7
33	—	—	—	—	3·68	7·2	—	—	—	—	—	—	—	—	—
34	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Table II (*cont.*).

Organism	P 90			P 37			Ad 89			Ad 87			P 69		
Days	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H
0	5·37	0·11	3·9	4·97	0·11	3·9	5·32	0·11	3·95	5·24	0·10	3·95	5·34	0·10	4·1
1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—	5·06	0·34	3·8	—	—	—
4	4·06	0·34	3·9	4·02	0·34	3·4	4·58	0·42	3·7	—	—	—	5·45	0·16	3·8
5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	—	—	—	—	—	—	—	—	—	3·28	0·46	3·2	—	—	—
7	2·23	0·54	3·9	2·52	0·72	3·3	2·98	1·15	3·65	—	—	—	3·66	0·32	3·0
8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9	1·51	0·61	3·95	1·47	1·04	3·5	—	—	—	1·87	0·40	3·25	—	—	—
10	—	—	—	—	—	—	2·20	0·35	3·3	—	—	—	1·63	0·62	2·8
11	0·93	0·82	4·6	—	—	—	—	—	—	—	—	—	—	—	—
12	—	—	—	—	—	—	1·35	3·04	4·0	0·87	0·40	4·1	1·67	0·62	2·8
13	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
14	0·43	1·01	6·0	0·21	1·34	4·9	—	—	—	—	—	—	—	—	—
15	—	—	—	—	—	—	0·65	4·40	4·5	0·36	0·45	6·0	0·82	0·88	3·0
16	—	—	—	0·14	1·42	5·5	—	—	—	—	—	—	—	—	—
17	0·19	1·07	6·75	—	—	—	—	—	—	0·23	0·34	6·5	—	—	—
18	—	—	—	0·05	1·41	5·7	0·23	4·54	4·5	—	—	—	0·42	1·01	3·0
19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
20	0·06	1·22	7·35	—	—	—	—	—	—	0·09	0·53	7·1	—	—	—
21	—	—	—	0·02	1·44	5·9	0·09	4·49	4·6	—	—	—	0·18	1·01	3·0
22	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
23	0·02	1·22	7·6	0·00	1·82	5·7	—	—	—	0·05	0·54	7·15	—	—	—
24	—	—	—	—	—	—	0·00	4·56	4·5	—	—	—	0·03	1·12	3·0
25	0·02	1·00	7·65	—	—	—	—	—	—	—	—	—	—	—	—
26	—	—	—	—	—	—	0·00	4·68	4·5	0·02	0·62	7·25	0·00	1·23	3·6
27	—	—	—	0·00	1·68	6·5	—	—	—	—	—	—	—	—	—
28	0·06	0·98	8·2	—	—	—	—	—	—	0·00	0·56	7·9	—	—	—
29	—	—	—	—	—	—	0·00	4·77	4·5	—	—	—	0·00	1·23	—
30	—	—	—	0·00	1·84	6·7	—	—	—	—	—	—	—	—	—
31	0·06	0·91	8·4	—	—	—	—	—	—	—	—	—	—	—	—
32	—	—	—	—	—	—	0·00	4·08	4·65	—	—	—	—	1·31	—
33	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
34	—	—	—	0·00	1·84	6·6	—	—	—	—	—	—	—	—	—

then a steady rise to values varying with the different organisms between 6·6 (P 37) and 8·4 (P 90). All these organisms give varying yields of both mycophenolic acid and the products of smaller molecular weight. The curve for Ad 87 is slightly different in type, lying somewhat lower, and this organism gives no mycophenolic acid and but little of the other products. The curve for Ad 89 shows almost no change in p_H at all. This organism produces no mycophenolic acid but gives the greatest yield of the products of lower molecular weight. Finally the curve for P 69 is quite different, decreasing from the initial value of 4·1 to 2·8–3·0 and maintaining this p_H until the 24th day when a sudden rise occurs. The sugar has, however, completely disappeared before this increase in p_H is obtained. It was not found possible to obtain any of the described metabolic products from the material isolated by ether extraction of the P 69 metabolism fluid.

Change of sugar content.

The same general similarity of curves is seen when the rates of disappearance of sugar are plotted.

Bromine absorption.

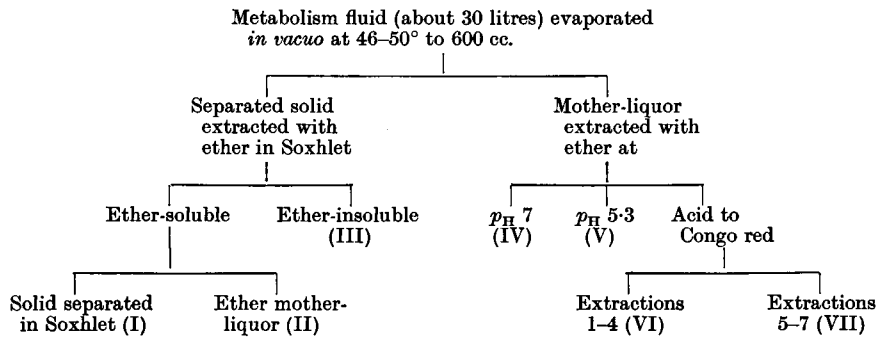
Perhaps the most interesting curves are obtained, however, by plotting the figures for the bromine absorption of the various metabolism fluids. Since the bromine absorption of fluids containing chiefly mycophenolic acid appears to be much smaller than that of those containing chiefly the products of lower molecular weight, it is possible to obtain from the bromine absorption figures some idea of the amount of total product, and of the relative amounts of the two types of products. Thus the highest bromine absorption was obtained with Ad 89, which produces no mycophenolic acid but gives the largest yield of the products of lower molecular weight. The lowest bromine absorption was obtained with Ad 87 which produced no mycophenolic acid and but little of the remaining products.

