CXXXIX. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XLIV. FULVIC ACID, A NEW CRYSTALLINE YELLOW PIGMENT, A METABOLIC PRODUCT OF *P. GRISEO- FULVUM* DIERCKX, *P. FLEXUOSUM* DALE AND *P. BREFELDIANUM* DODGE.

BY ALBERT EDWARD OXFORD, HAROLD RAISTRICK
AND PAUL SIMONART.

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

(Received March 29th, 1935.)

It has already been reported in Parts XIX and XXIX of this series that when *Penicillium griseo-fulvum* Dierckx is grown on liquid media containing glucose as sole source of carbon, and sodium nitrate as sole source of nitrogen, the following products are to be found in the metabolism solution when most of the glucose has been utilised: 6-methylsalicylic acid [Anslow and Raistrick, 1931]; gentisic acid, fumaric acid and mannitol [Raistrick and Simonart, 1933]. The factors governing the formation of these products are temperature of incubation and ratio of glucose to sodium nitrate. The presence of the two phenolic acids mentioned above causes the metabolism solutions to give respectively a purple or blue coloration with ferric chloride. Further experiments have now shown that when the nitrogen source is changed from NO_3^- to NH_4^+ the course of the metabolism of the glucose is entirely changed. The coloration given with ferric chloride is now no longer purple or blue but green, and in place of the above two colourless phenolic acids there is formed a much more complex yellow phenolic acid, of empirical formula $\text{C}_{14}\text{H}_{12}\text{O}_8$, which gives a deep green colour with aqueous ferric chloride, and for which we propose the name *fulvic acid*. The yield of fulvic acid from this organism is however small and its isolation in a pure state from the metabolism solution is very difficult. The reaction given by fulvic acid with ferric chloride and also certain of its other properties are very similar to those of the complex mould product citromycetin, $\text{C}_{14}\text{H}_{10}\text{O}_7$, produced by species of *Citromyces* [Hetherington and Raistrick, 1931], hitherto the only mould metabolic product known to give an intense green colour with ferric chloride.

Subsequently to the above observations on *P. griseo-fulvum* our colleague, Mr G. Smith, has found that two other species of *Penicillium*, viz. *P. flexuosum* Dale and *P. Brefeldianum* Dodge¹ give rise to metabolic solutions giving intense green colorations with ferric chloride, when grown in test-tubes on Raulin-Thom medium containing glucose and tartaric acid as sources of carbon, and ammonium tartrate, phosphate and sulphate as sources of nitrogen. We have isolated the metabolic product responsible for these reactions and have found that

¹ This name has recently been altered to *Carpenteles Brefeldianum* by Shear [1934].

it is in fact fulvic acid in each case. Furthermore, the yield, especially with *P. Brefeldianum*, is very much larger than with *P. griseo-fulvum*, the greater part of the fulvic acid being found however in the mycelium in the case of *P. Brefeldianum*.

A further point of interest is that the metabolism solution from *P. flexuosum* also contains 6-methylsalicylic acid, but this acid was not detected as a metabolic product of *P. Brefeldianum*. The fact that both *P. griseo-fulvum* and *P. flexuosum* give rise to fulvic and 6-methylsalicylic acids is not surprising in view of the close morphological relationship of these organisms. Thom [1930] places *P. griseo-fulvum* in section 5 of the *Asymmetrica* division of species of *Penicillia*, viz. among the *Asymmetrica-funiculosa*, with possible affinities with the next section, viz. the *Fasciculata*, in which he places *P. flexuosum*.

The relationship between these organisms and *P. Brefeldianum* appears however to be remote. Dodge [1933] has recently made a careful study of the last-named organism, which was first isolated by Dr Rhoda Benham in America from the human alimentary tract, and reports that in its conidial stage it belongs to the *Monoverticillata* section of *Penicillia* (as do *Citromyces* species) with the further note that in this stage it may possibly belong to Thom's *Asymmetrica* section. It is of great interest that *P. Brefeldianum* readily produces perithecia, quite unlike those of species in the *P. luteum* series but comparable with those described long ago by Brefeld [1874] as typical of the so-called *P. glaucum* Link, an organism which no later worker has been able to isolate and the very existence of which has been doubted by many mycologists. Dodge, in fact, is of the opinion that *P. Brefeldianum* is morphologically very near to the classical *P. glaucum* described by Brefeld. It may be noted here that *P. javanicum* van Beijma [van Beijma, 1929] which is morphologically very close to *P. Brefeldianum*, and which also forms similar perithecia, appears to metabolise glucose in a medium containing NH_4NO_3 as source of nitrogen in an entirely different way [Lockwood *et al.*, 1934], to yield citric acid, a product which was not detected among the metabolic products of *P. Brefeldianum*. We intend at an early date to study in detail the metabolism of *P. javanicum* when grown on Raulin-Thom medium and also the metabolism of both organisms when grown on Czapek-Dox medium with sodium nitrate as sole source of nitrogen.

The mycelium of *P. griseo-fulvum* has been shown to contain a colourless, crystalline, nitrogenous substance (m.p. 165° decomp.) while the mycelium of *P. Brefeldianum* yields, in addition to fulvic acid, a different nitrogenous compound, as colourless crystals, m.p. $132\text{--}135^\circ$. The description of these two metabolic products is reserved for a later communication.

In this paper we propose to record the isolation, properties, derivatives and reactions of fulvic acid, together with the evidence for the empirical formula we have assigned to it, viz. $\text{C}_{14}\text{H}_{12}\text{O}_8$. The question of its structural formula is left for a later communication.

EXPERIMENTAL.

Organisms used.

(a) *P. griseo-fulvum* Dierckx. L.S.H.T.M. Catalogue No. P 38. Received from Prof. Ph. Biourge, University of Louvain, December 1929, as No. B 34.

(b) *P. flexuosum* Dale. L.S.H.T.M. Catalogue No. P 129. Received from Centraalbureau voor Schimmelcultures, Baarn, August 1931. Supplied by Dale.

(c) *P. Brefeldianum* Dodge. L.S.H.T.M. Catalogue No. P 157. Received from Baarn, September 1933. Supplied by Dodge.

Section A. Metabolic products of P. griseo-fulvum grown on media containing glucose and tartaric acid as sources of carbon, and ammonium salts as sources of nitrogen¹.

The standard media used in this laboratory are Czapek-Dox medium (glucose, 50 g.; NaNO_3 , 2.0 g.; KH_2PO_4 , 1.0 g.; KCl , 0.5 g.; MgSO_4 , $7\text{H}_2\text{O}$, 0.5 g.; FeSO_4 , $7\text{H}_2\text{O}$, 0.01 g.; distilled water, 1 litre), and Raulin-Thom medium (glucose, 50 g.; tartaric acid, 2.7 g.; ammonium tartrate, 2.7 g.; diammonium hydrogen phosphate, 0.4 g.; K_2CO_3 , 0.4 g.; MgCO_3 , 0.27 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.17 g.; ZnSO_4 , $7\text{H}_2\text{O}$, 0.05 g.; FeSO_4 , $7\text{H}_2\text{O}$, 0.05 g.; distilled water, 1 litre).

Preliminary experiments in test-tubes indicated that when the mould was grown on Raulin-Thom medium, the metabolism solution, after a few days' incubation, gave a green colour with ferric chloride. The optimum temperature for the production of the substance responsible for the coloration appeared to be about 20° , but there still remained 3.28 % of glucose after 42 days' incubation. The medium, initially at p_{H} 4.1, became much more strongly acid during the incubation period, *e.g.*, in the instance quoted above the p_{H} was below 2.8 after 42 days' incubation. On the other hand, when the mould was grown on Czapek-Dox medium at the same temperature the metabolism solution was slightly alkaline when all the glucose had been utilised, and gave a purple colour with FeCl_3 . Hence it obviously became desirable to find out which constituents of Raulin-Thom medium are essential for the formation of the product giving the green coloration with FeCl_3 .

A comparison of the respective compositions of Raulin-Thom and Czapek-Dox media shows that the former contains NH_4^+ , CO_3^{--} , Zn^{++} and tartaric acid, all absent from the latter, whilst the latter contains Na^+ , Cl^- and NO_3^- all absent from the former. Experiments were therefore conducted with the following four media:

- A. Czapek-Dox medium omitting KCl but with ZnSO_4 added.
- B. Czapek-Dox medium omitting NaNO_3 but with NH_4NO_3 added.
- C. Czapek-Dox medium omitting NaNO_3 but with ammonium tartrate added.
- D. Czapek-Dox medium with K_2CO_3 added.

After 14 days' incubation at 20° , only the metabolism solutions from B and C gave the green ferric chloride reaction. It appeared therefore that replacement of NO_3^- by NH_4^+ was responsible for the new mode of metabolism (tartrate not being essential), and different sources of NH_4^+ were next tried, *viz.* ammonium tartrate, sulphate and phosphate at concentrations of 1, 2, 3, 4 and 5 g. per litre. The results indicated that ammonium tartrate at a concentration of 5 g. per litre was the best source of NH_4^+ . Finally a number of media were made up containing the salts of Czapek-Dox medium but with ammonium tartrate (5 g. per litre) replacing sodium nitrate, and with varying concentrations of glucose up to 10 %. The tubes were incubated for 30 days at 21° . The results are summarised in Table I and show that good production of phenolic substances took place on media containing 3–10 % glucose. In all cases a dark green colour was obtained with FeCl_3 .

Isolation of the metabolic product responsible for the green coloration with ferric chloride. The medium selected for large scale work had the following composition: glucose, 50 g.; ammonium tartrate, 5 g.; KH_2PO_4 , 1.0 g.; KCl , 0.5 g.; MgSO_4 ,

¹ The results in this section are taken from a thesis presented in 1934 by Dr P. Simonart for the degree of Ph.D. in the University of London.

Table I.

% glucose at start	Apparent % glucose by polarimeter at end	Br absorption value at end. mg. per ml.	Final p_H
1	—	0.93	7.1
2	0.21	2.05	4.1
3	0.64	2.53	3.5
4	1.70	2.40	3.2
5	2.70	2.29	3.2
6	3.66	2.46	3.1
7	4.80	2.44	3.2
8	5.88	2.48	3.2
9	7.06	2.43	3.2
10	7.80	2.56	3.3

7H₂O, 0.5 g.; FeSO₄, 7H₂O, 0.01 g.; distilled water, 1 litre. One hundred and fifty 1 litre flasks each containing 350 ml. of this medium were sterilised by steaming on each of three successive days, sown with a suspension of spores of *P. griseo-fulvum* (obtained from 15-day old cultures at 30° on beer-wort agar in Roux bottles) and incubated at room temperature (19–21°) for 50 days. The mycelium and the filtered metabolism solution, which gave an intense green colour with FeCl₃, were then dealt with separately. The mycelial constituents of *P. griseo-fulvum* grown on Czapek-Dox medium and on the above modification of this medium will form the subject of a separate paper. In this place it will be sufficient to say that both mycelia contained considerable amounts of an ether-soluble, colourless, crystalline nitrogenous substance (M.P. 165° decomp.) the isolation of which in a crude state (M.P. about 150°) was mentioned by Anslow and Raistrick [1931] in their first biochemical study of this mould; and that the mycelium from the modified Czapek-Dox medium appeared not to contain any of the phenolic substance (fulvic acid) present in the metabolism solution.

Estimations carried out on an average sample of the metabolism solution gave the following results: apparent glucose content (by polarimeter) 1.82 %; Br absorption value (Koppeschaar's method) 2.1 mg. per ml. The metabolism solution was evaporated to small volume *in vacuo* below 50°, made strongly acid to Congo red by addition of 2N H₂SO₄ and extracted 12 times with an equal volume of chloroform. The extract was dried over anhydrous sodium sulphate and the solvent removed *in vacuo* at 40°. The brown, gummy residue was treated with a large volume of boiling water, the aqueous extract boiled with a little decolorising charcoal and filtered. On cooling, 0.42 g. of yellow needles separated, M.P. 241° decomp. The substance was further purified by crystallisation from ethyl acetate. Analysis and mixed M.P. with specimens of fulvic acid produced by *P. flexuosum* and *P. Brefeldianum* will be found later (p. 1111).

Section B. Metabolic products of P. flexuosum Dale grown on Raulin-Thom medium.

A preliminary investigation of the course of metabolism of glucose by this mould was made exactly as described in Part XXXIX of this series [Oxford and Raistrick, 1934] for *P. crateriforme* Gilman and Abbott. A quantity of Raulin-Thom medium (for composition see p. 1104) was distributed in 350 ml. lots in 49 one-litre conical flasks. After sterilisation each flask was sown with a spore suspension of *P. flexuosum* and incubated at 24°. Single flasks were removed periodically and tests made on the filtered metabolism solution. The quantitative results so obtained are summarised in Table II.

Table II.

Period of incubation days	Apparent % glucose by polarimeter	% glucose by Shaffer-Hartmann method	p_H	Titrateable acidity ml. N/10 per 10 ml.	Br absorption value mg./ml.
0	5.35	5.20	3.9	2.5	0.10
3	4.70	—	3.5	3.7	0.30
(complete mycelial felt)					
6	2.76	2.70	2.8	4.6	0.49
9	1.23	1.21	3.0	2.6	0.68
13	0.50	0.50	3.6	0.8	0.88
16	0.25	—	4.2	—	1.00
22	Practically nil	Present	5.6	—	1.20
27	Nil	Nil	6.3	—	1.22

The only qualitative results of note were the following: the green coloration with ferric chloride was perceptible on the third day, of moderate intensity on the ninth day and a deep emerald green on the thirteenth and subsequent days. Tartrate was still present on the ninth day but absent later. A moderate yellow precipitate was given with normal lead acetate on the 13th and subsequent days. The metabolism solution in the later stages was pale yellowish brown, deepening to reddish brown in colour on the twenty-second day; when brought to p_H 7 a deeper orange-brown or red colour was obtained. The mycelial felt, coherent at first, began to disintegrate about the twenty-second day and marked autolysis had set in by the twenty-seventh day. It is evident that the mould grows, and metabolises glucose, rapidly, but the yield of dry mycelium (5.1 g. per flask) was not excessively large. Solvent extraction of this mycelium yielded a minute amount of fulvic acid, but none of the nitrogenous substance, M.P. 165° , found in the mycelium of *P. griseo-fulvum*.

Isolation of fulvic acid from the metabolism solution. 44 flasks were removed after 16 days' incubation and the filtered metabolism solution was evaporated to 400 ml. *in vacuo* below 40° . A dark-coloured precipitate (A) separated during the evaporation (4.6 g.), which was found to contain the greater part of the fulvic acid produced by the mould. A minute amount of fulvic acid (M.P. 236° decomp.) was with difficulty obtained from the filtrate from this precipitate by a method similar to that previously described for *P. griseo-fulvum*, or by addition of normal lead acetate, treatment of the lead precipitate with dilute H_2SO_4 , extraction with ether and crystallisation from water.

The dry precipitate (A) was extracted with ether in a Soxhlet apparatus for 3 working days. About 1.5 g. of yellow material separated from the ethereal extract and was crystallised first from 20 ml. dioxan, then from 16 ml. dioxan. Yield: 0.4 g. of lemon-yellow rods; M.P. 242° decomp. The material in the thimble unextracted by ether still contained some fulvic acid, as was proved by extraction with boiling dioxan. No other crystalline substance was obtained from it.

Isolation of 6-methylsalicylic acid from the metabolism solution. The dark brown filtrate from precipitate (A) was made acid to Congo red by addition of 2N H_2SO_4 and extracted 3 times with an equal volume of ether. Evaporation of the dried ethereal extract yielded a brown gum (1.4 g.) which, by fractional crystallisation from water, was shown to contain a little fulvic acid together with small amounts of 6-methylsalicylic acid. This substance was obtained as colourless crystals which were purified by sublimation in a high vacuum to yield colourless prisms, M.P. 170 – 171° , alone or mixed with authentic 6-methylsalicylic acid, which gave a beautiful purple colour with aqueous ferric chloride.

Section C. Metabolic products of P. Brefeldianum Dodge grown on Raulin-Thom medium.

A preliminary survey carried out as outlined previously for *P. flexuosum* Dale indicated that the course of metabolism was very similar to that of the latter mould. Growth was fairly rapid with production of a thick tough mycelial felt which, after becoming depressed at the edges, eventually became waterlogged, sank and was replaced by a new similar surface felt. The turbid metabolism solution was brown in colour and was obtained clear only after repeated filtration through paper. Its p_H was always well on the acid side (4 or below) and the titratable acidity (ml. *N*/10 NaOH required to bring 10 ml. of metabolism solution to p_H 7) initially 2.5, rose to 5.8 on the sixth day and then fell to 1.0 on the twenty-seventh day when all the glucose had been metabolised. When brought to p_H 7 the metabolism solution became deep brownish red in colour (or even clear wine-red when made slightly alkaline), but the colour became lighter on making strongly alkaline. The bromine absorption value of the metabolism solution steadily increased with incubation and when all the glucose had been utilised was 2.40 mg./ml. At all times the glucose content as estimated polarimetrically was practically identical with the value obtained by the Shaffer-Hartmann method. At the end of the incubation period the only striking reactions given by the metabolism solution were the following: an intense emerald-green coloration with aqueous or aqueous-alcoholic ferric chloride, given definitely on the sixth day and later becoming more striking; a heavy, brown precipitate with mercuric chloride solution; a heavy, yellow precipitate with normal lead acetate; reduction of $AgNO_3$ on standing. At p_H 7 the ferric chloride coloration is brown, becoming green on addition of a drop or two of dilute HCl.

