LXXI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XLII. THE METABOLIC PRODUCTS OF *ASPERGILLUS TERREUS* THOM. A NEW MOULD METABOLIC PRODUCT—TERREIN.

BY HAROLD RAISTRICK AND GEORGE SMITH.

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.

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THE name *Aspergillus terreus* is used by Thom and Church [1918; 1926] to cover a series of strains which have a number of well-marked morphological features in common, but which show a certain amount of variation in colour and type of growth. The conidial colour may range from pale cinnamon to fairly deep brown; the texture of the surface growth may vary from smooth velvety to definitely floccose, whilst the reverse of the colony and the agar may vary in colour from pale or bright yellow to fairly dark brown. Thom and Church state that *A. terreus* is common in soil and decaying vegetable matter throughout the United States, but has not been received by them from any European source. Smith [1928] found this organism as a latent infection of cotton yarns, occurring fairly often on American cottons and very frequently on all types of Egyptian cottons; also as a cause of serious damage to exported cloths, particularly dhooties.

The present communication is an account of experiments which have been carried out with five strains of *A. terreus*, obtained from different sources and covering approximately the range of variation shown by different strains in the series. When grown at 24° on the well-known Czapek-Dox solution with 5 % of glucose as the sole source of carbon, two of the strains, No. 45 and Ac 100, produce in the solution a new mould metabolic product which we have named *terrein* ($C_8H_{10}O_3$, M.P. 127°).[1] The yield of terrein appears to reach a maximum when the sugar in solution has been reduced to approximately 1 %. Succinic acid has also been identified as a metabolic product of No. 45, but is formed in small amounts only. From the metabolism solutions of the remaining three strains (No. 3, No. 37 and Ac 24) no terrein has been isolated but only small amounts of oxalic acid, succinic acid or both. The strain Ac 100 is of special interest since it produces, in addition to the new product terrein, appreciable quantities of citrinin, a substance hitherto believed to be formed only by *Penicillium citrinum* Thom [Hetherington and Raistrick, 1931]. The other four strains also give rise to fairly large amounts of substances which, like citrinin, are precipitated by acidification of the metabolism solutions. From none of these acid precipitates has any citrinin been obtained and so far no other substance has been isolated in a state of purity.

[1] At a meeting of the Biochemical Society held at Oxford on May 5th, 1934, a paper was read in which this substance was described as "terreic acid" [Raistrick and Smith, 1934]. It has since been found that a highly purified sample has p_H 6·8 in 1 % aqueous solution, and work on the constitution of terrein, at present in progress, has shown that the substance does not contain a true acidic group. Hence the change of name.

Thom and Church [1918] state "With the accumulation of material however, we find ourselves with a series of related strains (of *A. terreus*) rather than a single organism....Whether some of them may ultimately be separated as varieties, upon physiological grounds, is not determined". We suggest that if as the result of future research the *A. terreus* series be subdivided into a number of named species, the production of terrein when grown on Czapek-Dox glucose medium at 24° be diagnostic for one of these named species.

Experimental.

The organisms.

Relevant details of the five strains of *A. terreus* used in this investigation are given in Table I.

Table I.

Catalogue no.	Source	Isolated by	Cultures on Czapek-Dox agar: Surface colour	Reverse colour	Texture
No. 3	Mildewed cloth	G. Smith 1926	Cinnamon	Deep yellow	Smooth velvety
No. 37	Egyptian cotton	G. Smith 1926	Cinnamon	Greenish yellow	Smooth velvety
No. 45	American cotton	G. Smith 1926	Brownish	Brown	Slightly floccose
Ac 24	British National Collection of Type Cultures No. 981	Thom and Church (Washington 144)	Brownish	Deep brown	Slightly floccose
Ac 100	F. T. Brooks, Cambridge	Hansford. Identified by Miss Church	Brown	Deep brown	Definitely floccose

Cultural conditions.