Ferric chloride tests.

The metabolism solution (5 cc.) with all the organisms except P 69, on addition of $FeCl_3$ gives initially a yellow colour, becoming dirty yellow (3rd to 4th day), pale brown (5th to 6th day), golden brown (7th to 9th day), brownish-purple (10th to 12th day), crimson (13th to 16th day), and finally a deep blue-crimson colour. With P 69, the colour change is slower and only reaches the golden brown stage. The various colours are given at slightly different times with different organisms, and the days stated must only be regarded as a general indication of the time taken.

Separation of metabolic products.

The remaining 90 flasks were removed when the sugar had disappeared and worked up in bulk for the various metabolism products. The products were first separated from the metabolism fluid in a series of fractions which were eventually examined individually. The general scheme of separation of the fractions is as follows:



The combined filtered metabolism fluid for one species (usually from 90 flasks) was evaporated *in vacuo* to 600 cc. and allowed to stand at 0° for 2 days.

(*a*) The *separated solid* was then filtered off, washed with water, dried *in vacuo*, weighed and extracted continuously with ether in a Soxhlet. A considerable amount of colourless crystalline material separated in the Soxhlet flask (= fraction I) and the ether mother-liquors on removing the ether and drying gave a semi-crystalline solid (= fraction II). The ether-insoluble material (III) consisted of salts and was not further investigated.

(*b*) The *mother-liquor* (now about 700 cc.) was then adjusted to p_H 7 (by addition of about 40–60 cc. *N* NaOH) and extracted four times with an equal volume of ether. The extracted material, after removing the ether and drying to constant weight gave fraction (IV). The aqueous mother-liquor was then adjusted to p_H 5·3 (using 20–50 cc. *N* HCl) and extracted twice with an equal volume of ether. The extracted material after removing the ether and drying gave fraction (V). Finally the aqueous mother-liquor was made acid to Congo red (by addition of 25–30 cc. conc. HCl) and extracted (*a*) four times with an equal volume of ether, the extracted material giving a red colour with $FeCl_3$ (fraction VI), (*b*) three further times with ether, the material so extracted giving a brown colour with $FeCl_3$ (fraction VII). The dry weights of all these fractions are summarised in Table III. In those cases where more than one experiment was carried out with the same organism the weights for all the experiments are added together.

Table III.

Dry weights (g.) of fractions I–VII for the various species.

Organism	No. of flasks	I	II	IV	V	VI	VII
P 75	1100	52·73	13·44	76·16	71·36	364·94	42·45
M 3 (1)	90	6·22	2·08	7·65	4·70	27·01	0·67
M 3 (3)	360	47·96	8·28	17·94	9·08	47·54	4·24
M 3 (4)	90	7·90	1·38	5·78	1·21	9·50	0·54
P 151	90	8·46	3·37	2·12	1·65	9·63	
A 11	90	6·20	1·09	3·64	—	18·50	0·55
S 30	90	4·69	4·49	4·27	2·51	34·30	
P 84	90	3·16	1·05	1·95	2·09	2·50	
P 99	90	4·02	0·87	1·50	1·77	21·25	0·95
D 8	180	0·87	0·71	10·80	2·04	32·89	0·65
P 90	90	0	0·34	3·30	1·12	2·45	1·42
P 37	90	0	1·88	2·07	1·57	12·13	0·76
Ad 89	80	0	0·62	—	—	39·35	4·80
Ad 87	90	0	1·86	—	—	2·37	0·62
P 69	90	0	1·50	6·27	—	16·09	
P 75 (Czapek-Dox) 4570 cc.	—	0	0·03	0·85	0·90	1·93	

Since 10 flasks contained 175 g. of sugar at the beginning of the experiment and the flasks were taken off when the sugar had disappeared, the yields of these fractions in relation to the sugar utilised can readily be computed from the above figures.

Isolation of products from fractions I–VII.

Fraction I consists of almost pure mycophenolic acid; fractions II and IV are grossly impure mycophenolic acid and were worked up for this product by taking advantage of the insolubility of its dipotassium salt in alcohol; fractions V, VI and VII contain only traces of mycophenolic acid, and consist of a series of products of lower molecular weight, $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ and $C_8H_6O_6$.

A. *Isolation and yield of mycophenolic acid.* In the early experiments the organism P 75 was always used and the mycophenolic acid fraction I isolated. Fraction I in all cases was practically pure mycophenolic acid, and one or two recrystallisations from boiling water or aqueous alcohol yielded a pure product, M.P. 141°, giving with ferric chloride a pure blue colour in alcohol, and a blue-violet colour in water, and corresponding exactly in properties and analysis with Alsberg and Black's mycophenolic acid. The acid obtained using the other organisms was in each case shown to be identical in properties and mixed M.P. with the P 75 product.