Since the yield of fulvic acid given by *P. Brefeldianum* is considerably larger than with *P. griseo-fulvum* or *P. flexuosum*, *P. Brefeldianum* was used for the preparation in quantity of fulvic acid. The following are the results for a typical large-scale experiment: 100 flasks, each containing 350 ml. of medium, were taken after 33 days' incubation (residual glucose, nil) and the mycelium was separated from the bulk of the metabolism solution by filtration through muslin (weight of dried mycelium, 496 g.). The turbid filtrate was filtered through paper and the dark brown, slimy precipitate (product A, 15 g.) collected. The final clear brown filtrate was evaporated to about 400 ml. *in vacuo* below 50° and left in the cold room for several days. The brown, partly crystalline precipitate which separated was filtered off, washed with water and dried (product B, 42 g.). Further acidification of the final filtrate threw down a little tarry material from which no crystalline product could be isolated. Repeated ethereal extraction of the acid filtrate from this tar yielded a brown gum from which a minute amount of fulvic acid was eventually isolated by repeated crystallisation from water (charcoal) and finally from aqueous dioxan. The various aqueous mother liquors from these crystallisations were evaporated down in stages, but no product was obtained which gave other than a green coloration with ferric chloride. Hence 6-methylsalicylic acid was almost certainly absent.

Further batches of 100 flasks each gave very similar results save that the weight of dry mycelium fluctuated somewhat, *e.g.* weights of mycelium from successive batches were 452; 496; 371; 184; 351 g.; the probable explanation being that the last 3 batches were given longer periods of incubation (40–50 days), *i.e.* until several days after all the glucose had disappeared, with resulting partial autolysis of the mycelial felt. The yield of fulvic acid appeared not to be adversely affected thereby, but in these instances product B was small, its counter-

part being the precipitate obtained by acidification of the mother liquor from product B (previously a tar).

Isolation and separation of fulvic acid from other metabolic products. The isolation of fulvic acid in quantity and in a high state of purity proved to be difficult for many reasons:

(a) Fulvic acid is only very sparingly soluble in hot ether or chloroform. To extract all the fulvic acid from the mycelium, or from product B, by use of these solvents in a Soxhlet apparatus requires weeks or even months.

(b) The crude fulvic acid so obtained cannot be satisfactorily purified from solvents such as hot ethyl acetate or dioxan, in which it is moderately soluble, because the accompanying nitrogenous substance, M.P. 132–135°, is very similar to fulvic acid in solubilities and crystalline shape.

(c) Even when the fulvic acid has been separated from this nitrogenous substance and other neutral substances by solution in aqueous sodium bicarbonate, the product recovered on acidification contains other phenolic acids, which are more sparingly soluble in dioxan than is fulvic acid and cannot be removed with certainty by crystallisation from that solvent. It is necessary first to extract the fulvic acid with boiling ethyl acetate in which the other phenolic acids are practically insoluble.

(d) The most efficient method of purification, *viz.* conversion into the potassium salt and recrystallisation of the latter, is impracticable on a large scale since the solubility of this salt in boiling methyl alcohol is only of the order of 0.1 %.

The following paragraphs give an account of the method finally adopted for the separation of the metabolic products and the purification of the crude fulvic acid. The mycelium and products A and B were worked up separately.

Treatment of the mycelium. The dried mycelium was finely powdered and extracted with light petroleum (B.P. 50–60°) in a large Soxhlet apparatus for two working days. This treatment removes fats and sterols, in which the mycelium is particularly rich. Thus, if the extract is evaporated to small bulk and allowed to stand for several weeks in the cold room, a copious crystalline precipitate of ergosterol (long needles) and, probably, ergosteryl esters (groups of small crystals) is produced; or if the extract is evaporated to dryness and treated with cold alcohol [Oxford and Raistrick, 1933] a relatively large amount of insoluble ergosteryl esters is obtained. The defatted mycelium was then similarly extracted with ether until it appeared to be entirely exhausted of ether-soluble material. This process required at least 40–50 working days and the mycelium was re-ground and redistributed among the thimbles after about 20 days' extraction. The yellowish brown solid crust which separated from the ether on the walls of the flasks was removed periodically, and the solvent itself was likewise renewed every 5 or 6 days, the ethereal mother liquors being kept and later combined, evaporated to dryness and worked up separately. The material which separated from the ether during the first 2 or 3 days' extraction was worked up separately, since it contained the bulk of a new, colourless, crystalline compound containing nitrogen, M.P. 132–135°.

Purification of crude fulvic acid. The crude fulvic acid so obtained (15–20 % by weight of the mycelium) was finely powdered and treated (in 50 g. lots in a large filtrate jar) with stirring, with excess of cold, nearly saturated sodium bicarbonate solution. After the effervescence had subsided, the mixture was allowed to stand for several hours, filtered and the dark-coloured filtrate acidified with 2N HCl and then kept in the cold room overnight. The impure fulvic acid, now free from neutral substances, was collected, well washed with

water and dried *in vacuo* over solid KOH. It was further worked up in 40 g. lots, each being extracted many times with about 1600 ml. boiling ethyl acetate until the last extract deposited no crystalline material, or a mere trace of a sandy powder on long standing. Four or five extractions were usually necessary. The first was always deep red in colour, and subsequent extracts were orange-red, orange and finally pale yellow or brown. Fulvic acid was deposited in a hard cake of large brown or brownish yellow prisms on the bottoms and walls of the flasks, and since crystallisation was slow, 4 or 5 days were allowed before proceeding to the next stage. Detachment of the deposit by scraping with a spatula is a laborious task and it is better and quicker to decant off the supernatant liquid, wash once or twice with a little ethyl acetate, and dry *in situ* in a stream of air. Tared one-litre conical flasks were used for these crystallisations. Second and third crops of more impure fulvic acid were obtained by evaporating the ethyl acetate mother-liquors in stages and were separately crystallised from dioxan.

The volume of dioxan per g. required for the final crystallisation depends on the purity of the fulvic acid at this stage. If the acid is moderately pure, 10–12 ml. per g. will be required, but if still highly coloured and impure, 6 ml. per g. will be sufficient to bring the material into solution. The mixture of dioxan and crystals was heated to boiling with constant stirring in an oil-bath, boiled for a few minutes and filtered immediately. To the filtrate was added about 10 % of its volume of water and the whole, when cold, was left in the cold room for at least 2 days. Fulvic acid then separated as a yellow, sandy, crystalline powder (very small rods) which showed no tendency to adhere to the walls of the flask. The precipitate was collected, washed with dioxan containing 10 % of water, then with ethyl acetate and dried in air. Second, third, fourth and final crops were obtained by diluting successive mother liquors each time with 20 % of water and allowing to stand for several days in the cold room. The melting point and colour of each crop were noted. In general the lighter the colour, the higher the decomposition point when heated in a M.P. tube at a standard rate. The limiting values of the decomposition point were taken as 235° and 248°. Material melting or decomposing below 235° was again recrystallised from dioxan.

Treatment of product A. Product A was treated in a similar manner. In one instance, the proportion of the crystalline nitrogenous substance, M.P. 132–135°, was exceptionally large. The material remaining unextracted by ether after some 3 weeks' extraction was triturated with sodium bicarbonate solution. The filtrate contained some fulvic acid which was purified as previously described.

Treatment of product B. Product B yielded, by long-continued ethereal extraction, a pale, yellowish brown solid which was almost completely soluble in sodium bicarbonate solution. The unextracted part (after some weeks' extraction) was re-ground and the extraction continued with chloroform. After a few days, nothing further appeared to be extracted by this solvent, but this was due, not to removal of all the fulvic acid, but to the caking together of the solid in the thimble. The extraction was therefore discontinued and the residual solid treated with sodium bicarbonate *etc.*, as previously described. The presence of potassium hydrogen tartrate was noted in this residual solid, and a colourless specimen of this salt was obtained by repeated crystallisation from boiling water.

Recovery of dioxan. All dioxan used in the large scale work described above was purified by the method of Eigenberger [1931; see also Oxford, 1934] and was free from aldehydes and acetals. The final aqueous dioxan mother-liquors were combined and the greater part of the solvent was distilled off. The distillate was treated as crude, technical dioxan and submitted to the full process of purification.

The greater part of the dioxan used was recovered in this way in a pure state.

Yield of purified fulvic acid: 140 g. from 300 flasks, i.e. from 5250 g. glucose.

Section D. The colourless crystalline nitrogenous compound, m.p. 132–135°, from the mycelium of P. Brefeldianum.

On p. 1108 it is stated that the material separating from the ethereal extract obtained during the first 2 or 3 days' extraction of the mycelium was kept separate from the material from later extractions. The weight of this material (by extraction of mycelium from 300 flasks) was 41.4 g. of which some 15 g. were insoluble in aqueous NaHCO_3 . This latter material was repeatedly crystallised from dioxan (in which it is even more sparingly soluble than fulvic acid) and was finally obtained almost colourless but not crystalline by deposition from hot ethyl acetate solution. It melted at 132–135° with slight softening from about 129° onwards, the melting point remaining unchanged after a further crystallisation from dioxan. The melt set on cooling to a turbid glass. The substance contains neither sulphur nor phosphorus. (Found (Schoeller), C, 73.35, 73.24; H, 12.42, 12.29; N, 2.1 %; Methoxyl, nil. $\text{C}_{40}\text{H}_{79}\text{O}_5\text{N}$ requires C, 73.42; H, 12.18; N, 2.1 %.) The substance is of a waxy consistency and can be obtained crystalline only from dioxan, e.g. from hot aqueous dioxan it separates immediately in the form of tufts of curved, microscopically small, pointed hairs; but from hot anhydrous dioxan it separates first of all in tiny oily droplets, becoming partly crystalline in 24 hours and completely crystalline after several days in the form of radiating clusters of very small, straight needles, quite similar in shape to the crystals obtained when fulvic acid separates from alcohol. It separates in tiny droplets (in appearance not unlike yeast cells) from chloroform, acetone or ethyl acetate, but these do not become crystalline even on long standing. The material separating from these solvents can however be readily collected on a Büchner funnel and in bulk is indistinguishable from the crystalline form.

The substance is moderately readily soluble in cold alcohol, but quite insoluble in boiling water. It does not give the Molisch test for carbohydrates. It gives no coloration with ferric chloride in aqueous alcoholic solution. It is quite neutral, being insoluble in both dilute acids and alkalis, even on boiling. It gives no coloration with strong sulphuric acid and appears, in fact, to be a very stable and non-reactive substance. Further work is in progress on this substance and will be reported at a later date.

Section E. Fulvic acid and its derivatives.

Properties of fulvic acid. Fulvic acid is very sparingly soluble in the cold in all organic solvents, e.g. at 12°, even dioxan dissolves less than 1 % of the acid, which is not sufficient for an accurate determination of molecular weight by the cryoscopic method. It may be crystallised from boiling dioxan (15 parts), acetone or ethyl acetate (100–150 parts), ethyl alcohol (250–300 parts), toluene (very sparingly soluble even at B.P.), and from water when somewhat impure, pure fulvic acid being almost insoluble in boiling water. The appearance of the crystals obtained varies with the solvent used, the rate of cooling, the amount of water present and on other factors, e.g. from dioxan (with or without added water), it usually crystallises in lemon-yellow rods, sometimes curved, or in small rectangular prisms, or in parallelepipeds, sometimes with bluntly pointed ends; from acetone, in massive lemon-yellow prisms; from moist ethyl acetate in relatively large golden plates; from water in pale yellow plates, characteristically twinned; from absolute alcohol in clusters of very pale yellow, slender, pointed

needles; from aqueous alcohol at 0° in slender, segmented, rectangular prisms; from toluene in very small, flat crystals of indefinite shape. From acetone or ethyl acetate it sometimes happens that massive yellow prisms are found side by side with much paler slender rods. In spite of this diversity of crystalline appearance, there can be no doubt of the essential homogeneity of the product prepared in quantity as previously described, since when regenerated from the recrystallised potassium salt, the product behaves similarly when recrystallised from the above solvents. Crystals of fulvic acid tenaciously retain many solvents, and air-dried, or even vacuum-dried, specimens do not always give results in accordance with the formula $C_{14}H_{12}O_8$. Drying at 138° in a stream of dry, oxygen-free nitrogen, or in a high vacuum at 100–110° is in many cases necessary to remove the last traces of solvent. The analytical results for various specimens of fulvic acid are summarised in Table III. Fulvic acid contains no methoxyl groups and no nitrogen.

Table III.

Source and treatment	% C	% H
1. From <i>P. griseo-fulvum</i>	54.43	4.10
Crystallised from H_2O . Dried to constant weight <i>in vacuo</i> at 100°	54.41	4.02
2. From <i>P. flexuosum</i>	54.24	4.01
Crystallised from dioxan. Dried to constant weight <i>in vacuo</i> at 110°	54.09	3.98
3. From <i>P. Brefeldianum</i>		
Crystallised from toluene		
(a) Dried <i>in vacuo</i> at ordinary temperatures	54.44	4.06
(b) Dried in N_2 at 138°	54.42	4.07
Theoretical for $C_{14}H_{12}O_8$	54.53	3.93

0.1134 g. of fulvic acid from *P. flexuosum*, dissolved in 13.57 g. dioxan, gave a depression in F.P. of 0.148°; hence, molecular weight = 268 (theory for $C_{14}H_{12}O_8$ = 308). This estimation is not very satisfactory because of the slight solubility of fulvic acid in cold dioxan but full confirmation of the formula assigned, *viz.* $C_{14}H_{12}O_8$, was obtained by the preparation and analysis of the trimethyl and triethyl derivatives, and the monoethyl ester (see later).

Pure fulvic acid, after crystallisation from any solvent, always begins to darken at about 195°, becomes gradually darker as the temperature is raised, begins to soften at about 236° and melts with vigorous effervescence to a deep brown liquid at about 246°, the actual softening and decomposition points varying a little with the rate of heating. Citromycetin ($C_{14}H_{10}O_7$, $2H_2O$), which shows certain resemblances to fulvic acid, melts at 285° whilst a mixture of citromycetin and fulvic acid melted at 227°. The mixed melting-points of specimens of fulvic acid produced by all three moulds were as follows: product from *P. griseo-fulvum* (crystallised from ethyl acetate) + product from *P. flexuosum* (crystallised from dioxan), softens at 238°, melts with decomp. at 244°; product from *P. griseo-fulvum* + product from *P. Brefeldianum* (crystallised from dioxan), softening 236°, decomp. 243°; products from *P. flexuosum* and *P. Brefeldianum*, softening 235°, decomp. 245°. Further confirmation of the identity of all three specimens is afforded by the mixed m.p. of their trimethyl derivatives (see p. 1113).

Fulvic acid may be sublimed in a high vacuum at a bath temperature of 170°. The yellow sublimate melts at 242–243° (decomp.) but appears to have lost a molecule of water during the sublimation. (Found on different samples (Schoeller): C, 58.01, 57.63; H, 3.70, 3.48 %. Mol. wt. (in camphor), 329. $C_{14}H_{10}O_7$ requires C, 57.92; H, 3.47 %; Mol. wt. 290).

Fulvic acid titrates as a dibasic acid but the end-point is difficult to determine accurately because of the colour of the solution. 0.1902 g. from *P. Brefeldianum* dried to constant weight in N_2 at 138° dissolved in 10 ml. dioxan + 6 ml. water required 12.39 ml. $N/10$ NaOH for neutralisation to phenolphthalein; whence equivalent = 154. ($C_{14}H_{12}O_8$ titrating as a dibasic acid requires an equivalent of 154.)

Fulvic acid is optically inactive: 0.10 g. in 15 ml. dioxan in a 1 dm. tube gave a mean reading of only $+0.02^\circ$. It is soluble with difficulty in aqueous sodium acetate giving a very pale yellow solution, but is readily soluble in sodium bicarbonate (with effervescence), carbonate or hydroxide to give yellow to orange solutions. With $FeCl_3$ fulvic acid gives (a) a wine-red colour in absolute alcoholic solution; (b) a deep green in aqueous alcohol, becoming olive brown on addition of more $FeCl_3$; (c) a stable green colour in aqueous dioxan. Citromyccetin gives a dark emerald green colour under all these conditions. Unlike citromyccetin, fulvic acid does not yield a hydrobromide with HBr in glacial acetic acid, nor is a hydriodide formed when it is dissolved in hot hydriodic acid of specific gravity 1.7, since an aqueous extract of the substance crystallising out on cooling gives no precipitate with silver nitrate.

Potassium salt of fulvic acid. This salt was prepared by the method used by Hetherington and Raistrick [1931, p. 222] for the corresponding salt of citromyccetin. Fulvic acid (3 g.) was dissolved in boiling ethyl alcohol (750 ml.) and to the hot filtered solution was added an excess (90 ml.) of 5 % alcoholic potassium acetate solution. The potassium salt was precipitated immediately as a yellow jelly quickly crystallising to a mass of fine needles, which after standing overnight were collected, washed with alcohol, dried and recrystallised from boiling methyl alcohol, in which it is only very sparingly soluble.

The salt as prepared above was readily but not completely soluble in water and evidently contained traces of the free acid. (Found (Schoeller) on a sample dried to constant weight in a high vacuum at 80° , loss of weight (H_2O) 9.69 %; 9.42 %. $C_{14}H_{11}O_8K$, $2H_2O$ requires 9.43 % H_2O . Found on dried material, C, 48.40; H, 3.60; K, 10.21 %. $C_{14}H_{11}O_8K$ requires C, 48.52; H, 3.20; K, 11.29 %.) Hence, as with citromyccetin, the salt prepared by this method is essentially a monopotassium salt.