The culture medium used throughout was the well-known Czapek-Dox solution containing: glucose, 50 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·01 g.; distilled water, 1000 ml. Cultures were grown in conical flasks of 1 litre capacity, each containing 350 ml. of medium, and sown, after sterilisation, from cultures on Czapek-Dox agar slopes approximately 2 weeks old, one slope being used to sow 4 flasks. The temperature of incubation was 24°.

The course of metabolism.

Twenty flasks of medium were sown with each of the 5 strains, and single flasks were taken for analysis at intervals of 3 or 4 days. The experiment was continued for 35 days, by which time metabolism appeared to be complete in all cases. The examination included measurement of p_H colorimetrically, estimation of apparent residual glucose by polarimeter, estimation of bromine absorption

Table II.

R.S.P., % apparent residual sugar determined by polarimeter. B.A., bromine absorption in mg. Br per ml. Initial p_H, 4·1; initial glucose, 5·17 %; initial bromine absorption, 0·08 mg. Br per ml.

Days' incubation	No. 3			No. 37			No. 45			Ac 24			Ac 100		
	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	R.S.P.	B.A.
4	5·3	4·72	0·10	6·4	4·82	0·16	6·5	4·68	0·14	5·2	4·57	0·51	6·4	4·74	0·51
7	4·3	3·37	0·43	6·8	3·26	0·35	6·6	4·29	0·64	4·8	3·96	1·74	6·7	4·05	0·83
10	4·8	2·56	0·30	6·9	2·26	0·53	6·4	3·13	1·23	4·5	2·91	1·98	6·8	3·48	1·73
13	4·6	1·32	0·46	6·5	2·15	0·58	6·5	1·23	1·18	4·5	2·28	2·14	5·4?	1·53	1·92
17	5·6	0·36	1·34	6·4	1·35	0·64	6·4	1·44	4·22	5·0	1·26	3·01	6·6?	1·58	2·86
21	5·3	0·44	0·93	6·2	0·64	0·98	7·0	0·77	3·84	5·1	0·81	3·32	6·3?	0·96	4·09
24	6·5	0·31	1·15	6·2	0·28	1·68	6·6	0·69	3·76	5·2	0·69	3·46	6·3?	0·56	3·34
28	7·5	0·05	1·86	6·1	0·22	1·78	7·4	0·32	4·90	—	0·33	4·19	6·5?	0·30	4·41
32	7·3	0·05	2·00	6·5	0·19	1·84	7·8	0·62	5·41	—	—	3·36	—	0·10	3·88
35	8·4	0·05	2·56	7·3	0·15	2·10	7·3	0·23	4·30	—	0·18	3·50	—	0·14	4·77

by Koppeschaar's method [1876] and observation of the effect of adding $FeCl_3$ solution. After 2 weeks' incubation the reactions of the metabolism solutions with various metallic salts were tested. The results of the analyses are given in Table II.

With the strains No. 45, Ac 24 and Ac 100 the metabolism solution gradually became coloured an intense reddish brown. Estimation of p_H was thereby rendered difficult and, in the later stages of incubation, with Ac 24 and Ac 100 was quite impossible. For estimation of residual glucose the solutions from No. 45 and Ac 100 could be cleared by addition of a few drops of HCl and filtration. With Ac 24, however, this treatment did not appreciably lighten the colour and the figures are therefore only approximate.

The figures show that with all the strains there was some irregularity in growth, so that single flasks, though selected to be as representative of the whole as possible, could not be regarded as averages of a whole batch. With No. 3, No. 37, No. 45 and Ac 100 the appearance of the cultures was reasonably uniform. Ac 24 however showed great irregularity. In some flasks the solution remained yellow for a long time and the mycelium was thick, gelatinous and non-sporing. In others the solution quickly became intense brown, with the mycelium thinner, brittle and sporing freely.

The metabolism solutions of No. 3 and No. 37 did not at any stage give an appreciable reaction with $FeCl_3$. With No. 45, Ac 24 and Ac 100, $FeCl_3$ intensified the brown colour of the solutions and, with Ac 100 after 17 days, gave a precipitate. After 13 days' incubation solutions from all the strains reduced $AgNO_3$ rapidly in the cold, gave slight brownish precipitates with $HgCl_2$, $CuSO_4$ and uranium acetate, and heavy precipitates with neutral or basic lead acetate. Acidification gave precipitates in all cases, greatest in amount with No. 45 and Ac 100, and increasing with continued incubation.

Large-scale experiments.

For isolation and identification of products of metabolism each strain was grown in batches of 100 flasks. When the glucose was reduced to the desired figure (about 1 %) as determined by testing single pilot flasks, the combined metabolism solution was poured off and filtered. The mycelium was scraped out of the flasks, well pressed and washed and the filtered squeezings and washings added to the main filtrate. The whole was made slightly acid to Congo red by cautious addition of strong HCl (about 100 ml.) and the precipitate allowed to settle. The clear supernatant liquid was decanted, the residual precipitate filtered, well washed and dried *in vacuo* (fraction I). The supernatant liquid and filtrate were combined and evaporated *in vacuo*, with a bath temperature not exceeding 50°, to approximately 750 ml. Usually a further quantity of flocculent precipitate separated during evaporation, also a certain amount of black tar (fraction II) which has not been further examined. The concentrate was centrifuged (filtration was impracticable) until almost clear and the precipitate added to fraction I. The aqueous solution was exhaustively extracted with equal volumes of ether, 20 to 30 extractions being usually necessary. On evaporation the ether extracts gave fraction III. Only small amounts of syrupy material were obtained by subsequent extraction with $CHCl_3$.

Strain No. 3. Fraction I was a brown powder, weighing 29·4 g. from which no crystalline material has yet been obtained. The ether extracts, fraction III, gave on evaporation small amounts of crystals (1·34 g.) which were identified as oxalic acid by M.P., mixed M.P. and the usual chemical tests. Further evaporation of the ether mother-liquor gave only small amounts of reddish gum.

Strain No. 37. Fraction I was a bright yellow powder weighing 30 g. Extraction with ether gave thick syrups from which no crystalline material has been obtained. Ether extracts, fraction III, gave traces of oxalic acid and appreciable quantities of succinic acid, identified by analysis, M.P. and mixed M.P., amounting in one experiment to 8·5 g.

Strain No. 45. Fraction I was a buff-coloured powder, the weight of which in six successive experiments is given in Table III. It was extracted in a Soxhlet apparatus with several successive lots of ether until nothing further was dissolved, this requiring about 24 hours in all. The extracts, which were dark brown in colour, deposited on standing hard crusts of microcrystalline material which were filtered off and well washed with ether. Evaporation of the mother-liquors gave further amounts of apparently identical material, and was repeated until nothing but a thick, almost black syrup remained. The solid isolated was yellowish buff in colour and had M.P. 193–196° (decomp.). No satisfactory method of purification and no reliable criteria of purity have yet been found.

The ether extracts (30 extractions with an equal volume of ether were carried out) containing fraction III, on evaporation to small volume deposited long needles of crude terrein (fraction III*a*). The first ten ether extracts were combined. They were dark-coloured but gave clean, pale-coloured crystals, whilst extracts 11–30, which were also combined, were much paler but gave brownish crystals mixed with small amounts of sticky syrup. The ether mother-liquors were further evaporated, those from extracts 11–30 giving further small amounts of crude terrein (fraction III*a*), whilst those from extracts 1–10 gave crystals which softened at 115°, became red at 145–150° and finally melted about 170° (fraction III*b*). The crude terrein constituting fraction III*a*, M.P. 124–126°, was recrystallised from ether, in which it is only sparingly soluble, until of constant M.P., 127°. The pure material forms fine colourless needles, which are very difficult to free from the last traces of colour. Fraction III*b* was obviously a mixture, and was shown to consist of terrein (which is readily detected in mixtures on account of its reddening at 145–150°) and succinic acid.

In order to prove the presence of the latter, the mixture was dissolved in a minimum of water and excess of neutral lead acetate added. The precipitate was filtered off, well washed, suspended in water and decomposed by passing H_2S. The PbS was filtered off and the clear aqueous solution evaporated to dryness. The crystalline residue had M.P. 184°, unchanged on mixing with authentic succinic acid. This method does not give a complete separation, although it does result in the isolation of pure succinic acid, since lead succinate is not quantitatively precipitated unless the solution is first neutralised, and neutralisation is undesirable because of the instability of terrein. Terrein, still somewhat impure, was recovered by extraction of the lead acetate mother-liquors with ether.

The yields of products from six batches of Strain No. 45, each of 100 flasks, are given in Table III.

Table III.

Batch no.	Days' incubation	R.S.P. %	Crude precipitate (g.) Fraction I	Pure terrein (g.)	Mixture of terrein and succinic acid (g.)
1	29	0·4	46	8·5	2·76
2	33	0·2	52	9·3	0·31
3	26	0·7	38	13·1	3·70
4	27	0·5	38	13·0	0·54
5	26	0·38	80·5	4·5	1·64
6	23	0·9	31	17·3	2·05

Strain Ac 24. The acid precipitate (fraction I) was a brown powder, weight 22·9 g. The only crystalline product so far obtained from the ether extracts (fraction III) was succinic acid, 2·8 g. from 100 flasks.

Strain Ac 100. The acid precipitate, fraction I, was a yellowish green powder, weight 57·5 g. Extraction with ether gave a dirty yellow, micro-crystalline powder which melted at 170°, and decomposed in a manner characteristic of citrinin. Recrystallised from absolute alcohol it was obtained as long golden yellow needles, M.P. 173° (decomp.), mixed M.P. with an authentic sample of citrinin from *P. citrinum* Thom, 172° (decomp.). In absolute alcohol solution ($c=0·92$), $[\alpha]_{5461}^{23°}-43·1°$. (Hetherington and Raistrick [1931] give for citrinin $[\alpha]_{5461}-41·7°$, $-43·9°$.) Titration in aqueous suspension gave equiv. wt. 245, and in alcoholic solution 254. Estimation of molecular weight by depression of F.P. of dioxan gave 259. (Found (Schoeller): C, 62·36, 62·25; H, 5·59, 5·68 %. $C_{13}H_{14}O_5$ requires C, 62·37; H, 5·64 %. Mol. wt. 250.) A solution of the sodium salt gave on addition of $FeCl_3$ a heavy buff-coloured precipitate, soluble in excess of the reagent to give the clear deep iodine-brown solution characteristic of citrinin.

The statement by Hetherington and Raistrick [1931] that citrinin was apparently produced only by *Penicillium citrinum* Thom and was to be regarded as diagnostic for this organism, was based on the fact that no other of the hundreds of moulds tested at that time gave the typical reaction given by *P. citrinum* when $FeCl_3$ is added to the metabolism solution. Although the strain of *A. terreus* Ac 100 was actually one of the strains examined by Hetherington and Raistrick it is not surprising that citrinin was not detected by its colour reaction with $FeCl_3$, since this is almost entirely masked by the intense colour of the untreated metabolism solution and by simultaneous reactions between $FeCl_3$ and other metabolic products.

Because of the importance of this outstanding exception, the only one encountered in 13 years' work, it seemed desirable to repeat the experiment with *A. terreus* Ac 100 after rigorously re-checking the purity of our cultures. To this end the culture which was used as the starting point for the first 100-flask experiment with Ac 100 was plated out and showed no sign of contamination. Further, single spore cultures were made and these were used to sow the flasks in a second experiment. Citrinin was again obtained. There can thus be no doubt that citrinin is a metabolic product of at least one authentic strain of *A. terreus* Thom.