The mycophenolic acid of fractions II and IV was separated as the dipotassium salt. The method was tested quantitatively with a sample of P 75 fraction I. 1 g. of this fraction was dissolved in 15 cc. absolute alcohol, 6 cc. of 10 % alcoholic KOH were added and the mixture was allowed to stand 20 minutes. The precipitated dipotassium salt was filtered off, washed with absolute alcohol, dissolved in water and the free acid precipitated by addition of $2N$ H_2SO_4. After the acid had solidified, it was filtered off, washed and dried; wt. 0·95 g. Fractions II and IV in each experiment were therefore dissolved in a small amount of alcohol and the mycophenolic acid was separated in the above way. The acid so obtained after one recrystallisation from aqueous alcohol followed by one recrystallisation from boiling water was practically pure. The figures for the total yield of mycophenolic acid obtained (Table IV)

Table IV.

Organism	No. of flasks	Yield (g.) of mycophenolic acid isolated from Fraction I	Fraction II	Fraction IV	Total yield (g.) mycophenolic acid
P 75	1100	52·73	7·86	11·53	72·12
M 3 (1)	90	6·22	0·49	2·67	9·38
M 3 (3)	360	47·96	1·15	4·58	53·69
M 3 (4)	90	7·90	0·34	2·10	10·34
P 151	90	8·46	0·78	Trace	9·24
A 11	90	6·20	0·29	1·05	7·54
S 30	90	4·69	1·30	1·00	6·99
P 84	90	3·16	0·55	0·93	4·64
P 99	90	4·02	0·39	0·12	4·53
D 8	180	0·87	0·10	3·83	4·80
P 90	90	0·0	0·05	2·12	2·17
P 37	90	0·0	0·98	0·80	1·78
Ad 89	80	0·0	0·0	0·0	0·0
Ad 87	90	0·0	0·0	0·0	0·0
P 69	90	0·0	0·0	0·0	0·0
P 75 (Czapek-Dox)	—	—	—	0·49	0·49

consist of the combined weights of fraction I, together with those of the weights of acid recovered from the crude fractions II and IV.

Two samples of fraction V were also worked up by the alcoholic KOH method but were shown to contain only traces of mycophenolic acid, and this fraction was therefore worked up in the same way as fractions VI and VII.

Finally a small amount of material obtained by growing P 75 on tubes of Czapek-Dox instead of Raulin-Thom's medium was worked up by the alcoholic KOH method and shown to contain mycophenolic acid.

B. *Isolation and yield of the products* $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$, $C_8H_6O_6$. The material obtained in fractions V, VI and VII by the growth of the various organisms was first dried to constant weight *in vacuo* over sulphuric acid, then finely powdered and well mixed and aliquot portions used (*a*) for preliminary estimations, and (*b*) for the isolation of the metabolic products.

(*a*) *Preliminary estimations.* These determinations included the methoxyl content, proportion insoluble in water, a rough equivalent, optical activity and a rough determination of the dry weight of the precipitate obtained with Brady's 2:4-dinitrophenylhydrazine reagent (D.N.P.). The first two estimations should give an indication of the amount of mycophenolic acid still present since this substance is the only one possessing a methoxyl group and is almost insoluble in cold water. The fraction from Ad 89, however, did contain some methoxyl, but no mycophenolic acid could be isolated.

The equivalent would only be of value if the product had contained a large amount of $C_8H_6O_6$, a condition which was never obtained. The optical activity of all fractions was always zero. The substances $C_{10}H_{10}O_5$ and $C_{10}H_{10}O_7$ give precipitates with 2:4-dinitrophenylhydrazine, whilst the products $C_{10}H_{10}O_6$ and $C_8H_6O_6$ give no such precipitate in dilute solution. Hence the weight of the D.N.P. fraction is a measure of the combined amounts of $C_{10}H_{10}O_5$ and $C_{10}H_{10}O_7$ present.

Table V. *Results of preliminary experiments.*

Organism	Fraction	Methoxyl %	Insol. in 400 parts H_2O %	Wt. D.N.P. from 1 g. of fraction and its M.P. (decomp.)
P 75 (batch 10)	VI	1·62	5·3	0·88 (199–201°)
P 75 (batches 1–8)	VII	—	3·3	0·55 (197°)
M 3 (1)	VI	1·90	3·5	0·90 (200–207°)
M 3 (3)	VI	—	6·0	0·91 (207°)
M 3 (4)	VI	—	25·0	0·68 (206°)
P 151	VI + VII	—	1·9	1·02 (202–205°)
A 11	VI	1·80	24·0	0·73 (197–203°)
S 30	VI + VII	2·40	0·0	1·03 (206–211°)
P 84	VI	—	—	(199–204°)
P 99	V + VI + VII	1·3	3·0	0·96 (202–206°)
D 8 (batches 1 + 2)	VI	1·0	0·0	0·88 (195–205°)
P 90	V + VI + VII	—	37·0	0·66 (205–207°)
P 37	VI + VII	—	4·3	1·19 (206–208°)
Ad 89	VI + VII	1·5	4·6	0·70 (200–202°)
Ad 87	VI + VII	—	—	(194–200°)
P 75 (Czapek-Dox tubes)	V + VI + VII	—	—	(190–202°)
P 75 + M 3 (3) + D 8 + P 37 + M 3 (4) + M 3 (1) + P 84 + P 151 + S 30	V	—	—	0·67 (204–206°)

