CCCVII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

LII. ISOLATION, PROPERTIES AND CONSTITUTION OF TERRESTRIC ACID (ETHYLCAROLIC ACID), A METABOLIC PRODUCT OF *PENICILLIUM* *TERRESTRE* JENSEN.

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IN an attempt to identify certain strains of *Penicillium* isolated from New Zealand cheddar cheese and received under the label of *P. puberulum* Bainier, Birkinshaw & Raistrick [1936] were led to examine biochemically certain strains in the *P. terrestre* Jensen series. These moulds, with the exception of one strain, when grown on a Raulin-Thom glucose medium, produced in fair quantity a hitherto undescribed metabolic product for which the name *terrestrial acid* is proposed.

Terrestrial acid, $C_{11}H_{14}O_4$, is a colourless, crystalline acid showing many resemblances in properties to the substituted tetronic acids obtained by Clutterbuck *et al.* [1934] from *P. Charlesii* G. Smith, and in particular to carolic acid. This similarity is evident in the following résumé of the properties of terrestrial acid.

1. It gives an orange colour with aqueous ferric chloride, indistinguishable from that given by carolic acid, and with sodium nitrite it yields on standing a violet colour, a test which is indicative of the tetronic acid group. Like carolic acid it is extremely soluble in $CHCl_3$.

2. It is dextrorotatory and the molecule shows no active hydrogen in anisole but yields one atom in pyridine, although titrating sharply in water as a monobasic acid.

3. On bromination in 50% aqueous acetic acid it gives *d*-bromomethyl-tetronic acid identical with that obtained from carolic acid under similar conditions.

4. On acid hydrolysis it yields carbon dioxide and acetoin, but the other product is not butyrolactone as obtained from carolic acid but a *l*-hexanolactone. On oxidation with nitric acid this lactone yields succinic acid and on heating with hydriodic acid it is racemized and is then identical with *n*-hexanolactone obtained by synthesis. Part of the lactone is reduced by the hydriodic acid yielding *n*-hexanoic acid. It is clear therefore that the lactone obtained on hydrolysis is the *l*-isomeride of *n*-hexanolactone, which so far as we are aware has not hitherto been obtained in optically active form.

It thus appears that terrestrial acid hydrate must have the constitution of α -(*l*- γ -hydroxy-*n*-hexanoyl)-*l*- γ -methyltetronic acid (I), whilst the anhydrous form by analogy with carolic acid [Clutterbuck *et al.* 1935] will be represented by structure II. These forms differ from the corresponding forms of carolic

acid (III and IV) only in the replacement of the γ -hydroxybutyryl residue by the l - γ -hydroxy- n -hexanoyl residue. Terrestrial acid may therefore be regarded as ethylcarolic acid, the ethyl group replacing a hydrogen atom of carolic acid in such a manner that another centre of asymmetry is created in the molecule.

I

II

III

IV

EXPERIMENTAL.

Cultures.

The following strains in the *P. terrestris* Jensen series were examined:

(a) L.S.H.T.M. Cat. No. Ad 4. Received via Cambridge labelled *P. glaucum* (cake) and distributed originally by the British National Collection of Type Cultures under the name of *P. glaucum* Link, Cat. No. 763. Isolated from mouldy cake in 1920.

(b) L.S.H.T.M. Cat. No. Ad 8. Isolated by J. H. V. Charles as a bench contamination at Ardeer in 1922.

(c) L.S.H.T.M. Cat. No. Ad 9. Isolated by J. H. V. Charles from cheese at Ardeer in 1922.

(d) L.S.H.T.M. Cat. No. Ad 18. Isolated by J. H. V. Charles from milk at Ardeer in 1923-4.

All these cultures were identified by Dr Charles Thom [Birkinshaw *et al.* 1931] as strains in the *P. terrestris* Jensen series.

Cultural conditions.

The culture medium used throughout was a Raulin-Thom medium of the following composition: glucose, 75 g.; tartaric acid, 4 g.; ammonium tartrate, 4 g.; $(\text{NH}_4)_2\text{HPO}_4$, 0.6 g.; K_2CO_3 , 0.6 g.; MgCO_3 , 0.4 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.25 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07 g.; distilled water to 1500 ml. This was distributed in 350 ml. amounts in a number of 1-litre conical flasks, sterilized, sown with a spore suspension, prepared from Czapek-Dox glucose agar slopes, of the organism studied and incubated at a chosen temperature until the glucose content, as determined by polarimeter, had fallen below 1%. In a preliminary experiment the yields of crude chloroform extract obtained from batches of 20 flasks of each strain when incubated at a mean temperature of 14° were Ad 4 (15 days, 0.50% of residual glucose) 5.67 g.; Ad 8 (16 days, 0.68% of residual glucose) 7.81 g.; Ad 9 (22 days, 0.89% of residual glucose) 4.67 g.; Ad 18 (21 days, 0.69% of residual glucose) 0.37 g. The chloroform extracts from

strains Ad 4, Ad 8 and Ad 9 all set to a crystalline mass on standing and were all shown to contain terrestrial acid, but that from strain Ad 18 did not crystallize and no terrestrial acid could be detected. Strain Ad 8, which gave the best yield in this experiment, was selected for use in the preparation of terrestrial acid.

