the mercurimetric method of Schales & Schales (1941). Tests were performed on both standard solutions and specimens of plasma. The results obtained are set out in Tables 1 and 2.

Results in the Schales & Schales method were calculated by comparison with a standard solution, the chloride content of which was 607 mg. sodium chloride/100 ml.

SUMMARY

A rapid and accurate method of estimating plasma chloride has been developed, using as a basis the silver iodate procedure of Haslewood & King (1936). The results obtained from this and the methods of Van Slyke & Hiller (1947) and Schales & Schales (1941) were in good agreement.

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Studies in the Biochemistry of Micro-organisms

82. USTIC ACID, A METABOLIC PRODUCT OF *ASPERGILLUS USTUS* (BAINIER) THOM & CHURCH

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The *Aspergillus ustus* group of Aspergilli, as defined by Thom & Raper (1945) in their *Manual*, includes only two species, *A. ustus* (Bainier) Thom & Church and *A. granulatus* Raper & Thom. *A. ustus* is regarded by Thom & Raper as a single species aggregate including a whole series of interrelated individual strains differing markedly in their general habit, colony coloration and other characteristics. Representatives of the *A. ustus* group are perhaps the most abundant of all Aspergilli in the soil.

In spite of the ubiquity of *A. ustus* little was known until recently of its biochemical activities. Kurung (1945) reported that culture filtrates from a strain of *A. ustus* grown on a modified Czapek-Dox medium containing potassium chloride (0.5 g./l.) had marked antibacterial activity. Hogeboom & Craig (1946), in a continuation of this work, isolated two crystalline metabolic products containing chlorine, $C_{21}H_{17}O_6Cl_3$ (m.p. 185–187°) and $C_{21}H_{18}O_6Cl_2$ (m.p. 214–216°). Doering, Dubos, Noyce & Dreyfus (1946), in a parallel investigation, described other chlorine-containing metabolites of the same strain of *A. ustus* and gave the name *ustin* to the predominant component. *Ustin* was shown to be identical with Hogeboom & Craig's substance melting at 185–187°, but was assigned the formula $C_{19}H_{15}O_5Cl_3$.

We have found that four different strains of *A. ustus*, when grown on Raulin-Thom medium, which does not contain any chlorides, yield culture filtrates

which give very intense colours with ferric chloride varying in shade from almost pure purple to purple-brown. In contrast, no comparable colours are given by culture filtrates of three of the same four strains of *A. ustus* when grown on Czapek-Dox medium. The metabolic product responsible for the purple colour has been isolated in a pure state and we propose the name *ustic acid* for it, since it has not been described previously. Pure *ustic acid* was isolated in yields of about 0.5 g./l. of culture filtrate from the best strain, grown on Raulin-Thom medium: the other three strains gave smaller yields. *Ustic acid*, $C_{11}H_{13}O_7$, forms colourless, rhombic prisms, m.p. 169–170° (decomp.). It has no optical activity. It contains one methoxyl group and is a strong acid giving a colourless crystalline potassium salt, $C_{11}H_{11}O_7K$. An aqueous solution of *ustic acid* gives an intense, stable, purple colour with aqueous ferric chloride; immediately decolorizes bromine water without the formation of a precipitate; does not give a precipitate with Brady's reagent (2:4-dinitrophenylhydrazine in 2N-hydrochloric acid) except after standing for some hours; and, at room temperature, rapidly reduces Fehling's solution, copper acetate solution and neutral copper sulphate solution with the formation of cuprous oxide in each case. On boiling with 2N-sulphuric acid, *ustic acid* is not decarboxylated, but is converted, with the loss of one molecule of water, into a colourless crystalline com-

pound, $C_{11}H_{10}O_6$, which may be conveniently described as *anhydro-ustic acid*, although it is, in fact, no longer acidic. This substance has very different properties from ustic acid. It gives a much less intense purple ferric chloride colour, does not reduce cold Fehling's solution and gives an immediate precipitate with Brady's reagent. Ustic acid cannot be regenerated from it by treatment of it with cold 2*N*-sodium hydroxide, which brings about deep-seated changes. It is of importance, however, since its formation explains the almost complete failure to obtain straight functional derivatives of ustic acid itself. Thus, methylation of ustic acid with diazomethane or with dimethyl sulphate and aqueous sodium hydroxide led to gums which could not be crystallized; with dimethyl sulphate and potassium carbonate in methanol a monomethyl ether, probably *ustic acid 4-methyl ether*, $C_{11}H_{11}O_6(OCH_3)$, was obtained; with dimethyl sulphate and potassium carbonate in acetone the *trimethyl ether* of *anhydro-ustic acid*, $C_{11}H_7O_6(OCH_3)_3$, insoluble in cold sodium hydroxide, resulted, identical in all its properties with the trimethyl ether obtained by direct methylation, with the same reagents, of *anhydro-ustic acid*. Similarly, while acetylation of ustic acid with acetic anhydride and anhydrous sodium acetate led only to an uncrystallizable gum, acetylation of either ustic acid or *anhydro-ustic acid* with acetic anhydride and pyridine yielded crystalline *triacetyl anhydro-ustic acid*, $C_{11}H_7O_3(O.CO.CH_3)_3$, which, unlike ustic acid itself, is insoluble in aqueous sodium bicarbonate. Finally, on treatment with Brady's reagent, ustic acid slowly gave and *anhydro-ustic acid* quickly gave the same *mono-2:4-dinitrophenylhydrazone*,

In all these derivatives except ustic acid 4-methyl ether a molecule of water is lost from ustic acid in their formation.