(*b*) *Method of separation of the fractions of crude* $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$, $C_8H_6O_6$. The bulk (a known aliquot) of the dried fraction (see Table VI) was dissolved in purified ether (26 cc. per g.), filtered from a small amount of amorphous material and to 13 parts of the boiling ethereal solution 14 parts of boiling light petroleum (B.P. 40–50°) were added. After maintaining at the boil for a minute or two, a small amount of precipitated tar was removed by filtering through two layers of fluted filter-paper into a tared round flask. The solvent was then removed, the last traces by heating *in vacuo* for 30 minutes at 50°. The residue, a yellow or pale brown gum, was dissolved in acetone (1·2 cc. solvent per g. of gum) and to the boiling solution 55 times its volume of boiling chloroform was added, the mixture shaken and put aside to crystallise overnight. In the earlier experiments, methyl alcohol was employed instead of acetone, but the use of this solvent was later avoided as it led to partial esterification. The greyish crystalline deposit (1st crop, crude $C_{10}H_{10}O_6$) was then collected, washed with a little chloroform, weighed, and its M.P. taken.

Table VI. *Dry weights of isolated fractions and isolated crystalline substances.*

Organism	Fraction used	$C_{10}H_{10}O_5$ + $C_{10}H_{10}O_7$ g.	$C_{10}H_{10}O_6$ g.	$C_8H_6O_6$ g.	$C_{17}H_{20}O_6$ from fractions I, II, IV g.	Total isolated crystalline sub-stances g.	Total dry wt. of fractions from which isolated g.
P 75	VI + VII	166·90	28·55	4·00	72·12	271·57	549·72
M 3 (1)	VI + VII	10·10	3·72	0·50	9·38	23·70	43·63
M 3 (3)	VI + VII	23·70	4·06	0·10	53·69	81·55	125·96
M 3 (4)	VI + VII	2·67	0·53	0·00	10·34	13·54	25·10
P 151	VI + VII	3·95	0·16	0·03	9·24	13·38	23·58
A 11	VI + VII	7·48	2·48	0·16	7·54	17·66	29·98
S 30	VI + VII	15·52	2·11	0·46	6·99	25·08	47·75
P 84	VI + VII	0·18	Trace	*	4·64	4·82	8·66
P 99	V + VI + VII	7·46	3·41	0·04	4·53	15·44	30·36
D 8	VI + VII	12·63	7·23	0·11	4·80	24·77	45·92
P 90	V + VI + VII	0·81	0·11	*	2·17	3·09	8·63
P 37	VI + VII	5·43	0·54	0·09	1·78	7·84	16·84
Ad 89	VI + VII	16·00	5·62	0·34	0·00	21·96	44·77
Ad 87	VI + VII	0·04	Trace	*	0·00	0·04	4·85
P 69	VI + VII	0·00	0·00	0·00	0·00	0·00	23·86
P 75 (Czapek-Dox)	V + VI + VII	0·23	0·25	0·00	0·49	0·97	3·71
P 75, M 3 (1), M 3 (3), M 3 (4), P 37, D 8, P 84, P 151, S 30	V	24·15	4·24	0·05	—	28·44	96·21

* In these cases the total amount of lead precipitate was so small that it would have been impossible to isolate the substance $C_8H_6O_6$ even if present.

The acetone-chloroform mother-liquor was then evaporated *in vacuo* at 50° in stages to $\frac{1}{2}$, $\frac{1}{5}$, $\frac{1}{12}$ of the original volume and finally to dryness. After each evaporation (except the last) the solution was allowed to stand 2 days for complete deposition and in this way crops 2, 3, 4 and 5 were obtained.

The second crop (relatively small) consisted mostly of $C_{10}H_{10}O_6$, and was dissolved in acetone and treated with 55 volumes of chloroform, the crystalline deposit being added to crop 1, and the residue by evaporation of the mother-liquor to crop 3. The third crop (relatively large) was then dissolved in 15 times its weight of cold water, filtering from a little tar if necessary, and *N* NaOH added (volume noted) until neutrality to litmus paper as external indicator was reached. Three times this volume of *N*/2 neutral lead acetate was then immediately added, the flask well shaken and allowed to stand overnight. The lead precipitate was then filtered off, dried *in vacuo* over sulphuric acid and weighed. The mother-liquor was acidified with dilute H_2SO_4, filtered from lead sulphate and extracted 5 times with half its volume of ether. The ethereal extract was dried over anhydrous Na_2SO_4 and evaporated at 50–60°. When distillation of ether had practically stopped, the residue which contained a considerable amount of acetic acid was poured into 50 volumes of light petroleum and the distilling flask washed out with a few cc. of ether. After standing for a few days, the precipitate (usually completely crystalline) was collected, washed with a little light petroleum, dried, weighed and its behaviour on heating carefully noted. The lead precipitate was then ground in a mortar with dilute (2*N*) sulphuric acid, filtered from lead sulphate, the filtrate extracted with ether and the ethereal extract worked up as above, the final product being usually a brownish gum. The crystalline fraction from the soluble lead salts consisted of a mixture of $C_{10}H_{10}O_7$ and $C_{10}H_{10}O_5$, and a separation was effected by fractional crystallisation from ethyl acetate-light petroleum, or chloroform-light petroleum. The gum from the lead precipitate was shown to contain the substance $C_8H_6O_6$, a considerable amount of tar, all the remaining $C_{10}H_{10}O_6$ and also amounts of $C_{10}H_{10}O_7$ and $C_{10}H_{10}O_5$, evidently carried down during the precipitation since the lead salts of the pure substances are readily soluble in cold water.

The fourth crop (relatively small) was similar in composition to crop 3 and was worked up similarly.

The fifth crop (final residue) contained nearly all the mycophenolic acid remaining, and also other tarry material insoluble in water. It was therefore extracted first with 25 times and then with 8 times its weight of cold water and the aqueous extracts filtered. The filtrates were worked up as with crop 3 and the residue on the filter was shown by crystallisation from chloroform and light petroleum to contain a small amount of mycophenolic acid.

The method was often further simplified by evaporating the acetone-chloroform mother-liquor in two stages only, first to one-fifth of the original volume and then to dryness. If the M.P. of the second crop showed it to be mostly $C_{10}H_{10}O_6$ it was added to the first crop; if the M.P. showed it to be mostly $C_{10}H_{10}O_7$, it was worked up as above.

The isolation of the substance $C_8H_6O_6$ was always difficult, partly because of the smallness in amount, but chiefly because of the impossibility of direct crystallisation from the gum recovered from the first lead precipitate. In

most instances, the material was put through a second lead acetate treatment, which led to enrichment in $C_8H_6O_6$. It was also found advantageous to use only the lead precipitate from the final residue.

The tar thrown down in the first stage of this separation was examined in the case of P 75 and shown to contain a little $C_8H_6O_6$ and $C_{10}H_{10}O_7$. It was further observed that if the crude fraction contains an abnormally large amount of $C_{10}H_{10}O_6$, the amount of acetone indicated is insufficient for complete solution, in which case more acetone is used to bring all the material into solution.

The total yield of these fractions is given in Table VI. In each case, the materials were isolated from the bulk (a known aliquot) of the fraction, and the actual figures obtained have been corrected so as to refer to the whole fraction. The weight of mycophenolic acid isolated is also given, and the total weight of isolated crystalline products is compared with the dry weight of the fractions from which they were derived.

Properties of the crystalline products.

I. *Mycophenolic acid.* This acid is almost insoluble in cold water, but crystallises beautifully in radiating needles from boiling water. Thus 5 g. of P 75 fraction I, M.P. 137–139°, on recrystallising from 3 litres of boiling water, gave 4·40 g. of dry acid, M.P. 141°. With ferric chloride it gave a pure blue colour in alcoholic solution (Alsberg and Black say a green colour) and a blue-violet colour in aqueous solution. It gives a heavy precipitate with bromine water but no reaction with bleaching powder solution. It does not reduce Fehling's solution or ammoniacal silver nitrate but is oxidised by permanganate immediately in the cold and is readily reduced catalytically by palladium and hydrogen at ordinary pressures and temperatures. It is very stable to boiling alkalis and acids. Mycophenolic acid dissolved in cold H_2SO_4 does not give a blue fluorescence in ultra-violet light. It is a very weak acid, being extracted almost completely at p_H 7, but titrates as a dibasic acid. It is optically inactive. The lead, copper and silver salts are insoluble in water and the acid may be readily separated by the insolubility of its dipotassium salt in alcohol. It contains a methoxyl group.

The following results were obtained by micro-combustion (Schoeller): C, 63·85, 63·80 %; H, 6·36, 6·33 %; OCH_3, 9·65, 9·36 %; mol. wt., 337, 341. $C_{17}H_{20}O_6$ requires C, 63·73 %; H, 6·30 %; OCH_3, 9·69 %; mol. wt., 320·2.

Also, 0·093 g. mycophenolic acid requires 5·49 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 169. (Theoretical for $C_{17}H_{20}O_6$ titrating as a dibasic acid, 160.)

II. *The substance $C_{10}H_{10}O_6$.* This substance is obtained pure by two recrystallisations from 15 parts of water of the first crop from acetone-chloroform. It forms colourless flat rhombs melting over the range 193–203° with softening, darkening and vigorous gas evolution at the latter temperature.

Micro-analysis (Schoeller): C, 52·95, 53·11 %; H, 4·72, 4·45 %; OCH_3, nil; mol. wt., 211. $C_{10}H_{10}O_6$ requires C, 53·07 %; H, 4·46 %; mol. wt., 226.

0·1057 g. required 7·5 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 141, but the end-point was very indefinite, the solution becoming brown. The substance is moderately soluble in cold ethyl and methyl alcohols and acetone, readily soluble in cold ethyl acetate, very sparingly soluble in chloroform. The lead, mercury and silver salts are sparingly soluble in water, whilst a solution of the sodium salt reduces copper sulphate in the cold in a few moments. The calcium and barium salts are readily soluble in water and the calcium salt is not precipitated by alcohol. With ferric chloride, the substance gives a stable reddish-purple colour in alcohol and a much redder colour in water, which fades in an hour.

With bromine water it gives a heavy precipitate. With concentrated sulphuric acid the substance on gently warming gives a series of colour changes from yellow-green to yellow-brown, red, deep port-wine colour, the colour being discharged on diluting with water. In dilute solution it does not give an immediate precipitate with 2:4-dinitrophenylhydrazine in 2*N* HCl. Permanganate is immediately decolorised in the cold, and bleaching powder solution gives an orange-red colour fading to yellow. The substance gives a pale green solution in cold concentrated H_2SO_4 which fluoresces blue in ultra-violet light. The colour gradually fades but the fluorescence remains.

III. *The substance* $C_{10}H_{10}O_7$. This substance along with the substance $C_{10}H_{10}O_5$ forms the fraction isolated from the soluble lead salts and is readily separated from the mixture by fractional crystallisation from ethyl acetate-light petroleum, or chloroform-light petroleum, the substance $C_{10}H_{10}O_5$ having the greater solubility. The substance $C_{10}H_{10}O_7$ melts at 125–135° with effervescence due to the loss of $1H_2O$.

Micro-analysis (Schoeller): C, 49·58, 49·51 %; H, 4·28, 4·15 %; OCH_3, nil; loss at 130–145°, 7·8 % (7·1 % H_2O, 0·7 % CO_2).

$C_{10}H_{10}O_7$ requires C, 49·57 %; H, 4·17 %; loss $1H_2O$, 7·4 %. 0·0951 g. required 7·15 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 133, but the end-point was again indefinite, the solution becoming brown. The dehydration product obtained by heating at 130–145° melts at 166–168°, but has not yet been satisfactorily obtained crystalline. When this product is crystallised from ordinary A.R. chloroform and light petroleum, it is obtained in well-defined crystals of a partially hydrated form, M.P. 166–168° (with previous softening at 116°) which analysed for $C_{10}H_8O_6$, 0·8 H_2O.

Micro-analysis (Schoeller): C, 50·27, 50·22 %; H, 4·19, 4·14 %; mol. wt., 206, 198. $C_{10}H_8O_6$ 0·8 H_2O requires C, 50·31 %; H, 4·05 %; mol. wt., 238.

When the product is crystallised from water, the original substance $C_{10}H_{10}O_7$ is obtained.

The substance $C_{10}H_{10}O_7$ is readily soluble in methyl and ethyl alcohols, ethyl acetate and acetone, fairly readily soluble in cold water, moderately soluble in cold chloroform and sparingly soluble in light petroleum. It crystallises from chloroform-light petroleum in well-formed diamond-shaped crystals. The lead salt is soluble in cold water, the silver salt sparingly soluble in cold, readily in hot water. The colour given with ferric chloride in alcoholic solution is indistinguishable from that with the substance $C_{10}H_{10}O_6$ under the same conditions, but differs from the latter in being stable and intensely purple-red in aqueous solution. The substance gives a heavy precipitate with bromine water and an immediate copious yellow crystalline precipitate with 2:4-dinitrophenylhydrazine in 2*N* HCl, but does not restore the colour to Schiff's reagent. It gives a pure, fairly stable, deep orange colour with bleaching powder solution.

With concentrated H_2SO_4 the colour reaction is very similar to that given by the substance $C_{10}H_{10}O_6$, except that it is somewhat more intense and more quickly developed on warming. Both substances give a brown colour on standing in the cold with H_2SO_4 containing a little $K_2Cr_2O_7$. With sufficient dichromate, the colour becomes green on warming. No coloration is observed, even on warming, with H_2O_2 in glacial acetic acid. The substance $C_{10}H_{10}O_7$ is more sensitive to alkali than the substance $C_{10}H_{10}O_6$. It dissolves in cold concentrated H_2SO_4 giving a pale yellow solution, but no fluorescence was obtained in ultra-violet light.

IV. *The substance* $C_{10}H_{10}O_5$. This substance can be distinguished from the substance $C_{10}H_{10}O_7$ by the fact that it first melts from 145 to 153° with effervescence and loss of water (and some CO_2), then almost immediately resets and again melts at 220–230° (decomp.), and also by its separation from water in large diamond-shaped crystals, whereas the substance $C_{10}H_{10}O_7$ separates from water in irregular aggregates of small crystals. Its separation from the substance $C_{10}H_{10}O_7$ is rendered difficult, not only by the similar solubilities of the substances and their salts, but also by the fact that a mixture containing but little $C_{10}H_{10}O_5$ behaves on melting very like the pure substance, the melt quickly resetting and remelting above 200°. The readiest separation is effected by fractional crystallisation from water.

The substance $C_{10}H_{10}O_5$ loses the elements of water on heating to give an entirely new substance $C_{10}H_8O_4$, which does not revert to the parent material on recrystallisation from water, as does the dehydration product of $C_{10}H_{10}O_7$.

Micro-analysis (Schoeller): C, 56·84, 57·28, 56·64 %; H, 4·64, 4·98, 4·68 %; loss at 150–170°, 9·4 % (7·3 % H_2O, 2·1 % CO_2).

$C_{10}H_{10}O_5$ requires C, 57·14 %; H, 4·76 %; loss of $1H_2O$, 8·6 %. 0·1703 g. required 12·75 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 134, but here again, as with the substances $C_{10}H_{10}O_7$ and $C_{10}H_{10}O_6$, the end-point was indefinite, the solution becoming brown.

With ferric chloride in aqueous or alcoholic solution, the substance behaves similarly to the substance $C_{10}H_{10}O_7$, the colour being perhaps a little more purple in shade. In alcohol, the colour with the substance $C_{10}H_{10}O_5$ is, however, markedly less stable. With bromine water it gives a heavy precipitate.

Like the substance $C_{10}H_{10}O_7$, this compound gives an immediate copious precipitate with 2:4-dinitrophenylhydrazine in 2*N* HCl.

With bleaching powder solution a deep brownish-red colour is obtained, accompanied by formation of a precipitate.

With concentrated H_2SO_4, there is no coloration in the cold, but on warming a pale permanganate-purple colour develops, distinct from the brownish-red given by the substance $C_{10}H_{10}O_7$. The solution in cold H_2SO_4 shows a blue fluorescence in ultra-violet light.

The substance $C_{10}H_8O_4$ is formed, as previously stated, by heating the substance $C_{10}H_{10}O_5$ at 150–170°, and after crystallisation from water, gave the following figures.

Micro-analysis (Schoeller): C, 62·61, 62·81 %; H, 4·29, 4·30 %; OCH_3, nil; $C_{10}H_8O_4$ requires C, 62·50; H, 4·20 %.

0·0530 g. required 2·70 cc. *N*/10 NaOH for neutralisation to phenolphthalein (sharp end-point) corresponding to an equivalent of 196. Theoretical for $C_{10}H_8O_4$ titrating as a monobasic acid, 192. On acidifying the titrated solution, the compound was recovered unchanged.

The substance $C_{10}H_8O_4$ differs from the substance $C_{10}H_{10}O_5$ in its sparing solubility in cold water and chloroform, and can be recrystallised from boiling water. It forms glistening platelets, M.P. 246–250°, with darkening after 230°. It gives a stable deep purple colour with $FeCl_3$ in alcoholic solution and gives no precipitate with 2:4-dinitrophenylhydrazine in 2*N* HCl. With concentrated H_2SO_4 it does not give a colour on gently warming, and with bleaching powder solution it gives a transient orange-red colour. The solution in cold H_2SO_4 shows a blue fluorescence in ultra-violet light.

V. *The substance* $C_8H_6O_6$. This substance was isolated in small yield after a long series of crystallisations from ethyl acetate-light petroleum, carried out on the crude material isolated from the lead precipitate. It crystallises in well-formed hexagonal tablets with slow, and in flint-shaped leaflets with rapid, crystallisation. It melts at 188–190° with effervescence, resets and remelts at 206–210°.

Micro-analysis (Schoeller): C, 48·56, 48·56 %; H, 3·11, 3·18 %; OCH_3, nil; mol. wt., 174, 166. $C_8H_6O_6$ requires C, 48·48 %; H, 3·05 %; mol. wt., 198.

0·1283 g. required 13·68 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 94 (theoretical for $C_8H_6O_6$ titrating as a dibasic acid, 99). The substance was recovered unchanged by acidifying the titrated solution.

It is readily soluble in cold water and in most organic solvents except light petroleum and benzene. With aqueous or alcoholic ferric chloride it gives a stable deep red colour with perhaps a trace of purple. With dilute ferric chloride and dilute alcoholic solutions the colour is brown, but with dilute aqueous solutions it is reddish-purple. It gives no precipitate with bromine water. With bleaching powder solution and a dilute solution of the acid in water, a transient magenta colour is obtained. It does not give a blue fluorescence in H_2SO_4. No precipitate is obtained with 2:4-dinitrophenylhydrazine in 2*N* HCl.

At its first melting-point the substance (per mol.) loses 0·73 mol. H_2O and 0·37 mol. CO_2.

Growth of the organisms on Czapek-Dox solution.

Although in tube experiments, growth of these organisms and the formation of products (as shown by colour reactions) proceeded satisfactorily, greater difficulty was experienced in obtaining the colour reactions on Czapek-Dox solution in flasks than with Raulin-Thom's solution in flasks. As the latter

medium contains, in addition to glucose, considerable amounts of tartaric acid, it was necessary to decide whether the metabolic products arise from tartaric acid, or are true metabolism products of glucose. An experiment on Czapek-Dox solution (glucose being the only source of carbon) was therefore carried out in tubes. 699 tubes, each containing 10 cc. of sterlised Czapek-Dox solution, were sown with a loopful of a spore suspension of *P. brevi-compactum* Dierckx (P 75) and were incubated at 24°. The tubes were periodically tested and after 30 days were taken off. After filtration, 4570 cc. of filtrate, brownish-yellow in colour and showing a green fluorescence, were obtained. The residual glucose by polarimeter was 0·07 %, the bromine absorption 2·20 mg. per cc., the p_H 8·2, and the solution gave a plum colour with ferric chloride. The solution was then evaporated *in vacuo* and worked up as with the routine experiments. The three metabolism products, $C_{17}H_{20}O_6$ (mycophenolic acid), $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$, were shown to be present, and the failure to detect the substances $C_{10}H_{10}O_5$ and $C_8H_6O_6$ was probably due to the smallness of the amount of product. The products $C_{17}H_{20}O_6$, $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$ are therefore certainly, and the products $C_{10}H_{10}O_5$ and $C_8H_6O_6$ are probably, metabolic products of glucose and the presence of tartaric acid is not essential for their formation.

