24. Studies in the Biochemistry of Micro-organisms

70. Stipitatic Acid, $C_8H_6O_5$, a Metabolic Product of *Penicillium stipitatum* Thom

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Penicillium stipitatum Thom was described as a new species by Emmons [1935]. It grows well on the ordinary solid culture media giving yellow, orange or even reddish orange colonies. The most marked morphological characteristic of the fungus is its very free production of yellow ascocarps.

We have found that when this species is grown on the well-known Czapek-Dox solution the metabolism solution gives a characteristic deep red colour with ferric chloride. The metabolic product which is responsible for this colour has been isolated in considerable yield and, since it has not been described previously, we propose to name it *stipitatic acid*.

Stipitatic acid, $C_8H_6O_5$, forms cream-coloured needles, M.P. 302–304° (decomp.). It is optically inactive, contains no *C*-methyl group and has three active hydrogen atoms (Zerewitinoff). It titrates as a dibasic acid, the neutral aqueous solution of the disodium salt being deep yellow in colour. It forms two different but isomeric diacetates, one with acetic anhydride and anhydrous sodium acetate, the other with acetic anhydride and conc. H_2SO_4, both of which are soluble in aqueous sodium acetate. The former titrates as a monobasic, the latter as a dibasic, acid. The following crystalline and well-defined derivatives of stipitatic acid have also been prepared.

(*a*) Two isomeric trimethyl derivatives (mol. wt: 240–246. Theor. for $C_8H_3O_2.(OCH_3)_3=224$) which are formed by the action of diazomethane in ether. These are both neutral substances and are insoluble in cold dilute aqueous NaOH.

(*b*) A dimethyl derivative which is formed either by heating stipitatic acid with methanol containing 3% HCl or by treatment of its disodium salt with methyl iodide. This derivative is readily soluble in dilute NaOH but insoluble in $NaHCO_3$.

(*c*) A monomethyl derivative which is formed by treating the acid with methyl sulphate and KOH (excess) in methanol. This derivative titrates as a dibasic acid.

(*d*) Monobromostipitatic acid, $C_8H_5O_5Br$, a dibasic acid which gives a neutral trimethyl derivative with diazomethane.

The formation of the above derivatives proves the presence in stipitatic acid of two hydroxyl groups and indicates the probable presence of one carboxyl group. The presence of one carboxyl group was established by heating stipitatic acid with copper chromite and quinoline, a method commonly used for the decarboxylation of carboxylic acids [Adkins & Connor, 1931; Kinney & Langlois, 1931]. One molecule of CO_2 is evolved and a substance, $C_7H_6O_3$, is formed which titrates as a monobasic acid and gives a striking blood red precipitate with $FeCl_3$. It is thus clear that, of the five oxygen atoms present in stipitatic acid two are in the form of hydroxyl groups, one of which has more strongly developed acidic properties than the other, and two as a carboxyl group.

The function of the remaining oxygen atom did not become clear for some time since tests with a large number of reagents for aldehyde and ketone groups which were carried out on stipitatic acid and on a number of the derivatives mentioned above gave uniformly

negative results. Paradoxically, however, positive tests for a ketonic group (though still negative for an aldehyde group) were obtained on the mixture of substances produced by the *reduction* of stipitatic acid either at room temperature with hydrogen and platinum oxide or by boiling with zinc and glacial acetic acid. One of the products of the catalytic reduction of stipitatic acid is a tetrahydrostipitatic acid, $C_8H_{10}O_5$, which was isolated as the mono-2:4-dinitrophenylhydrazone and the main product of the reduction with zinc and acetic acid is a ketone, C_6H_8O, which was also isolated as a mono-2:4-dinitrophenylhydrazone. Hence it follows that the fifth oxygen atom in stipitatic acid is present as a masked ketonic group giving none of the typical ketonic reactions but becoming unmasked on reduction and then reacting in a normal fashion. A fact of some potential importance in relation to the structural formula for stipitatic acid is that the total titratable acidity of the products of the catalytic reduction of stipitatic acid is only half that of stipitatic acid itself, although no CO_2 is formed during reduction.

Finally, perhaps the most surprising reaction of stipitatic acid is the ease with which, on fusion with KOH, it or its monomethyl derivative is converted, in very good yield, into the isomeric 5-hydroxy*iso*phthalic acid, i.e. 5-hydroxy-1:3-dicarboxybenzene.