As mentioned previously, ustic acid rapidly reduces copper sulphate in neutral aqueous solution at room temperature with the formation of cuprous oxide. The oxidation product of ustic acid so formed was isolated in good yield as a colourless crystalline substance, $C_{11}H_{10}O_7.H_2O$, which may be described as hydrated dehydro-ustic acid or, as will be shown below, more conveniently as *dehydro-ustic acid*. This compound, which has the properties of a hydrated $\alpha\beta$ -diketone, proved to be more amenable to treatment than ustic acid itself so that most of the structural investigations were carried out on it. Dehydro-ustic acid gives the same intense purple colour with aqueous ferric chloride as does ustic acid, but, unlike ustic acid, very quickly gives a precipitate with Brady's reagent and, of course, no longer reduces copper salts. Its aqueous solution at neutral or alkaline reactions is bright yellow in colour, the colour being discharged on acidification. On heating

at its melting point, 124–125°, it loses a molecule of water and the resulting clear yellow gum, which does not recrystallize on standing, may be crystallized from undried benzene with the reformation of the colourless hydrated form, but we have not succeeded in obtaining crystalline anhydrous dehydro-ustic acid. However, on methylation of dehydro-ustic acid with dimethyl sulphate and anhydrous potassium carbonate in acetone, there is formed the *methyl ester* of *dimethyldehydro-ustic acid*, $C_{11}H_7O_4(OCH_3)_3$, in lemon-yellow crystals, m.p. 119–120°, which recrystallize on cooling. This substance, on hydrolysis with dilute acid, yields *dimethyldehydro-ustic acid*, $C_{11}H_8O_5(OCH_3)_2$, in colourless prisms from water, m.p. 152–153°, which also recrystallize on cooling and remelt at the same temperature. It will be seen that neither of these methyl derivatives contains the molecule of water which is lost on heating hydrated dehydro-ustic acid. Neither of the methyl derivatives gives any colour with ferric chloride.

The precipitate formed on the addition of Brady's reagent to an aqueous solution of dehydro-ustic acid consists essentially of *dehydro-ustic acid mono-2:4-dinitrophenylhydrazone*,

which was obtained as yellow prisms from ethyl acetate-benzene. On heating dehydro-ustic acid with *o*-phenylenediamine in ethanol it gives a crystalline *quinoxaline*,

which is readily soluble in cold aqueous sodium bicarbonate and in 2*N*-hydrochloric acid.

Degradation experiments with dehydro-ustic acid led to the following results:

(1) The dimethyl ether of dehydro-ustic acid, on oxidation with hot alkaline potassium permanganate, yielded 3:4:6-trimethoxyphthalic acid, identical with a synthetic specimen prepared by Bargellini's (1914) method.

(2) On oxidation with alkaline hydrogen peroxide at room temperature, dehydro-ustic acid gave one molecule of a volatile acid, consisting essentially of acetic acid, and an 80% yield of a crystalline substance which is the unknown 4:6-dihydroxy-3-methoxyphthalic acid. This substance, on methylation with dimethyl sulphate and potassium carbonate in acetone, also yielded 3:4:6-trimethoxyphthalic acid.

(3) On heating with copper chromite in quinoline, dehydro-ustic acid readily gave one molecule of carbon dioxide and, in fairly good yield, crystalline *decarboxydehydro-ustic acid*, $C_{10}H_{10}O_5$. The same products are formed when dehydro-ustic acid is heated with 2*N*-sulphuric acid, though the reaction is slow and incomplete (48% carbon dioxide after 6.7 hr. boiling). Decarboxydehydro-ustic acid forms

yellow prisms which do not contain the molecule of water present in dehydro-ustic acid. It is not acidic and cannot be extracted from ethyl acetate solution with aqueous sodium bicarbonate. Its aqueous solution gives an immediate yellow precipitate with Brady's reagent, as does dehydro-ustic acid itself, but, in contrast to the latter which gives an intense stable purple colour with ferric chloride, decarboxydehydro-ustic acid gives with this reagent a blue colour changing rapidly to brown and finally to green. Decarboxydehydro-ustic acid is believed to be 3:5-dihydroxy-2-methoxyphenyl methyl diketone.

(4) On oxidation with alkaline hydrogen peroxide, decarboxydehydro-ustic acid gave, in good yield, a crystalline oxidation product which was identified as the previously unknown 3:5-dihydroxy-2-methoxybenzoic acid, as follows. (a) On methylation it gave 2:3:5-trimethoxybenzoic acid identical with a synthetic specimen. (b) On ethylation it gave 3:5-diethoxy-2-methoxybenzoic acid identical with a synthetic specimen.