Ethyl fulvate. Various attempts to make methyl fulvate failed. These included the following: boiling the K salt with methyl iodide in dry methyl alcohol (no action); boiling the acid with 3 % methyl alcoholic HCl (mostly unchanged); boiling with saturated methyl alcoholic HCl (no crystalline product isolated). A beautifully crystalline ethyl ester was however obtained, though in small yield, by the following method. Fulvic acid (1 g.) was boiled with saturated ethyl alcoholic HCl (150 ml.) for $2\frac{1}{2}$ hours. Most of the alcohol was then distilled off, and addition of water to the residue precipitated a brown amorphous solid from which nothing crystalline could be obtained. The aqueous mother-liquor from this amorphous solid deposited a considerable amount of yellow, crystalline material overnight in the cold room. This was collected, dried and extracted with a little boiling benzene. The residue, after many crystallisations from aqueous alcohol, yielded some 30 mg. of colourless, slender, silky needles; m.p. $230-234^\circ$ (decomp.), after darkening from 200° onwards, but the effervescence is not so vigorous as with the parent substance.

(Found (duplicates by independent analysts): C, 57.11, 57.31; H, 4.86, 4.98; OEt, 13.88 %. $C_{14}H_{11}O_7 \cdot OC_2H_5$ requires C, 57.14; H, 4.80; OEt, 13.41 %.) Hence the substance is monoethyl fulvate. It gives a green coloration with ferric chloride in aqueous alcoholic solution. It dissolves immediately in aqueous

sodium bicarbonate to a colourless solution, and addition of HCl then precipitates the unchanged ester. Its solution in glacial acetic acid (colourless) develops a yellow colour and a greenish blue fluorescence on addition of a few drops of concentrated HCl. Concentrated HBr however produces only a yellow colour and no fluorescence. Corresponding experiments with the parent acid gave neither colour nor fluorescence.

Trimethyl derivative of fulvic acid. Fulvic acid (0.8 g.) suspended in dry ether was treated with an ethereal solution of diazomethane prepared from nitroso-methylurethane (6 ml.). Evolution of N_2 was very slow and the acid did not appear to go into solution. The reaction mixture was kept overnight and the insoluble part was then collected. It consisted of almost colourless crystals (0.6 g.), which gave no reaction with ferric chloride in alcoholic solution, and was purified by two crystallisations from benzene (200 ml.) containing a little light petroleum, and then from dioxan (2.5 ml. per 100 mg.). From either solvent it formed colourless, microscopic crystals, m.p. 192° , with effervescence and some darkening. (Found on material dried to constant weight *in vacuo* over P_2O_5 (duplicates by independent analysts): C, 58.18, 58.32; H, 5.09, 5.21; OCH_3 , 26.39, 27.02 %. $C_{14}H_9O_5(OCH_3)_3$ requires C, 58.26; H, 5.18; OCH_3 , 26.59 %.)

The above trimethyl derivative was prepared by the same method from specimens of fulvic acid yielded by all three moulds, and the same compound was obtained in each case as the following melting points and mixed melting points show.

A. Derivative from fulvic acid from *P. griseo-fulvum* (crystallised from aqueous dioxan): m.p. $186-188^\circ$.

B. Derivative from fulvic acid from *P. flexuosum* (crystallised from benzene-light petroleum): m.p. $188-192^\circ$.

C. Derivative from fulvic acid from *P. Brefeldianum*, described above; m.p. 192° .

Mixture of A + C: m.p. $187-190^\circ$.

Mixture of B + C: m.p. 188° .

Like fulvic acid, this derivative is sparingly soluble in all solvents. It is not sufficiently soluble in cold dioxan to allow an accurate cryoscopic determination of its molecular weight. It is slightly soluble in boiling water giving a solution with a striking blue-violet fluorescence. It gives no coloration with ferric chloride and is slowly soluble in cold dilute alkali. Addition of a few drops of concentrated HCl to the colourless solution of the trimethyl derivative in glacial acetic acid produces a pale yellow colour and an intense blue fluorescence. Concentrated HBr gives a more intense yellow colour but no fluorescence.

The same product is obtained by methylation of fulvic acid in sodium bicarbonate solution with dimethyl sulphate.

Triethyl derivative of fulvic acid. Fulvic acid (0.8 g.) was treated with ethereal diazoethane prepared from nitrosoethylurethane (6.5 ml.). The reaction was vigorous and all the material soon dissolved. After standing overnight, a colourless substance (0.5 g.) had separated. This was collected and twice crystallised from benzene (50 ml.) and light petroleum (50–100 ml.). It formed colourless aggregates of microscopic crystals; m.p. $172-174^\circ$ with effervescence to a red liquid which did not reset on cooling. (Found (duplicates by independent analysts): C, 61.04, 61.28; H, 6.31, 6.36; OEt , 33.6, 35.2 %. $C_{14}H_9O_5(OC_2H_5)_3$ requires C, 61.20; H, 6.17; OC_2H_5 , 34.4 %.) 0.0781 g. in 5.41 g. dioxan depressed the f.p. of the latter by 0.180° . Hence mol. wt. = 377; $C_{20}H_{24}O_8$ requires mol. wt. 392.

The properties of the triethyl derivative are very similar to those of the trimethyl derivative, save that it is more soluble in certain solvents such as

benzene and dioxan. It gives no colour with FeCl_3 . It gives a yellow colour and a brilliant blue fluorescence with concentrated HCl and glacial acetic acid.