The yield of citrinin could not be estimated accurately since, as shown by Hetherington and Raistrick, it cannot be recovered quantitatively from very impure crude samples. In the first experiment 5·5 g. of pure material were obtained. In the second experiment, in which the crude acid precipitate was much darker in colour, only 3·5 g. were isolated.

Ether extracts of the metabolism solution, fraction III, gave crude terrein on evaporation. It was purified by recrystallisation from ether until practically colourless and of sharp and constant M.P. In the first experiment referred to above, final glucose 0·50 %, 4·8 g. of pure terrein were obtained. In the second experiment referred to above, final glucose 1 %, 6·1 g. of pure terrein were obtained. No succinic acid was isolated in either experiment.

General properties of terrein.

The substance crystallises from ether or acetone in colourless needles, M.P. 127°. The melt resets instantly on cooling and melts again at the same temperature. On heating to higher temperatures it begins to turn yellow at 145°,

without any other visible change. The colour deepens as the temperature rises, becoming deep red at about 175°, and if held for some time at this temperature the melt does not solidify on cooling.

(Found (Schoeller): sample *ex* ether, C, 62·37, 62·33; H, 6·59, 6·53 %; OCH_3, nil; N, nil. Sample *ex* acetone, C, 62·33; H, 6·46 %. $C_8H_{10}O_3$ requires C, 62·31; H, 6·54 %.)

Mol. wt., by depression of F.P. of dioxan = 157·5. $C_8H_{10}O_3$ requires 154·1.

Optical rotation. In 1 % solution in water $[\alpha]^{20°}_{5461}+185°$, $[\alpha]^{20°}_{5790}+168·5°$. In 2 % aqueous solution $[\alpha]^{21°}_{5461}+183·4°$, $[\alpha]^{21°}_{5790}+167·2°$.

Active hydrogen (Roth). In anisole at 28°, 1·60, 1·67 atoms; at 95°, 1·85, 1·84 atoms; in pyridine at 28°, 2·06 atoms of active hydrogen.

Solubilities at room temperature were estimated approximately by adding the desired solvent from a micro-burette to 0·1 g. of finely powdered substance and shaking vigorously until solution was just complete. Solubilities, as g. substance in 100 ml. of solvent are: water, 11·9; alcohol, 10·5; acetone, 5·75; chloroform, 0·75; ether, 0·32; benzene, 0·02; light petroleum, nil.

Reactions. A 1 % aqueous solution is neutral to litmus and has p_H 6·8. Addition of NaOH turns the solution first yellow, then brown and finally dark brown on standing. The neutral aqueous solution gives no reactions with salts of Ag, Ca, Ba, Hg″, Cu″, Fe‴, U, Ni or Pb. Fehling's solution is reduced instantly in the cold, bromine is rapidly absorbed without giving a precipitate, acid $KMnO_4$ is instantly decolorised, and Brady's reagent (2:4-dinitrophenylhydrazine in 2*N* HCl) produces a turbidity almost immediately and gives a bright red precipitate on standing overnight. An alkaline solution of terrein reduces $AgNO_3$ rapidly in the cold, reduces iodine instantly and immediately discharges the colour of alkaline phenolphthalein. The constitution of this interesting substance will be the subject of a future communication.

SUMMARY.

1. Five strains belonging to the group species *Aspergillus terreus* Thom have been grown at 24° on Czapek-Dox solution with glucose as sole source of carbon, and their metabolic products have been examined.

2. Two of the five strains give rise to a new mould metabolic product *terrein*, $C_8H_{10}O_3$, M.P. 127°, the isolation and properties of which are described.

3. One of these strains produces, in addition to terrein, citrinin, $C_{13}H_{14}O_5$, the crystalline yellow colouring matter which has previously been obtained only as a metabolic product of *Penicillium citrinum* Thom.

4. Of the three strains that do not produce terrein, one gives succinic acid, one oxalic acid, while the third strain gives a mixture of these two acids.

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