Methods are given in the experimental part of this paper for the synthesis of 2:3:5-trihydroxybenzoic acid, 2:3:5-trimethoxybenzoic acid, 3:5-diethoxy-2-hydroxybenzoic acid and 3:5-diethoxy-2-methoxybenzoic acid.

DISCUSSION

Structure of dehydro-ustic acid (VI) and ustic acid (X)

The molecular structure (VI) which we propose for dehydro-ustic acid was deduced from the following experimentally established facts:

(1) Ustic acid and dehydro-ustic acid each contain one methoxyl group.

(2) Dehydro-ustic acid dimethyl ether, on oxidation with alkaline potassium permanganate yields 3:4:6-trimethoxyphthalic acid. Hence it follows that dehydro-ustic acid must contain the skeleton structure (I), in which one of the hydroxyl groups is methylated.

(3) Anhydro-ustic acid, on treatment in the cold with aqueous sodium hydroxide, gives a small yield of a monomethyl ether of 3:4:6-trihydroxyphthalic acid which, as will be shown later, is 4:6-dihydroxy-3-methoxyphthalic acid (VII). The same compound is given in 80 % of the theoretical yield, together with one molecule of a volatile acid which is almost entirely acetic acid, when dehydro-ustic acid is oxidized with alkaline hydrogen peroxide. Hence structure (I) may be expanded to either structures

(II) or (III), since the only other alternative, structure (IV), could not yield both a substituted phthalic acid and acetic acid each in yields approaching the theoretical.

(4) A decision between (II) and (III) was made as follows. Dehydro-ustic acid, $C_{11}H_{10}O_7$, on heating with copper chromite and quinoline loses one molecule of carbon dioxide and gives a fairly good yield of decarboxydehydro-ustic acid, $C_{10}H_{10}O_5$, which is a neutral diketone. Decarboxydehydro-ustic acid, on oxidation with alkaline hydrogen peroxide, gives one molecule of acetic acid and, in good yield, 3:5-dihydroxy-2-methoxybenzoic acid, structure (V). Hence dehydro-ustic acid must have the skeleton structure (II), in which the C_3 side chain must be unbranched because of the formation of one molecule of

acetic acid on hydrogen peroxide oxidation. We therefore propose structure (VI) for anhydrous dehydro-ustic acid. Since structure (V) was confirmed by synthesis it follows that the monomethyl ether of 3:4:6-trihydroxyphthalic acid described in paragraph (3) above must have structure (VII), and, if structure (VI) is accepted for dehydro-ustic acid, then decarboxydehydro-ustic acid must have structure (VIII).

The fact that dehydro-ustic acid (VI) has been obtained crystalline only as the compound $C_{11}H_{12}O_8$ may be explained by assuming that this formula is more correctly written as $C_{11}H_{10}O_7 \cdot H_2O$ since one molecule of water is readily lost on heating and the resulting anhydrous form is a yellow gum which, on recrystallization, is rehydrated to the colourless, crystalline form. Further, all the derivatives of dehydro-ustic acid, i.e. its dimethyl ether, dimethyl

ether methyl ester, mono-2:4-dinitrophenylhydrazones and quinoxaline are derivatives of the anhydrous form $C_{11}H_{10}O_7$.

(5) Since dehydro-ustic acid, $C_{11}H_{10}O_7$, is formed in almost quantitative yield by treating ustic acid, $C_{11}H_{12}O_7$, with cold neutral copper sulphate solution it is reasonable to assume that, under these very mild conditions, no deep-seated change takes place. This reaction involves the loss of two hydrogen atoms by ustic acid and, bearing in mind the similarities and differences in the properties of ustic acid and dehydro-ustic acid already described, is readily explained by postulating that the $-\text{CO}.\text{CO}.\text{CH}_3$ or $-\text{C}_3\text{H}_5\text{O}_2$ side chain in dehydro-ustic acid arises from a similarly placed $-\text{C}_3\text{H}_5\text{O}_2$ side chain in ustic acid. Hence we propose structure (IX) for ustic acid and it now remains to discuss the nature of this $-\text{C}_3\text{H}_5\text{O}_2$ side chain.

An unbranched side chain $-\text{C}_3\text{H}_5\text{O}_2$ derived from $-\text{CO}.\text{CO}.\text{CH}_3$ by reduction may have one of the following five forms: (a) $-\text{CHOH}.\text{CO}.\text{CH}_3$; (b) $-\text{CO}.\text{CHOH}.\text{CH}_3$; (c) $-\text{C}(\text{OH})=\text{C}(\text{OH}).\text{CH}_3$; (d) $-\text{CH}-\text{C}(\text{OH}).\text{CH}_3$; (e) $-\text{C}(\text{OH}).\text{CH}.\text{CH}_3$, or an

equilibrium mixture of two or more of these possible tautomers depending on the conditions obtaining when any particular test is carried out. It must be admitted that none of our experimental evidence enables us to decide clearly between them, but this evidence for and against each form may be summarized as follows:

(i) