CLXXXIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

LXIII. ITACONIC ACID, A METABOLIC PRODUCT OF A STRAIN OF *ASPERGILLUS TERREUS* THOM

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THE study of the metabolic processes of different cinnamon to brown strains in the *Aspergillus terreus* Thom series has formed the subject of a number of communications from these laboratories. Raistrick & Smith [1935] reported that two strains out of five grown on Czapek-Dox solution gave a hitherto undescribed mould metabolic product—terrein, $C_8H_{10}O_3$—which was shown by Clutterbuck *et al.* [1937, 2] to be 4-propenyl-2-hydroxy-3:5-oxido*cyclo*pentane-1-one. One of these two strains gave, in addition to terrein, considerable quantities of citrinin, $C_{13}H_{14}O_5$, a crystalline yellow colouring matter previously reported as a metabolic product of *Penicillium citrinum* Thom by Hetherington & Raistrick [1931]. Of the other three strains of *A. terreus* one gave succinic acid, another oxalic acid, while the third strain gave a mixture of these two acids. In a later communication Raistrick & Smith [1936] showed that one of the five strains previously examined by them, which produced terrein also, metabolized almost the whole of the chloride supplied as KCl in the Czapek-Dox solution and gave rise to two new chlorine-containing metabolic products, geodin $C_{17}H_{12}O_7Cl_2$ and erdin $C_{16}H_{10}O_7Cl_2$, the molecular constitutions of which have been investigated [Clutterbuck *et al.* 1937, 1; Calam *et al.* 1939].

The main purpose of the present communication is to give the results of an investigation of the metabolic products of a recently isolated but indubitable strain of *A. terreus*, L.S.H.T.M. Cat. No. Am. 1. This strain, when grown on Czapek-Dox solution, gives none of the metabolic products described above, nor does it utilize to any appreciable extent any of the KCl present in the medium, but instead it produces considerable quantities of itaconic acid.

This acid has only once previously been reported as a mould metabolic product, nor indeed, so far as we are aware, has it been reported from any other biological source. Kinoshita [1931, 1] described a new species of *Aspergillus A. itaconicus*, which he isolated from the juice of salted plums. This green species, which is quite distinct from *A. terreus*, is exceptional in that it will only grow well on media of high osmotic pressures such as concentrated sugar solutions and produces very large amounts of itaconic acid on media containing KNO_3 as nitrogen source and 25% of sucrose. Smaller yields of itaconic acid are formed on 25% glucose solutions [Kinoshita, 1931, 2]. When *A. itaconicus* was grown on sucrose or glucose solutions containing $CaCO_3$, appreciable amounts of calcium citrate and calcium gluconate were formed in addition to calcium itaconate, and

Kinoshita therefore concluded that the course of the metabolism of sugar by *A. itaconicus* may be represented as

$$\text{Sugar} \longrightarrow \text{gluconic acid} \longrightarrow \text{citric acid} \xrightarrow{-H_2O} \text{aconitic acid} \xrightarrow{-CO_2} \text{itaconic acid},$$

although no aconitic acid could be detected in the metabolism solution.

Our experiments, while giving no clue as to the stages intermediate between glucose and itaconic acid, offer no support for the view that citric acid is an intermediate product in the formation of itaconic acid by our strain of *A. terreus*. Thus in a large scale experiment, with an incubation period of 25 days and using the ordinary Czapek-Dox 5% glucose medium, the amounts of itaconic and citric acids present were estimated at intervals of a few days. The amounts of itaconic acid increased steadily from the fifth day of incubation to the end of the incubation period but at no time during the whole of the experiment could any citric acid be detected by the pentabromoacetone method. At the end of the incubation period, when all the glucose had been metabolized, the metabolism solution was drained as carefully as possible from the mycelium and was replaced in separate flasks by the following series of substrates: (1) Czapek-Dox salts solution without glucose or $NaNO_3$. (2) Czapek-Dox salts solution as in (1) and containing in separate flasks 1% of the following: glucose, citric acid, malic acid, pyruvic acid, acetic acid, malic + acetic acids, pyruvic + acetic acids. Glucose was the only substrate which gave yields of itaconic acid which were substantially larger than those obtained in the Czapek-Dox salts solution control.

Finally, the metabolisms of five other strains of *A. terreus*, three of which have not been examined previously, were investigated. None of these strains utilized any appreciable amount of the KCl supplied in the medium, three strains produced succinic acid, one oxalic acid and one fumaric acid. Amorphous precipitates were produced on acidification of the metabolism solution from all strains at the end of the incubation period, often in considerable amounts, but in no case could any crystalline substance be isolated from these precipitates.

Experimental

History of culture used for production of itaconic acid

The strain of *Aspergillus terreus* used, L.S.H.T.M. Cat. No. Am. 1, was isolated in 1935 by Mr G. Smith from American cotton yarn. It is without question a strain of *A. terreus* and forms typical, velvety, cinnamon-coloured colonies on Czapek-Dox agar.

Cultural conditions

The medium used was a Czapek-Dox solution of the following composition: 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, 1 l. 350 ml. quantities of this solution were placed in a number of 1-litre conical flasks plugged with cotton wool which were sterilized, inoculated with a spore suspension of *A. terreus*, Am. 1, and incubated in the dark at 24°.

Exp. 1

In this experiment 97 flasks were used. At intervals during the incubation period single flasks were taken off, the contents filtered, and the following estimations were carried out on the filtrate.

(1) Residual glucose by polarimeter.

(2) Chlorine in solution as inorganic chloride by the method described by Raistrick & Smith [1936].

(3) Total chlorine in solution. 50 ml. of the filtrate were made alkaline with a few drops of NaOH and evaporated to dryness in a nickel crucible. The residue was fused with a mixture of NaOH (10 g.) and Na_2O_2 (5 g.). The cooled melt was acidified with HNO_3 and the chloride estimated by titration with $N/10$ $AgNO_3$ by the Volhard method.

The results are given in Table I and prove that this strain of *A. terreus*, unlike strain No. 45 [Raistrick & Smith, 1936] which gives the chlorine-containing substances geodin and erdin, produces little, if any, water-soluble organic compounds containing chlorine.

Table I

Incubation period days	Residual glucose %	Inorganic chloride as KCl %	Total chlorine as KCl %
0	5·00	0·050	0·050
9	3·50	0·052	0·052
16	1·53	0·052	0·053
23	0·39	0·051	0·055
30	0·00	0·046	0·048
36	0·00	0·052	0·052
Av. of 93 flasks			

At the end of the incubation period (36 days) the metabolism solution in the remaining 93 flasks was separated by filtration and the mycelium was washed with water, dried and weighed (wt. 440 g. = 4·73 g. per flask). The yellow metabolism solution and washings were combined, acidified to Congo red with conc. HCl, kept for 2 days and filtered from a yellow amorphous solid (3·5 g.). The filtrate was neutralized to litmus with NaOH, evaporated *in vacuo* to 700 ml., and a small amount of amorphous material was separated by centrifuging. On acidifying the clear supernatant liquid with HCl, itaconic acid separated as a mass of dark brown crystals, wt. 62·5 g. The itaconic acid remaining in solution (33 g.) was extracted with ether in a continuous extractor as almost colourless crystals. The combined crops (95·5 g. ≡ 5·9 % of glucose metabolized) were purified by crystallizing from ethyl acetate and itaconic acid was finally obtained as large colourless prisms, M.P. 164–167°, not depressed on admixture with an authentic specimen. (Found: C, 46·15, 46·20; H, 4·81, 4·99; equiv. by titration, 65·6. Calc. for $C_5H_6O_4$: C, 46·15; H, 4·65 %; equiv. as a dibasic acid, 65·0.) 1·192 g. of the acid, shaken in aqueous solution with a palladium-charcoal catalyst, absorbed 204 ml. of hydrogen at N.T.P. (calc. 205 ml.). On ether extraction of the solution 0·9 g. of methylsuccinic acid was obtained, M.P. 108–111°, (lit. 111–112°). (Found: equiv. by titration, 65·6. Calc. for $C_5H_8O_4$ titrating as a dibasic acid, 66·0.)

Exp. 2

In this experiment, in which 108 flasks each containing 350 ml. of Czapek-Dox solution were used, a more extensive examination was made of the course of metabolism. Individual flasks were taken off at frequent intervals and the metabolism solution was separated by filtration from the mycelium which was washed with water, dried *in vacuo* and weighed. The filtrate and washings were made up to 500 ml. The following estimations were carried out on aliquot portions and the results are given in Table II in which all figures are calculated as for 1 flask.

(1) Residual glucose by polarimeter.
(2) Residual glucose by the Shaffer-Hartmann method.
(3) *p*H colorimetrically.
(4) Acidity by titration with $N/10$ NaOH to phenolphthalein.

(5) Permanganate reduction. This somewhat empirical estimation was carried out as follows: $N/10$ $KMnO_4$ was gradually added to 20 ml. of the metabolism solution, acidified with 10 ml. of $2N$ H_2SO_4. The end-point, which was not very well defined, was taken when decoloration of the permanganate ceased to be immediate.

(6) Bromine absorption by the Koppeschaar method [1876].

(7) Itaconic acid. 100 ml. of the metabolism solution were evaporated *in vacuo* to 5–10 ml., acidified with HCl and centrifuged until clear. The supernatant was extracted continuously with ether and the residue obtained on removing the solvent was sublimed in a high vacuum at 100–110°. The sublimate, consisting of almost pure itaconic acid, was weighed and its identity proved by crystallization from ethyl acetate-light petroleum, M.P. and mixed M.P.

(8) Citric acid. The method used is described in Allen, *Commercial Organic Analysis* (Churchill, 1924), **1**, pp. 751–2. The citric acid was isolated as the barium salt which was converted into pentabromoacetone and this was collected and weighed. Tests on artificial metabolism solutions, containing glucose and mixtures of various organic acids, including itaconic acid, showed that the method is accurate and specific for citric acid.

Table II

Incubation period days	Mycelium g.	Residual glucose: Polarimeter %	Residual glucose: Shaffer-Hartmann %	pH	Acidity $N/10$ NaOH ml.	$KMnO_4$ $N/10$ ml.	Br absorption: As Br mg.	Br absorption: As itaconic acid mg.	Itaconic acid isolated mg.	Citric acid estimated mg.
0	0	3·51	3·46	4·2	26·4	0·0	60	0	—	—
5	0·87	3·82	3·02	6·3	20·8	17·5	200	113	—	—
8	1·67	2·58	2·40	6·4	26·7	39·0	230	138	—	—
11	2·94	1·70	1·69	6·5	14·5	80·0	375	256	—	Nil
15	4·27	0·73	0·79	6·6	8·2	102·5	530	382	75	Nil
19	5·50	0·24	0·23	5·2	48·6	222·5	960	730	615	Nil
22	5·35	0·11	0·16	5·9	16·1	191·3	790	594	400	Nil
25	5·32	0·04	0·08	5·2	44·7	200·0	990	755	707	Nil

After 25 days' incubation the contents of 100 flasks were filtered and the filtrate and mycelium washings were evaporated *in vacuo* at 40–50° to a volume of about 1400 ml. The concentrated solution was made acid to Congo red with conc. HCl (100 ml.) diluted to 1800 ml. with water and kept overnight. The precipitate formed was difficult to separate but was removed by filtration through kieselguhr. The clear filtrate was extracted with ether in a continuous extractor and gave crude itaconic acid (70·7 g. ≡ 4·0 % of glucose metabolized), which was purified by crystallization from ethyl acetate-light petroleum. A portion was converted into the phenacyl ester [Rather & Reid, 1919] which after recrystallizing from aqueous methanol melted at 78–80°; the M.P. was not depressed on admixture with a specimen of the phenacyl ester of authentic itaconic acid, which also melted at 78–80°. Rather & Reid give the M.P. as 79·5°.

Formation of itaconic acid on different substrates

At the end of the incubation period (25 days) in exp. 2, 24 flasks, chosen at random, were divided into 8 groups of 3 flasks each. The metabolism solution was carefully drained off, so as to disturb the mycelium as little as possible, and was used along with the metabolism solution and mycelium washings from the remaining 76 flasks for the isolation of itaconic acid, as described above. Into each flask, in each group of 3 flasks, were then poured 350 ml. of each of the

following solutions, previously sterilized, the operation being carried out in such a way as to leave the mycelium, as far as possible, floating on the surface of the liquid. (1) Czapek-Dox solution from which was omitted the glucose and sodium nitrate, i.e. 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, 1 l. (2) The same solution of salts as in (1) together with (*a*) 1 % glucose; (*b*) 1 % citric acid; (*c*) 1 % malic acid; (*d*) 1 % pyruvic acid; (*e*) 1 % acetic acid; (*f*) 0·66 % malic acid + 0·33 % acetic acid; (*g*) 0·6 % pyruvic acid + 0·4 % acetic acid. Solutions 2 (*b*)–2 (*g*) were brought to *p*H 4–5 by the addition of aqueous NaOH before sterilization. After incubation for 7 days at 24° the metabolism solution was separated by filtration, the mycelium pressed, dried and weighed. The following estimations were then carried out on the combined metabolism solution and pressings from the mycelium: (1) *p*H; (2) itaconic acid. The latter was isolated and weighed as described on p. 1491. (3) Residual substrate: (*a*) glucose—by polarimeter and Shaffer-Hartmann method; (*b*) citric acid—by pentabromoacetone method; (*c*) pyruvic acid—by precipitation and weighing as the 2:4-dinitrophenylhydrazone; (*d*) acetic acid—by distillation after acidification with phosphoric acid, and titration of the distillate. The residual malic acid was not estimated. The results obtained are given in Table III in which all results are expressed as for 1 flask.

Table III

Substrate	Substrate supplied g.	Substrate utilized g.	*p*H at Start	*p*H at Finish	Mycelium g.	Itaconic acid isolated g.	Excess itaconic acid formed over Czapek-Dox salt control g.
Glucose, 1 %	3·50	3·20	5·0	4·6	5·88	0·437	0·378
Citric acid, 1 %	3·50	2·20	4·4	5·0	5·46	0·095	0·036
Malic acid, 1 %	3·50	—	4·3	6·0	5·18	0·122	0·063
Pyruvic acid, 1 %	3·50	1·23	4·2	7·1	5·13	0·066	0·007
Acetic acid, 1 %	3·50	0·25	4·2	4·9	4·55	0·047	—
Malic acid, 0·66 %	2·34	—	4·2	4·8	4·71	0·063	0·004
Acetic acid, 0·33 %	1·17	0·60					
Pyruvic acid, 0·60 %	2·10	0·42	4·2	6·0	4·35	0·050	—
Acetic acid, 0·40 %	1·40	0·30					
Czapek salts	—	—	5·0	5·8	5·13	0·059	—

The results prove that with pyruvic acid, acetic acid and mixtures of malic acid and acetic acid and of pyruvic acid and acetic acid no itaconic acid is formed, while with citric acid or malic acid the amounts of itaconic acid formed are very small compared with the amounts formed from glucose, being about 10 % of the yield from glucose in the case of citric acid and 17 % in the case of malic acid. The yield of itaconic acid obtained from glucose was 11·8 % of the glucose metabolized.

The metabolism of other strains of Aspergillus terreus

History of strains used. Relevant details of the five strains of *A. terreus* used in this part of the work are given in Table IV.

Table IV

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
Am. 2	American cotton	G. Smith, 1935	Cinnamon	Greenish yellow	Slightly floccose
Ac. 107	Laboratory contaminant at Ardeer	J. H. V. Charles, 1926	Cinnamon	Reddish brown	Slightly floccose
E. M. J.	Binding of old vol. of *Edin. Med. Journal*	H. H. Stroud, 1938	Sand brown	Yellowish brown	Smooth velvety

Table V

A. terreus strain Cat. No.	Incubation period days	No. of flasks	Residual glucose %	Mycelium per flask g.	Total Cl as KCl %	Inorganic Cl as KCl %	Acid precipitate	Ether extract I	Neutral precipitate	Ether extract II	Ether extract III
Unsown	—	—	5·00	—	0·050	0·050	—	—	—	—	—
No. 3	29	97	0·15	5·12	0·043	0·043	Not weighed	13·0 g. brown glass	17 g. brown solid giving 7 g. viscous oil on ether extraction	Small amount of light yellow oil	Brown oil, 11·8 g., giving 2·25 g. oxalic acid
No. 37	38	89	0·05	5·48	0·049	0·048	23·5 g.	18·2 g. dark brown glass	Phosphates only	0·95 g. brown glass	Brown sticky semi-solid, 20·25 g., giving 3·35 g. fumaric acid
Am 2	34	90	0·14	2·71	0·044	0·044	1·2 g. black floccules	Not extracted	6·65 g. black solid, not extracted	1·35 g. black tar	Black crystals, 8·2 g., gave 3·0 g. succinic acid
Ac 107	36	94	0·00	3·31	0·052	0·048	5·9 g. black solid	Not extracted	Phosphates only	Viscous oil, 0·5 g.	Oil and crystals, 3·9 g., giving 0·72 g. succinic acid
E. M. J.	37	93	0·24	5·05	0·058	0·051	68 g. solid	19·1 g. brown glass	Phosphates only	Brown viscous oil, 0·79 g.	Brown glass and crystals, 14·9 g.; 0·9 g. succinic acid
Am 1 (exp. 1)	36	93	0·00	4·73	0·052	0·052	Yellow solid, 3·5 g.	Not extracted	22·7 g. amorphous brown solid, not extracted	Not extracted	Itaconic acid, 95·5 g.

Cultural conditions and examination of metabolic products. 100 one-litre flasks each containing 350 ml. of Czapek-Dox 5 % glucose solution were sterilized and sown with a spore suspension of the strain of *A. terreus* under investigation, and were then incubated at 24°. At intervals of a few days the residual glucose, total chlorine in solution and inorganic chloride in solution were estimated. However, since none of the strains investigated metabolized any appreciable amount of the chloride present, only the figures at the end of the incubation period are given in Table V. When the glucose had been almost completely metabolized the flasks were removed from the incubator, the mycelium separated by filtration, washed, dried and weighed. The metabolism solution was now made acid to Congo red with conc. HCl and the amorphous precipitate was filtered off (Acid precipitate, col. 8, Table V). In those cases (Strains Nos. 3 and 37 and E.M.J.) where filtration was difficult a weighed amount of kieselguhr was added and the dried kieselguhr and precipitate were exhaustively extracted with ether in a Soxhlet apparatus. Removal of ether gave ether extract I (col. 9). In all cases the acid filtrate was made neutral to litmus with caustic soda and evaporated *in vacuo* at 40–50° to about 1 l. The material which separated and consisted in most cases of inorganic phosphates was filtered off and dried (Neutral precipitate, col. 10). The neutral filtrate was then extracted with ether giving ether extract II (col. 11) and was finally acidified to Congo red and re-extracted with ether giving ether extract III (col. 12). The corresponding figures for strain Am. 1 are added to Table V for comparison.

It is obvious from the results given in Table V that, since none of the six strains examined metabolizes inorganic chloride to any appreciable extent, none of them produces the chlorine-containing substances geodin or erdin. This conclusion was confirmed by tests for chlorine on the acid precipitate and on ether extracts I, II and III. In all cases a negative result was obtained.

No crystalline substance could be isolated, in any instance, either from the acid precipitate or from ether extracts I or II. Ether extract I is acidic in nature and contains alkoxyl groups. Thus ether extract I from strain No. 3 had an equivalent, by titration, of 405 and a methoxyl content, by the Zeisel method, of 6·9 %; while the corresponding figures for strain No. 37 were 458 and 8·9 % and those for strain E.M.J. were 402 and 8·4 %.

The only crystalline metabolic products obtained from any of the strains examined were separated from ether extract III, and with each strain a relatively simple organic acid was isolated from this fraction. The variable nature of these acids, from strain to strain, is of some interest from the point of view of relationship between morphological characteristics and biochemical activities within the boundaries of a mycological "series" of strains of a species of fungus. Thus of the six strains examined one strain gave itaconic acid, one strain oxalic acid, three strains succinic acid and one strain, No. 37, fumaric acid. This strain had been examined previously by Raistrick & Smith [1935] who found that it then gave appreciable amounts of succinic acid and traces of oxalic acid but no fumaric acid was detected.

Summary

It has been shown that itaconic acid is a metabolic product of an indubitable strain of *Aspergillus terreus* Thom when this organism is grown on Czapek-Dox 5 % glucose solution. The evidence presented does not support the view that, with this mould, citric acid is an intermediate stage in the formation of itaconic acid from glucose. Five other strains of *A. terreus* were also grown on Czapek-Dox 5 % glucose solution. None of them produced itaconic acid, three produced succinic acid, one fumaric acid and one oxalic acid.

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