Preparation of terrestrial acid.

Batches of 100 flasks of Raulin-Thom medium were sown with *P. terrestre* Jensen, strain Ad 8. The flasks were incubated at room temperature either in the laboratory or in underground vaults, where a lower temperature was obtainable. The best and most consistent yields were obtained at a mean temperature of about 20°, when the period of incubation was 15–16 days, the glucose content as determined by polarimeter having fallen in that time to 0.2–0.4 %. The yield of crude crystalline material (total chloroform extract) under these conditions was about 40 g. per batch of 100 flasks. A trial batch incubated at 24° gave a somewhat lower yield and the material did not appear to crystallize with the same facility. The experimental details of a number of preparations are given in Table I, in which all yields are calculated on a 100-flask basis.

Table I.

Incubation period days	Temperature (° C.)		Residual glucose by polarimeter	Final pH of metabolism solution	Titratable acidity ml. N/10 NaOH per 10 ml.	Yield of crude cryst. products g.
	Range	Average				
12	24	24	0.39	3.6	1.20	37.7
15	17–21	20	0.36	3.4	1.50	41.4
16	17–21	20	0.30	3.3	1.32	39.8
16	17–21	20	0.19	3.5	1.23	39.3
24	11–18	14	0.48	2.8	1.70	34.1
21	11–18	14	0.60	2.7	2.83	28.8
19	11–18	14	0.41	2.8	2.12	35.7
19	10–14	12	0.56	2.8	2.05	25.5
35	6–12	9	0.19	3.6	0.65	39.7
32	6–10	8	0.81	3.1	2.50	18.5

When incubation was finished the metabolism solution, which gave a reddish brown colour with FeCl_3 , was filtered from the mycelium, acidified with 10 ml. of conc. HCl per litre and extracted twice with about one-third of its volume of chloroform. The chloroform from the second extraction of each batch was used for the first extraction of a further batch. The procedure after acidification was exactly the same as that employed in the extraction of palitantin [Birkinshaw & Raistrick, 1936]. The chloroform was removed under reduced pressure and the extracts from the whole batch of 100 flasks were combined. The residual chloroform was allowed to evaporate spontaneously and in 2 or 3 days the syrup remaining set to a mass of crystals. It was usually necessary to break up the cake once or twice in order to get rid of the last traces of chloroform.

For purification the product was first crystallized from ether and then from light petroleum. Thus in a typical experiment the crude product (16 g.) was dissolved in ether (600 ml.) in two lots and allowed to crystallize. It deposited large prisms (9.22 g.) somewhat yellow in colour, m.p. 87–89°. A further crop of 2.42 g. of the same m.p. was obtained on evaporation of the ether to 50 ml. These crops were combined and boiled with light petroleum (1300 ml. B.P. 60–80°). The undissolved portion was again boiled with light petroleum (1600 ml.). This effected complete solution except for a very small dark-coloured residue. On chilling, 10.12 g. of colourless flattened needles were obtained, m.p. 89°.

which represented pure terrestrial acid. Since further small quantities were obtained from the mother-liquor, it is clear that most of the original chloroform extract of the metabolism solution consists of terrestrial acid.

Properties of terrestrial acid.

Terrestrial acid is a colourless, crystalline solid of m.p. 89° , and empirical formula $C_{11}H_{14}O_4$. (Found (Schoeller): C, 62.94, 62.86; H, 6.79, 6.63%. Mol. wt. cryoscopic in dioxan (Dr A. E. Oxford) 226. $C_{11}H_{14}O_4$ requires C, 62.84; H, 6.72%; mol. wt. 210.) In aqueous solution it titrates sharply as a monobasic acid. Thus 0.0532 g. required 2.52 ml. of 0.1 N NaOH for neutralization to phenolphthalein, giving an equivalent of 211. Calc. for $C_{11}H_{14}O_4$ (monobasic), 210. In aqueous solution it has $[\alpha]_{5461}^{20} + 61.1^{\circ}$ ($c = 0.53$). It is somewhat soluble in cold water and readily soluble in most organic solvents with the exception of light petroleum, which dissolves only a trace in the cold but more on heating. Like carolic acid it is extremely soluble in chloroform even in the cold, which explains the ease with which it is extracted from dilute aqueous solution by this solvent.