It must be admitted that, in spite of the large amount of experimental work which has been carried out on this substance, we have been unable up to the present to deduce an entirely satisfactory structural formula for it. Amongst other possibilities considered was a formyldihydroxybenzene carboxylic acid, $C_6H_2(OH)_2(CHO)COOH$, in which the normal reactions of the aldehyde group are masked. Such a compound might be 2-formyl-3:5-dihydroxybenzoic acid. This has been synthesized here [Birkinshaw & Bracken, 1942]. It was not identical with stipitatic acid and gave typical aldehyde reactions. The molecular constitution of stipitatic acid must therefore remain for the time being unsolved since, because of prevailing conditions, it has become necessary to postpone further work on the subject. It would seem, however, that, in spite of its relatively simple empirical formula, $C_8H_6O_5$, and the ease with which it can be transformed into a readily recognizable isomeric benzene derivative, i.e. 5-hydroxy*iso*phthalic acid, stipitatic acid may belong to a class of compounds hitherto not encountered among mould metabolic products. Puberulic acid, $C_8H_6O_6$, a metabolic product of *Penicillium puberulum* Bainier and *P. aurantio-virens* Biourge [Birkinshaw & Raistrick, 1932], the empirical formula for which differs from that of stipitatic acid only by one oxygen atom, may also be a compound of the same type since the two acids show certain similarities in general behaviour and offer similar resistance to attempts to elucidate their molecular constitution [Barger & Dorrer, 1934].

Experimental

History and description of culture

We are indebted to our colleague, Mr G. Smith, for the information given in this section.

Penicillium stipitatum Thom was described as a new species by Emmons [1935]. It was isolated from rotting wood in Louisiana and was sent to Dr C. Thom's laboratory by T. C. Sheffer. The culture used throughout this work, L.S.H.T.M. Cat. No. P 199, was obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland in October 1935 and was derived from Emmons's original strain. The fungus grows well on all the common culture media, at first pale bright yellow, floccose and somewhat funiculose, turning duller and often somewhat reddish in age; more or less granular in appearance due to the development of numerous perithecia; reverse and medium yellow, then dull orange and finally reddish brown. The most marked characteristic of the mould is its very free production of yellow ascocarps. Conidial production, on the other hand, is very scanty and is never sufficient to give the colonies any noticeable greenish tint.

Cultural conditions

Batches of 100 1-l. conical flasks were prepared, each containing 350 ml. of Czapek-Dox solution, i.e. glucose, 50·0 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. After sterilization, each flask was sown with a spore suspension, in sterile distilled water, of *P. stipitatum* Thom (L.S.H.T.M. Cat. No. P 199, prepared from mature cultures grown on beer-wort agar slopes). We are indebted to our colleague, Mr G. Smith, for the preparation of the cultures and the inoculation of the flasks.

The inoculated flasks were incubated in the dark at 24° for about 7 weeks. At the end of the incubation period the flasks were uniformly covered with a bright yellow felt which was separated from the metabolism solution by filtration, washed, dried and reserved for future investigation. The yellow-orange metabolism solution, usually still containing about 1·5 % of glucose (by polarimeter) gave a fine deep red colour with a little aqueous $FeCl_3$ and, with excess of this reagent, a browner colour usually accompanied by deposition of a copious brown precipitate on standing. Neutral lead acetate gave a bright yellow gelatinous precipitate and a clear, colourless supernatant liquid.

Details of a number of representative experiments are given in Table 1.

Isolation and purification of stipitatic acid

The filtered metabolism solution and mycelium washings from each batch of 100 flasks were evaporated *in vacuo* at 45–50° to about 2 l. The evaporated solution was kept in the cold room until the separation of a yellow-brown solid was complete, usually 2–3 days. This solid, which consists essentially of a crude magnesium salt of stipitatic acid, was separated by filtration, washed with cold water and ground with successive 200 ml. amounts of 2*N* HCl until substantially free from ash, two or three lots of acid being generally required. The ash-free residue consists of crude stipitatic acid. The HCl washings were combined with the main filtrate and the mixture, now acid to Congo red, was extracted five or six times with 2 l. of ether. The ether extracts were washed with water, dried over anhydrous $MgSO_4$, and evaporated to about 1/10 of the original volume when crude stipitatic acid separated and was collected. On evaporation to dryness the ether mother liquors gave a sticky residue containing very little stipitatic acid.

The crude stipitatic acids from the magnesium salt and from the ether extract were combined and purified by repeated crystallization from boiling water (with norite) about 100 ml. being required for each g. of crude acid. Pure stipitatic acid was finally obtained, in a 50–60 % yield of the crude product, in the form of fine, cream-coloured needles.

Details of yields obtained in a number of representative experiments, 100 flasks in each experiment, are given in Table 1.