Diacyl derivative of fulvic acid. To a solution of fulvic acid (6 g.) in glacial acetic acid (36 ml.) were added acetic anhydride (24 ml.) and anhydrous sodium acetate (7 g.), and the mixture was boiled for 20 minutes (oil bath at $140\text{--}150^\circ$); some darkening took place. It was then cooled and poured into ice-water, but nothing separated. Acidification with dilute HCl threw down a copious, pale yellow, sandy, crystalline precipitate which was filtered off immediately (5.4 g.). The product was purified by crystallisation from alcohol, then twice from chloroform-light petroleum, then from dioxan-light petroleum and finally from aqueous dioxan. It formed very pale yellow, minute, hexagonal prisms or square tablets which did not melt at 310° , but considerable darkening and shrinking occurred above 220° . It is almost insoluble in boiling benzene and carbon tetrachloride, sparingly soluble in boiling chloroform, moderately readily soluble in boiling ethyl alcohol or cold methyl alcohol, glacial acetic acid or dioxan. Its solution in aqueous dioxan or acetic acid has a marked blue fluorescence intensified by the addition of concentrated HCl . It is immediately soluble in aqueous NaHCO_3 or sodium acetate to yield almost colourless solutions with a marked blue fluorescence. It gives no colour with FeCl_3 in aqueous alcoholic solution. (Found (Schoeller): C, 57.77, 57.85; H, 3.89, 3.79 %. Mol. wt. (cryoscopic in dioxan), 319. $\text{C}_{18}\text{H}_{14}\text{O}_9$ (i.e. diacyl derivative of $\text{C}_{14}\text{H}_{10}\text{O}_7$) requires C, 57.74; H, 3.77 %. Mol. wt. 374.)

Titration: 0.2305 g. in 35 ml. methyl alcohol + 10 ml. H_2O required about 9 ml. $N/10$ NaOH for the first transient end-point to phenolphthalein, but a permanent end-point appeared to have been reached at 12.2 ml. $N/10$ NaOH . This corresponds to an equivalent of 189 (theoretical for $\text{C}_{18}\text{H}_{14}\text{O}_9$ titrating as a dibasic acid = 187). The acetyl derivative is readily hydrolysed by cold dilute NaOH as was shown in the following way: 0.1529 g. was dissolved in 20.0 ml. $N/10$ NaOH , and the solution kept at room temperature for $3\frac{1}{2}$ hours; the yellow colour became much intensified. Back-titration with $N/10$ H_2SO_4 then showed that 15.5 ml. $N/10$ NaOH had been used up, corresponding with an equivalent of 99 (theoretical for $\text{C}_{18}\text{H}_{14}\text{O}_9$ titrating as a tetrabasic acid, 94). Acidification of the titrated liquid then yielded 0.10 g. of almost pure fulvic acid (theoretical, 0.12 g.).

Acetyl estimation. 0.4418 g. was boiled for 6 hours with 50 ml. N H_2SO_4 . 300 ml. H_2O were then added and the liquid distilled at atmospheric pressure until only 50 ml. remained. This was repeated seven times until a constant, very small titration was obtained for volatile acid in the distillate. A net amount of volatile acid equivalent to 21.8 ml. $N/10$ NaOH was produced in all, the theoretical for $\text{C}_{18}\text{H}_{14}\text{O}_9$ yielding $2\text{CH}_3\cdot\text{CO}_2\text{H}$ being 23.6 ml. A parallel experiment showed that fulvic acid yielded no volatile acid at all under these conditions.

It will be noticed that the empirical formula for this acetyl compound $\text{C}_{18}\text{H}_{14}\text{O}_9$ corresponds to an empirical formula for the parent substance of $\text{C}_{14}\text{H}_{10}\text{O}_7$. The empirical formula we have assigned to fulvic acid, $\text{C}_{14}\text{H}_{12}\text{O}_8$, follows from the analysis of the trimethyl and triethyl derivatives, of the monoethyl ester and of the free acid dried to constant weight at 138° in nitrogen or at 110° in a high vacuum. On the other hand, fulvic acid on sublimation in a high vacuum at 170° gives analytical results agreeing well with the empirical formula $\text{C}_{14}\text{H}_{10}\text{O}_7$. Hence we believe that fulvic acid readily loses a molecule of water under certain conditions, e.g. acetylation or sublimation, and we hope to find the explanation for this fact as a result of experiments at present in progress on the molecular constitution of fulvic acid.

SUMMARY.

Penicillium griseo-fulvum Dierckx, *P. flexuosum* Dale and *P. Brefeldianum* Dodge when grown on a synthetic medium containing glucose, ammonium tartrate and mineral salts all give rise to a hitherto undescribed metabolic product for which the name fulvic acid is proposed. Fulvic acid, $C_{14}H_{12}O_8$, M.P. 246° (decomp.) is a crystalline yellow pigment. The following derivatives were prepared and are described: potassium salt, monoethyl ester, trimethyl, triethyl and diacetyl derivatives.

P. griseo-fulvum and *P. flexuosum* also produce 6-methylsalicylic acid.

Two different colourless crystalline compounds containing nitrogen have been isolated—one, M.P. 165° (decomp.), from the mycelium of *P. griseo-fulvum* and the other, M.P. 132 – 135° , from the mycelium of *P. Brefeldianum*.

REFERENCES.

- Anslow and Raistrick (1931). *Biochem. J.* **25**, 39.
van Beijma (1929). *Verhandl. Akad. Wetensch. Amsterdam, Afd. Natuurk.* [Tw. Sect.] **62**, [4], 16.
Brefeld (1874). *Botanische Untersuchungen über Schimmelpilze*, Heft 2. (Leipzig.)
Dodge (1933). *Mycologia*, **25**, 90.
Eigenberger (1931). *J. prakt. Chem.* **130**, 78.
Hetherington and Raistrick (1931). *Phil. Trans. Roy. Soc. Lond. B* **220**, 209.
Lockwood, Ward, May, Herrick and O'Neill (1934). *Z. Bakt.* **90**, 411.
Oxford (1934). *Biochem. J.* **28**, 1325.
—— and Raistrick (1933). *Biochem. J.* **27**, 1176.
—— — (1934). *Biochem. J.* **28**, 1321.
Raistrick and Simonart (1933). *Biochem. J.* **27**, 628.
Shear (1934). *Mycologia*, **26**, 104.
Thom (1930). *The Penicillia*. (Baillière, Tindall and Cox, London.)