In aqueous solution terrestrial acid gives an orange colour with ferric chloride, indistinguishable from that given by carolic acid. It gives no immediate colour with aqueous sodium nitrite but yields a lilac colour on standing which is not so intense as that given by carolic acid under these conditions. It does not reduce Fehling's solution even on heating, gives no precipitate with neutral lead acetate, but gives in aqueous solution a precipitate with mercuric chloride.

In a Zerewitinoff determination (Roth) terrestrial acid afforded no active hydrogen in anisole at 18° or 95° but gave 1 atom in pyridine at 18° .

Acid hydrolysis of terrestrial acid.

Terrestrial acid (0.5531 g.) was heated with 2N H_2SO_4 (20 ml.) under reflux in a stream of nitrogen. The bath was maintained at $135-140^{\circ}$. The issuing gas was passed through a bubbler containing 2:4-dinitrophenylhydrazine solution, then through standard baryta. Carbon dioxide was evolved which after 3 hours' heating was equivalent to 5.00 ml. of N HCl. After a further 2 hours only the equivalent of 0.17 ml. was obtained, giving a total of 5.17 ml. The calculated amount for 1 mol. of CO_2 is 5.27 ml. Hence 1 mol. of terrestrial acid yields 1 mol. of carbon dioxide on acid hydrolysis. There was only a trace of precipitate in the 2:4-dinitrophenylhydrazine reagent.

The hydrolysate was cooled and titrated. It used 0.20 ml. of N alkali in excess of the equivalent of the H_2SO_4 used for hydrolysis. The solution was then heated and the titration continued. Further alkali (2.25 ml.) was slowly used, the titration proceeding as for a lactone. The total organic acid present was therefore equivalent to 2.45 ml., whereas the calculated amount for 1 equiv. per mol. of terrestrial acid is 2.63 ml. This indicates that 1 mol. of lactone is formed per mol. of terrestrial acid.

The neutralized hydrolysate was distilled *in vacuo* 7 or 8 times to dryness, water being added after each distillation to make up the loss. The distillate was made up to 500 ml. It reduced Fehling's solution in the cold and gave a precipitate with 2:4-dinitrophenylhydrazine. By analogy with carolic acid it was considered that the reducing substance present was probably an acyloin, and a Wood-Ost determination was carried out. Calculated as acetoin the total amount present in the 500 ml. was 154 mg., the theoretical amount for 1 mol. obtainable from 1 mol. of terrestrial acid being 232 mg. The yield was thus 67%. Since some precipitate was present in the 2:4-dinitrophenylhydrazine

bubbler employed during hydrolysis and the Wood-Ost determination was performed at great dilution, which would tend to render it inaccurate, the result was considered to indicate that 1 mol. of terrestric acid gives rise to 1 mol. of acyloin.

Identification of the acyloin. A portion of the distillate containing the acyloin was treated with ferric chloride and distilled. The distillate was treated with 2:4-dinitrophenylhydrazine. It gave overnight a copious orange precipitate which was collected and recrystallized from nitrobenzene-toluene. It gave Neuberg's reaction for a bis-dinitrophenylhydrazone and had m.p. 321°. (Found (Weiler): C, 43.19, 43.06; H, 3.11, 3.22; N, 24.88, 25.30%. $C_{16}H_{14}O_8N_8$, the bis-dinitrophenylhydrazone of diacetyl requires C, 43.04; H, 3.16; N, 25.11 %.) The product showed no depression in m.p. when mixed with authentic diacetyl-bis-dinitrophenylhydrazone. Hence the acyloin formed on hydrolysis of terrestric acid is acetoin (acetylmethylcarbinol).

The residue from vacuum distillation of the acyloin was dissolved in water, acidified with H_2SO_4 and heated to 100°. After cooling it was continuously extracted with ether to remove the lactone. The ether was removed and the extracted lactone was treated with phenylhydrazine (0.3 ml.) and alcohol (1 ml.) and heated for 2 hours under reflux on the water-bath. On cooling a small amount of crystals separated. These were collected and washed with ether. m.p. 246° (decomp.). This was probably the bis-phenylhydrazone of diacetyl, which has m.p. 245° (lit.), but the amount was too small for analysis. After standing for 2 or 3 days no further crystals were observed, but on addition of ether a voluminous crop of crystals was produced, m.p. 92–93°. When recrystallized from ether the m.p. was 92°. (Found (Schoeller): C, 64.51, 64.63; H, 8.18, 8.18; N, 12.86, 12.70 %. $C_{12}H_{18}O_2N_2$ requires C, 64.82; H, 8.17; N, 12.61 %.) The product is thus the phenylhydrazide of a (probably γ -) hydroxyhexanoic acid.

Characterization of the lactone. In order to obtain a pure sample of the lactone for characterization, a further quantity (15 g.) of terrestric acid was hydrolysed with 2N H_2SO_4 (150 ml.) by heating for 3 hours in a stream of nitrogen. After cooling, 2N NaOH (190 ml.) was added and the mixture was again heated for $\frac{1}{2}$ hour. The slightly alkaline product was distilled *in vacuo* to remove acyloin and then extracted with ether to remove any neutral resinous matter which might have been formed. 2N H_2SO_4 (50 ml.) was then added and the mixture was heated to 100° to convert the acid into lactone. The lactone was extracted with ether and distilled at atmospheric pressure, giving 9 g. of a laevorotatory distillate. The distillation was repeated twice. On the third distillation the lactone showed a constant b.p. of 219° (uncorr.) and the rotation and refractive index were practically unchanged. It had the following properties: d_{20}^{20} 1.027, n_D^{20} 1.4393, $[\alpha]_{5461}^{20}$ –58.11°. The specific rotation was also determined in water $[\alpha]_{5461}^{20}$ –57.4° ($c=2.03$). (Found (Weiler): C, 63.06, 62.96; H, 8.72, 8.86 %. $C_6H_{10}O_2$ requires C, 63.11; H, 8.83 %.)

Oxidation of the lactone. The lactone (1 ml.) was heated with a mixture of conc. HNO_3 (13 ml.) and water (9 ml.) on the water-bath. A violent reaction occurred at first but this soon moderated. In about 2 hours the oxidation was apparently complete. The product was evaporated to dryness *in vacuo*, the crystals were dissolved in water, the solution was made slightly alkaline with ammonia, then acid with acetic acid. Calcium chloride was added to precipitate the oxalic acid. The filtrate on acidification with HCl and extraction with ether (continuous extractor) gave 0.32 g. of crystals which on vacuum sublimation nearly all sublimed giving a product of m.p. 186°. It showed no depression when

mixed with succinic acid. The equivalent by titration was 58.6; theoretical for $C_4H_6O_4$ (dibasic) 59.0. Thus succinic acid was produced on oxidation of the lactone with nitric acid. It seems probable therefore that the lactone must have the constitution $\gamma\gamma$ -dimethyl- or γ -ethyl-butyrolactone, and since it is optically active, only the latter would meet the case.

Racemization and identification of the lactone from terrestrial acid. A small sample of *n*-hexanolactone prepared from gluconolactone by heating under reflux with hydrogen iodide and red phosphorus [cf. Kiliani & Kleemann, 1884] was found to be optically inactive. It appeared probable therefore that the lactone from terrestrial acid would be racemized by this treatment. This was found to be the case. The optically active lactone (3.7 g.) was heated for 7 hours under reflux with hydrogen iodide (20 ml., sp. gr. 1.7) and red phosphorus (1 g.). The oil-bath was maintained at 150–160°. Water (60 ml.) was added to the cooled mixture which was then extracted with ether (600–700 ml.). The ether was washed with a little water, then with sodium thiosulphate to remove iodine and the acids were transferred to sodium carbonate solution, the lactone remaining in the ether. The aqueous carbonate on acidification gave an oily acid. The ether was removed on the water-bath and the residual lactone was heated for 1 hour on the water-bath with a few g. of granulated zinc and 10 ml. of conc. HCl diluted with an equal volume of water. The product was extracted with ether and treated with sodium carbonate as before. Again a small amount of oil smelling of *n*-hexanoic acid was obtained on acidification of the carbonate solution. This was added to the previous sodium carbonate product. The lactone recovered from the ether was distilled. The B.P. (214°) was probably a little low owing to the small amount recovered. The product was optically inactive and weighed 1.05 g. It was heated in alcohol with phenylhydrazine for conversion into the phenylhydrazone. The product began to crystallize after removal of the alcohol. The crystals were treated with ether, collected and recrystallized from ether. Plates, M.P. 101°. (Found (Weiler): C, 64.93, 64.72; H, 7.93, 8.15; N, 12.49, 12.84%. $C_{12}H_{18}O_2N_2$ requires C, 64.82; H, 8.17; N, 12.61%.) This product was identical with the phenylhydrazone obtained from *n*-hexanolactone prepared by the method of Fittig & Dubois [1890] from dihydrosorbic acid, which also had M.P. 101°. There was no depression in M.P. on mixing the two products.