Table 1

Incubation period days	Glucose by polarimeter %	*p*H	Titration *N* NaOH per 100 ml. of solution ml.	Bromine absorption (Koppeschaar) mg. of Br/ml.	Yield of crude product g.	Yield of pure stipitatic acid g.
48	1·49	4·2	3·64	3·21	21	10·5
50	1·47	4·1	3·74	2·80	21	10·2
55	1·30	4·0	2·98	2·81	23	13·0
49	1·56	4·0	3·60	3·93	38	20·0
48	1·43	4·0	3·80	3·70	30	15·0

General properties of stipitatic acid

The purest specimens of stipitatic acid crystallized from water in well-formed needles of a definite cream colour. Even on sublimation in a high vacuum at 190° a colourless product was not obtained, the sublimate consisting of light yellow needles. When placed

in a bath pre-heated to 280° the acid darkens, on further heating, at 295° and blackens and melts with evolution of gas at 302–304°. (Found: C, 52·61, 52·62; H, 3·45, 3·22 %; N, nil; Cl, nil; CH_3O, nil; equiv. by titration, 87·6. $C_8H_6O_5$ requires C, 52·73; H, 3·32 %; equiv., as a dibasic acid, 91·0.)

In a duplicate Zerewitinoff estimation carried out in pyridine at 21° and 15° respectively the acid gave methane corresponding to 3·3 and 3·0 active H atoms per mol.

It is optically inactive. 0·2537 g. dissolved in ethanol (25 ml.) gave, in a 2 dm. tube, a rotation of −0·01° in the mercury yellow light. It contains no *C*-methyl group as determined by the Kuhn-Roth method.

Stipitatic acid is only slightly soluble in cold water though moderately soluble on heating. Its solubility in ethanol is about 1 in 100 at room temperature, but it is almost insoluble in benzene and chloroform. It dissolves readily in alkalis, CO_2 being evolved with Na_2CO_3 or $NaHCO_3$, and the neutral aqueous solution of the disodium salt is strongly yellow in colour which makes accurate titration difficult. Curiously enough the acid is readily soluble in cold conc. HCl or HNO_3 and separates unchanged on pouring these solutions into water. With cold conc. H_2SO_4 it gives a colourless solution changing, on gentle warming, to a deep dirty brown.

With $FeCl_3$ in aqueous solution it gives an immediate deep red colour and then a rusty brown precipitate with excess of $FeCl_3$. In alcoholic solution the initial reaction is a deep red colour turning brownish green on addition of excess $FeCl_3$. An aqueous solution of the acid gives a bright yellow precipitate with lead acetate and a quickly fading deep orange colour with bleaching powder solution.

In spite of repeated attempts to detect aldehyde or ketonic groups in stipitatic acid only negative results were obtained. The reagents employed were: Schiff; 2:4-dinitrophenylhydrazine in 2*N* HCl [Brady & Elsmie, 1926]; cold ammoniacal $AgNO_3$; $KI + HgI_2$ [Doeuvre, 1927]; sodium pentacyanoaminoferroate + ammonium hydrosulphide [Feigl, 1934]; the salicylaldehyde reaction for the —$CH_2.CO.CH_2$— group [Täufel & Thaler, 1932]; no reaction took place on heating with aniline in an attempt to prepare the anil of stipitatic acid; no evidence of oxime formation could be obtained.

Derivatives of stipitatic acid

(I) *Acetyl derivatives*

(*a*) *With acetic anhydride and sodium acetate.* A mixture of stipitatic acid (1 g.) anhydrous sodium acetate (2 g.) and acetic anhydride (4 ml.) was heated in an oil bath initially at 85° but raised after some minutes to 105° to complete the acetylation. Only slight darkening occurred, and after the acid and sodium acetate had dissolved crystals soon began to separate. The mixture was cooled, diluted with water (10 ml.) to give a clear solution which, on acidification with 2*N* H_2SO_4 (15 ml.), quickly deposited crystals. These were collected, washed with water and dried. Wt. 1·41 g., M.P. 172·5°, unchanged on recrystallization from water from which the diacetate separates in cream-coloured needles which give only a greenish yellow colour with $FeCl_3$ in alcoholic solution. (Found: C, 54·06, 54·10; H, 3·85, 3·90; CH_3CO, 31·35 %; mol. wt. in camphor, 308, 327, decomposition occurring; equiv. by titration, 265. $C_{12}H_{10}O_7$, i.e. $C_8H_4O_3(O.CO.CH_3)_2$ requires C, 54·12; H, 3·79; $2CH_3CO$, 32·3 %; mol. wt. 266; equiv., titrating as a monobasic acid, 266.)

This acetate reacted vigorously with ethereal diazomethane but the reaction product was a syrup which was not obtained crystalline.