Discussion.

In a series of recent publications [Raistrick *et al.*, 1931] it was repeatedly found in quantitative examination by the carbon balance sheet method of the types of products formed from glucose by large numbers of species and genera, that each group of organisms has its own peculiar biochemical characteristics, while the different species in each group have similar characteristics. The grouping of the species agreed well with the grouping adopted by Thom and Church on morphological grounds.

The present paper may be regarded as the first serious attempt to test the truth of this conclusion in a detailed way, by following various chemical changes during metabolism and by investigation of the chemical nature of a number of the products of metabolism, using as a test series the group of organisms forming the *P. brevi-compactum* series.

The close biochemical relationship of the organisms dealt with has been amply verified. Thus there is a close similarity in the progress of the metabolic processes of the various organisms, *e.g.* in the rate of disappearance of glucose, the increase in bromine absorption of the metabolism fluids, the change in p_H and in the series of colours given by the fluids with ferric chloride after varying periods of incubation. More particularly this relationship is seen in the isolation from almost all the metabolism solutions of a series of products, $C_{17}H_{20}O_6$, $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ and $C_8H_6O_6$, the amounts varying with different organisms.

It is of interest in this connection to note that the total amount of mycophenolic acid isolated seems to vary with the length of time during which

the culture has been kept in artificial cultivation. Thus the largest yields of mycophenolic acid were obtained with the organisms M 3 (1), M 3 (3), M 3 (4) and P 151. Of these, the first three were freshly isolated from spoiled Italian maize, the fourth being received from Prof. Biourge, its history being unknown to us. Alsberg and Black [1913] report that, while a culture of *P. stoloniferum* freshly isolated from Italian maize produced considerable amounts of mycophenolic acid, Dr Thom's type culture of this same organism, which had been kept in artificial cultivation for a considerable time, failed to produce any mycophenolic acid, though in its place was found quite a different substance or mixture of substances, the nature of which was not further investigated. We have had the same experience. Thus our culture, Ad 89, which was obtained through the courtesy of Dr Charles Thom, is the one which was used by Alsberg and Black [1913] for the preparation of mycophenolic acid, and yet in our hands it did not give a trace of mycophenolic acid, but did give the largest yield of the products of lower molecular weight. It seems obvious, therefore, that after almost 20 years in artificial cultivation, this culture had also lost its power of producing mycophenolic acid. Unfortunately, with the remaining cultures, their history is not sufficiently well known to say how long they have been kept in artificial cultivation, although many of them have actually been kept for some time, but the above facts suggest that the power of the organism to produce mycophenolic acid is gradually lost during cultivation over long periods on artificial media.

Moreover, the product mycophenolic acid is not known to be produced by organisms of any other group or series and may therefore be regarded as specifically obtained from organisms of the *P. brevi-compactum* series.

Investigation of the structural relationships of all the various products is now in hand, and it appears probable that their interrelationship will be established and further work may indicate the order of their appearance. In this connection it is interesting to note, from the examination of the series of colours given by the metabolism medium with ferric chloride, that the production of mycophenolic acid takes place almost certainly in the later stages of the metabolic processes.

Summary.

The metabolism of 15 species or strains in the *Penicillium brevi-compactum* Dierckx series has been examined, and mycophenolic acid $C_{17}H_{20}O_6$, together with the following new mould metabolic products $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ and $C_8H_6O_6$ have been isolated. All these products in varying amounts are given by 12 of these species. Two of the three remaining organisms, strains of *P. stoloniferum* Thom and *P. Biourgeianum* Zaleski, although giving no mycophenolic acid, gave in the first case considerable amounts, and in the second case small amounts of the other metabolic products, whilst the third species, *P. aurantio-griseum* Dierckx var. *Poznaniensis* Zaleski gave none of any of these products.

The best yields of mycophenolic acid are obtained using cultures freshly isolated from natural sources, and such cultures, after keeping over long periods (20 years) in artificial cultivation, tend to lose their power to form mycophenolic acid, but do not lose their power to form the products of smaller molecular weight.

The grouping on morphological grounds of these species in one series is therefore supported by their biochemical characteristics. Thus mycophenolic acid is produced, so far as is known, only by organisms of this series, and is obtained with 12 of the 15 organisms available. Moreover, during the metabolism of these strains, the rate of disappearance of sugar and the changes of p_H, of bromine absorption and of colour reaction with ferric chloride, all show great similarity, the only exception being *P. aurantio-griseum* Dierckx var. *Poznaniensis* Zaleski.

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