Treatment of the acidic portion. The acid extracted by sodium carbonate was treated with zinc and HCl exactly as in the case of the lactone in order to remove combined iodine. The product was extracted with ether, washed with sodium thiosulphate and retransferred to sodium carbonate solution. The aqueous solution was acidified and extracted with ether. The product was esterified with diazomethane in ether. The ester after removal of ether was treated with 10 ml. of aqueous ammonia saturated in a freezing mixture and left for several days with occasional shaking. Crystals were obtained; these were collected and weighed 0.27 g. M.P. 99°. After recrystallization from light petroleum the M.P. was raised to 100°. (Found (Weiler): C, 62.78, 62.60; H, 11.09, 10.87; N, 12.54, 12.34%. $C_6H_{13}ON$ requires C, 62.55; H, 11.39; N, 12.16%.)

This substance was *n*-hexanoamide since it showed no depression in M.P. when mixed with synthetic material prepared in the same manner from *n*-hexanoic acid. The synthetic substance crystallized in large plates, M.P. 101°. The formation of *n*-hexanoic acid by reduction of the lactone affords further proof of the constitution of the lactone obtained by hydrolysis of terrestrial acid as *n*-hexanolactone.

Bromination of terrestric acid in 50% aqueous acetic acid.

The conditions were exactly similar to those employed in the bromination of carolic acid [Clutterbuck *et al.* 1935]. Terrestric acid (1.05 g.) dissolved in 50 % aqueous acetic acid (20 ml.) was treated slowly with the theoretical amount ($20.1 \text{ ml.} \equiv 2\text{Br}_2$) of a solution of bromine (2 ml.) in 50 % acetic acid (80 ml.). The product was evaporated in high vacuum over caustic potash. After 4 days' drying the residue (1.57 g.) consisted of crystals and syrup. The crystals were collected and washed with benzene and then with light petroleum. The product (0.53 g.) melted at $169\text{--}171^\circ$ decomp. Further separation occurred in the mother liquor. On recrystallization from benzene the product had m.p. 180° (decomp.), unchanged on sublimation. It gave an immediate strong purple colour with sodium nitrite solution and a red colour with ferric chloride solution and showed no depression of m.p. when mixed with *d*- α -bromo- γ -methyltetronic acid obtained from carolic acid. The m.p. of that acid is stated as 172° , but it was found to melt at the same temperature as the newly isolated product when heated under the same conditions. As the m.p. is really a decomposition point it depends to some extent on the rate of heating. The rotation of the acid in water was determined in a micropolarimeter tube: $[\alpha]_{5461}^{20} = +8.0^\circ$ ($c=2.5$). (Found (Schoeller): C, 31.26; H, 2.79; Br, 41.05 %. Equivalent by titration 196. Bromomethyltetronic acid, $\text{C}_5\text{H}_5\text{O}_3\text{Br}$ requires C, 31.10; H, 2.61; Br, 41.40 %. Equivalent (if monobasic) 193.) Since this product is undoubtedly *d*- α -bromo- γ -methyltetronic acid it affords further proof that the acyloin isolated from the products of acid hydrolysis of terrestric acid is actually acetoin.

Thus it has been established that terrestric acid contains a methyltetronic acid nucleus linked to a *l*- γ -hydroxy-*n*-hexanoyl residue. In view of the remarkably close similarity in general properties between terrestric and carolic acids, the constitutions of the two acids must be closely related. The same arguments as developed by Clutterbuck *et al.* [1935] for the constitution of carolic acid may therefore be applied *mutatis mutandis* to terrestric acid, which has thus the constitution of an ethylcarolic acid formulated in the hydrated and anhydrous forms as I and II (p. 2195) respectively.

SUMMARY.

Terrestric acid, $\text{C}_{11}\text{H}_{14}\text{O}_4$, a hitherto undescribed mould metabolic product, is formed on Raulin-Thom glucose medium by three different strains in the *Penicillium terrestre* Jensen series. Its isolation, properties and breakdown products are described. It has been shown to be an ethyl derivative of carolic acid, a metabolic product of *Penicillium Charlesii* G. Smith. Terrestric acid hydrate has been shown to be α -(*l*- γ -hydroxy-*n*-hexanoyl)-*l*- γ -methyltetronic acid.

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