(*b*) *With acetic anhydride and conc.* H_2SO_4. Stipitatic acid (0·5 g.) was dissolved in 3 ml. of a cold mixture of acetic anhydride (10 ml.) and conc. H_2SO_4 (0·5 ml.) and the solution was kept at room temperature for 3 days. The resulting pale greenish yellow solution was poured into water (15 ml.) and the crystalline precipitate which quickly

separated was collected, washed with water and dried. Wt. 0·37 g. This diacetylstipitatic acid, which was crystallized from ethyl acetate, forms pale yellow needles, M.P. 176–178° (decomp.), depressed to 160–162° on admixture with the diacetylstipitatic acid, M.P. 172·5°, described in section I (*a*) above. It gives a pale olive brown colour with $FeCl_3$ in alcoholic solution. (Found: C, 54·10, 53·95; H, 3·99, 3·84; CH_3CO, 28·7 %; equiv. by titration, 128. $C_{12}H_{10}O_7$ requires C, 54·12; H, 3·79; $2CH_3CO$, 32·3 %; equiv., titrating as a dibasic acid, 133.)

II. *Methylated derivatives of stipitatic acid*

(*a*) *Monomethyl derivative.* Stipitatic acid (1 g.) was suspended in methanol (10 ml.) and to the heated mixture there were added in small amounts and at intervals during about 30 min. methyl sulphate (10 ml.) and 20 % KOH (40 ml.). The reaction was kept permanently alkaline and when all the reagents had been added the mixture was boiled for a further 30 min. to hydrolyse any ester present. The cooled solution was now acidified with conc. HCl and the precipitated methyl derivative was collected, washed with water and dried. Wt. 0·82 g., M.P. 245–255°, raised to 273° (decomp.) (wt. 0·34 g.) by two crystallizations from methanol. This monomethyl derivative of stipitatic acid forms cream-coloured needles which give a deep reddish brown colour with $FeCl_3$ in alcoholic solution, almost indistinguishable from that given by stipitatic acid itself. (Found: C, 54·82, 54·99; H, 4·06, 4·22; CH_3O, 15·6, 15·9%; equiv. by titration, 97·4. $C_9H_8O_5$ requires C, 55·11; H, 4·11; $1CH_3O$, 15·8 %; equiv. titrating as a dibasic acid, 98.)

During the titration of this derivative with $N/10$ NaOH to phenolphthalein the solution remained almost colourless until half of the final volume of NaOH had been added. It then took on a strong yellow colour which remained almost to neutrality.

(*b*) *Dimethyl derivative.* A solution of stipitatic acid (0·5 g.) in methanol (50 ml.) containing 3 % of HCl was refluxed for 8 hr. There was considerable darkening in colour. Most of the methanol was removed by evaporation *in vacuo*. The residue was diluted with water (200 ml.), neutralized by the careful addition of aqueous $NaHCO_3$ and the resulting yellow, turbid solution was extracted with ether. The washed and dried ethereal extract, on removal of the solvent, gave a solid (0·1 g., M.P. 146–148°) which was purified by crystallization from methanol giving pale yellowish needles, M.P. 163–165°. (Found: C, 57·46, 57·50; H, 4·86, 4·93; CH_3O, 28·2 %. $C_{10}H_{10}O_5$, i.e. $C_8H_4O_3(OCH_3)_2$ requires C, 57·12; H, 4·80; $2CH_3O$, 29·5 %.)

This dimethyl derivative is insoluble in cold aqueous $NaHCO_3$, readily soluble in N NaOH and gives a reddish brown colour with $FeCl_3$ in alcohol. The same substance was prepared by heating the disilver salt of stipitatic acid with methyl iodide, the identity of the two products being evident from the fact that they have the same M.P. and show no depression in M.P. on mixing.

(*c*) *Trimethyl derivatives.* An excess of ethereal diazomethane (from 5 ml. of nitrosomethylurethane) was added to a suspension of stipitatic acid (1 g.) in a little dry ether. The initial vigorous evolution of nitrogen ceased after about an hour and the methyl derivative which separated on the sides of the flask was then collected. Wt. 0·43 g., M.P. 173–174°. This product which was purified by crystallization from acetone, in which it is not very soluble, is referred to as trimethylstipitatic acid A. It forms cream-coloured, long needles, M.P. 189–190°. The ethereal filtrate from the crude trimethylstipitatic acid A was evaporated to dryness to remove residual diazomethane, washed with a little ether to remove a trace of oily material, and the undissolved residue (0·40 g., M.P. 122–130°) was purified by crystallization from light petroleum, B.P. 60–80°. Trimethylstipitatic acid B separates from this solvent in small, light yellow needles, M.P. 126–128°, unchanged on sublimation in a high vacuum at 105°. (Found: (*a*) On trimethylstipitatic acid A. C, 58·82, 58·78; H, 5·52, 5·39; N, nil; CH_3O, 40·4 %; mol. wt. in camphor 241, 245. (*b*) On trimethylstipitatic acid B. C, 58·48, 58·62; H, 5·16, 5·27; CH_3O, 41·5 %; mol. wt.

in camphor, 240, 246. $C_{11}H_{12}O_5$, i.e. $C_8H_3O_2(OCH_3)_3$ requires C, 58·92; H, 5·40; $3CH_3O$, 41·5%; mol. wt. 224.)

Neither of these trimethyl derivatives is soluble in dilute NaOH or gives an appreciable colour with $FeCl_3$ in alcohol. Neither of them gives a positive test with Schiff, Doeuvre, Brady or Feigl's reagents nor do they reduce ammoniacal $AgNO_3$.

III. *Monobromostipitatic acid*

Stipitatic acid appears to give a loose addition compound with bromine in aqueous solution and does not react with bromine in glacial acetic acid solution. In acetic acid containing about 20% of water monobromostipitatic acid is formed in good yield.

A *N* solution of bromine in glacial acetic acid, in slight excess of the amount required for 2 atoms of bromine per mol. of stipitatic acid, was added to a solution of stipitatic acid (1·82 g.) in glacial acetic acid (450 ml.). The mixture showed no change in colour after 2 hr., but on addition of water (100 ml.) the colour quickly became much lighter. After a further hour the solution was evaporated to small bulk *in vacuo* and the yellow solid which separated was collected (2·06 g.) and crystallized from methanol. It may also be purified, but less conveniently, by crystallization from water. *Monobromostipitatic acid* forms pale yellow blunt-ended needles, M.P. 275°, with blackening and gas evolution. (Found: C, 36·90, 37·01; H, 2·06, 1·93; Br, 30·2, 30·4%; equiv. by titration (poor end-point), 124·1. $C_8H_5O_5Br$ requires C, 36·80; H, 1·93; Br, 30·6%; equiv., titrating as a dibasic acid, 130·5.)

An alcoholic solution of the acid gives with $FeCl_3$ a red colour similar to that given by the parent substance but somewhat deeper in tint. The bromine in the molecule is not converted into a form precipitable with $AgNO_3$ by either boiling *N*/4 H_2SO_4 or *N*/4 NaOH but boiling dilute HNO_3 effects a vigorous oxidation with the formation of HBr.

Trimethylbromostipitatic acid. Monobromostipitatic acid reacts vigorously with ethereal diazomethane to give the trimethyl derivative. Yellow plates *ex* acetone, M.P. 175°, which sublime without decomposition in a high vacuum at 130–135° and give no appreciable colour with $FeCl_3$. (Found: C, 43·66; H, 3·71; Br, 26·1; CH_3O, 30·2%. $C_8H_2O_2Br(OCH_3)_3$ requires C, 43·57; H, 3·66; Br, 26·4; $3CH_3O$, 30·7%.)

Decarboxylation of stipitatic acid

A mixture of stipitatic acid (0·5013 g.), copper chromite catalyst (0·2 g.) [Adkins & Connor, 1931] and quinoline (7 g.), contained in a small flask fitted with an air condenser, was heated in an oil bath. A slow stream of CO_2-free nitrogen was passed through the apparatus and the issuing gases were bubbled through *N*/10 aqueous barium hydroxide. CO_2 began to be evolved at a bath temperature of 200° and the bath was maintained at 220°. After 2 hr. CO_2 was evolved equivalent to 60%, and after 4 hr. 98% of the theoretical required for the evolution of 1 mol. CO_2 per mol. of stipitatic acid. The contents of the flask were then cooled, acidified with 2*N* HCl (100 ml.) and extracted with ether. On removal of the solvent a solid (0·20 g.) remained which was purified by the addition of 4–5 vol. of light petroleum to its acetone solution. This treatment precipitated a small amount of flocculent material which was separated and rejected, and the filtrate, on evaporation to low bulk, deposited micro-crystals, 0·11 g., M.P. 210°, which were sublimed twice in a high vacuum at 140°. The cream-yellow sublimate, consisting of small elongated plates or flattened needles, melted at 227–228° and gave a striking blood red precipitate with $FeCl_3$ in aqueous solution. (Found: C, 61·01, 61·05; H, 4·32, 4·40%; equiv. by titration, 132. $C_7H_6O_3$ requires C, 60·85; H, 4·38%; equiv., titrating as a monobasic acid, 138.) The substance gives a bright yellow solution in dilute NaOH. It gives no precipitate with 2:4-dinitrophenylhydrazine in 2*N* HCl.

Potash fusion of stipitatic acid

A mixture of stipitatic acid (1 g.), KOH (5 g.) and water (2 ml.) was heated in a metal bath. The temperature was raised slowly and at 240° (bath temperature) a further 5 g. of KOH were added to increase the fluidity of the yellow, viscous mass. The yellow colour remained up to 300° when it changed to a light brown but no charring occurred. After 10 min. at 300° the melt was cooled, dissolved in water and acidified with HCl. On chilling, crystals separated (0·36 g., M.P. 275°) and were collected. Treatment of the filtrate is described below. The crystals were neutralized with NaOH, the solution decolorized with norite and acidified. The colourless crystals which separated proved to be 5-hydroxy-*iso*phthalic acid, M.P. 304°, not depressed on admixture with an authentic specimen prepared from *iso*phthalic acid [Beyer, 1882; Storrs & Fittig, 1870]. Both the fusion product and the synthetic specimen gave only a yellow colour with $FeCl_3$ in ethanol. (Found: C, 52·74, 52·85; H, 3·27, 3·54 %. Calc. for $C_8H_6O_5$: C, 52·73; H, 3·32 %.)

The identity of the acid was confirmed by methylation with ethereal diazomethane. After removal of the solvent and excess of diazomethane the solid residue was sublimed in a high vacuum at 75°. The colourless crystalline sublimate of *5-methoxydimethyl-isophthalate* melted at 109°. A specimen prepared in the same way from synthetic 5-hydroxy*iso*phthalic acid melted at the same temperature, as did a mixture of the two specimens. (Found: C, 58·84, 58·87; C, 5·47, 5·47; CH_3O, 40·8 %. $C_6H_3(OCH_3)(COOCH_3)_2$ requires C, 58·92; H, 5·40; $3CH_3O$, 41·5 %.)

The acid filtrate from the 5-hydroxy*iso*phthalic acid was extracted with ether. The ether extract was freed from carboxylic acids by shaking with aqueous $NaHCO_3$. On removal of the solvent the small amount of crystalline residue was sublimed. The sublimate, colourless needles, M.P. 103°, proved to be slightly impure resorcinol. It gave the same colour reactions as resorcinol with $FeCl_3$ and with sodium hypochlorite and on admixture with resorcinol the M.P. was intermediate between that of the fusion product and pure resorcinol, 110°.

The carboxylic acids extracted by $NaHCO_3$ proved to be a further amount of 5-hydroxy-*iso*phthalic acid (0·04 g.) and a trace of oxalic acid.

Fusion of the monomethyl derivative of stipitatic acid (M.P. 273°, 0·25 g., see p. 246) with KOH (2·5 g.) and water (1 ml.) for 15 min. at 300–320° gave an almost quantitative yield (0·23 g.) of only slightly impure 5-hydroxy*iso*phthalic acid.

Fusion of monobromostipitatic acid with KOH led to the isolation of about 10 % of 5-hydroxy*iso*phthalic acid together with a complex mixture of acids which were probably phenolic in nature. From this mixture small amounts of solids were isolated of varying M.P. and $FeCl_3$ reactions (violet-blue and green) none of which were, however, satisfactorily characterized.

Reduction experiments with stipitatic acid

(1) *Catalytic reduction.* A solution of stipitatic acid (1 g.) in ethanol (100 ml.) was shaken in an atmosphere of hydrogen with 0·2 g. of platinum oxide catalyst [Voorhees & Adams, 1922]. The primary quick absorption of hydrogen lasted 40 min. when 4·64 mol. H_2 (corr. for blank) were absorbed per mol. of stipitatic acid. The residual gas in the absorption apparatus was bubbled through standard baryta but no CO_2 was detected. The catalyst was removed by filtration. 5 ml. of the clear colourless alcoholic filtrate required 2·76 ml. *N*/10 NaOH for neutralization to phenolphthalein.. Since 5 ml. of the same solution before reduction required 5·92 ml. *N*/10 NaOH for neutralization it is clear that approximately half of the titratable acidity has disappeared during reduction and that this reduction in acidity is not due to the liberation of CO_2. On removal of the solvent *in vacuo* from the rest of the alcoholic filtrate there remained an oil which gave negative tests with Schiff's and Doeuvre's reagents and did not reduce ammoniacal $AgNO_3$ but

which gave a precipitate with 2:4-dinitrophenylhydrazine in 2*N* HCl, a strongly positive Zimmermann test as modified by Callow *et al.* [1938], a red colour with sodium nitroprusside and NaOH, and quickly reduced Fehling's solution in the cold. It seems clear, therefore, that the crude reduction product contains a substance or substances which, while failing to give reactions typical of a CHO group, does give a number of reactions typical of a CO group.

A second experiment, in which 1 g. of stipitatic acid was reduced under conditions as nearly identical as possible with those described above (4·7 mol. H_2 absorbed per g.mol. of stipitatic acid), showed that a complex mixture of substances is formed during the reduction. The hydrogenated oil (1·04 g.) was divided into neutral and acidic fractions by partition between ether and aqueous $NaHCO_3$. The *neutral fraction* consisted of an oil (0·18 g.) which gave a crude 2:4-dinitrophenylhydrazone, M.P. 120–140°, which could not be crystallized. The *acidic fraction* (0·53 g.; equiv. by titration 180, cf. equiv. of stipitatic acid, 91) was also an oil. Part of this material was treated with a solution of 2:4-dinitrophenylhydrazine in 2*N* HCl. The crude yellow dinitrophenylhydrazone (M.P. 175–177°) was separated by filtration, washed, dried and extracted with hot benzene. The insoluble portion was purified by recrystallization from ethanol when a homogeneous product was obtained consisting of fine orange yellow needles, M.P. 218–221° (decomp.), which gave a deep red colour with alcoholic NaOH indicative of a monodinitrophenylhydrazone. (Found: C, 46·00; H, 4·07; N, 15·5 %. $C_{14}H_{14}O_8N_4$, i.e. monodinitrophenylhydrazone of $C_8H_{10}O_5$ requires C, 45·88; H, 3·86; N, 15·3 %.) This product is thus a monodinitrophenylhydrazone of a tetrahydrostipitatic acid, $C_8H_{10}O_5$. Hence it follows that by the catalytic reduction of stipitatic acid a CO group, previously inactive towards 2:4-dinitrophenylhydrazine, becomes reactive towards this reagent.

Catalytic reduction of stipitatic acid with hydrogen, using 5 % palladized $BaSO_4$ as catalyst, was slow. A small yield of an oily reduction product was obtained but this was not characterized.

(2) *Zinc and glacial acetic acid.* Powdered zinc (20 g.) was added over a period of 1 hr. to a boiling solution of stipitatic acid (2 g.) in glacial acetic acid (400 mol.). The heating was then continued for a further 30 min. and the hot solution was filtered. The clear filtrate was cooled, diluted with water (1 l.) and Brady's reagent (600 ml.) was added. A yellow solid slowly separated and was collected next day. Wt. 1·5 g.; M.P. 120–140°. This crude material, which is a mixture of dinitrophenylhydrazones, was fractionated by the addition of light petroleum to its solution in ethyl acetate. In this way the main constituent, amounting to almost half of the crude product, was isolated and was purified by crystallization from a mixture of pyridine and light petroleum. It was thus obtained as fine orange yellow needles, M.P. 188–189°, which gave a red colour with alcoholic NaOH indicative of a monodinitrophenylhydrazone. (Found: C, 51·65, 51·70; H, 4·43, 4·37; N, 20·5 %. $C_{12}H_{12}O_4N_4$, i.e. monodinitrophenylhydrazone of C_6H_8O requires C, 52·16; H, 4·38; N, 20·3 %.) In a second experiment the acetic acid solution from the reduction of 1 g. of stipitatic acid was evaporated to low bulk *in vacuo* and was dried *in vacuo* over solid KOH. A brown uncrystallizable oil was thus obtained which gave a negative Schiff's reaction but a strongly positive Zimmermann reaction. Its solution in aqueous $NaHCO_3$ became blue on standing.

(3) *Other reducing agents.* Stipitatic acid is readily reduced by Clemmensen's method (zinc amalgamated with mercury in 5*N* HCl), by sodium amalgam at room temperature, or by heating with HI and red phosphorus. In each case the reduction product was an oil which had an equivalent of 188 in the Clemmensen reduction product and gave a deep blue colour in aqueous $NaHCO_3$ with the product from HI and red phosphorus.

Oxidation experiments with stipitatic acid

Attempts were made, with a large variety of oxidizing agents, to isolate identifiable products which still contained a considerable portion of the stipitatic acid molecule. All these attempts failed since, even with the mildest oxidizing agents tried, deep-seated degradation of the molecule took place. Only small fragments of the molecule were identified and the results obtained will, therefore, not be given in full detail.

(1) *Aqueous alkaline hypoiodite at room temperature.* About 10 atoms of iodine were absorbed per g. mol. of stipitatic acid. Much iodoform was produced.

(2) *Aqueous periodic acid at room temperature or at 0°.* About 7 atoms of oxygen were absorbed per g.mol. of stipitatic acid. When aqueous solutions of stipitatic acid and periodic acid are mixed the following characteristic colour changes take place: from colourless to amethyst to orange brown to intense iodine brown. No free iodine is present, however, as the solution gives no reaction with starch paper. Ether extraction of the oxidation mixture led to the isolation of a pungent smelling oil. The only oxidation products identified were oxalic acid and glyoxal as the *bis*-2:4-dinitrophenylhydrazone by chromatographic analysis of the mixed dinitrophenylhydrazones (Dr R. A. Webb).

(3) *Boiling 2N HNO_3.* Vigorous gas (containing CO_2) evolution took place. The resulting deep yellow-orange solution gave with Brady's reagent an orange-red precipitate. This could not be crystallized but appears to contain a *bis*dinitrophenylhydrazone as it gave a violet colour with alcoholic NaOH.

(4) *Lead tetraacetate in glacial acetic acid.* Only partial oxidation took place owing to the sparing solubility of the lead salt of stipitatic acid.

(5) *Dakin's reagent (alkaline hydrogen peroxide).* No reaction at room temperature. On warming, the colour darkened to orange brown but there was no evolution of gas and 85 % of the stipitatic acid was recovered unchanged.

(6) *Ozone in water.* Ozonized oxygen was passed for 3 days through a suspension of stipitatic acid (0·4943 g.) in water (50 ml.) and then through standard baryta. CO_2 equivalent to 1 mol./mol. of stipitatic acid was evolved. (Found: 5·38 ml. *N* acid. Required for 1 mol. CO_2, 5·43 ml. *N*.) The titratable acidity of the clear solution, initially 5·43 ml. *N*, increased to 9·24 ml. *N*, after ozonization. About 25 % of the titratable acidity (9·24 ml. *N*) consists of volatile acids and 75 % of non-volatile acids of which half was isolated as oxalic acid. Malonic acid also appeared to be present.

(7) *Ozone in ethyl acetate.* The only breakdown product identified was oxalic acid (dihydrazide, M.P. and mixed M.P. 240–242°). There was also present an unidentified ketonic substance giving a *bis*dinitrophenylhydrazone.

Oxidation experiments with derivatives of stipitatic acid

(1) *Monomethyl derivative with aqueous alkaline hypoiodite.* About 10 atoms of iodine absorbed per mol. Much iodoform was produced.

(2) *Dimethyl derivative and trimethyl derivative, M.P. 189°, with $KMnO_4$ in acetone.* Only oxalic acid was isolated.

(3) *Trimethyl derivative,* M.P. 189°, *with silver oxide in water.* Gave a small amount of a yellow, crystalline, but unidentified substance, *M.P.* 275° (decomp.), with sublimate formation.

SUMMARY

Penicillium stipitatum Thom when grown on Czapek-Dox 5 % glucose solution gives rise to a considerable amount of a hitherto undescribed mould metabolic product which has been named *stipitatic acid*, $C_8H_6O_5$. A number of derivatives and breakdown products have been described, notably the formation in good yield of the isomeric 5-hydroxy*iso*-phthalic acid on potash fusion. It has been shown that of the five oxygen atoms present

in the molecule, two are present as a carboxyl group, two as hydroxyl groups one of which is strongly acidic, and one as a masked CO group. The molecular constitution of stipitatic acid has not been satisfactorily established but the acid is believed to belong to a class of compounds not previously encountered among mould metabolic products, except possibly for puberulic acid from *P. puberulum* Bainier [Birkinshaw & Raistrick, 1932].

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REFERENCES

Adkins, H. & Connor, R. [1931]. *J. Amer. chem. Soc.* **53**, 1091.
Barger, G. & Dorrer, O. [1934]. *Biochem. J.* **28**, 11.
Beyer, B. [1882]. *J. prakt. Chem.* [2], **25**, 470.
Birkinshaw, J. H. & Bracken, A. [1942]. *J. chem.* (in the Press).
Birkinshaw, J. H. & Raistrick, H. [1932]. *Biochem. J.* **26**, 441.
Brady, O. L. & Elsmie, G. V. [1926]. *Analyst*, **51**, 77.
Callow, N. H., Callow, R. K. & Emmens, C. W. [1938]. *Biochem. J.* **32**, 1312.
Doeuvre, J. [1927]. *Bull. Soc. Chim.* [4], **41**, 1145.
Emmons, C. W. [1935]. *Mycologia*, **27**, 128.
Feigl, F. [1934]. *Mikrochemie*, **15**, 183.
Kinney, C. R. & Langlois, D. P. [1931]. *J. Amer. chem. Soc.* **53**, 2189.
Storrs, H. E. & Fittig, R. [1870]. *Liebigs Ann.* **153**, 283.
Täufel, K. & Thaler, H. [1932]. *Hoppe-Seyl Z.* **212**, 256.
Voorhees, V. & Adams, R. [1922]. *J. Amer. chem. Soc.* **44**, 1397.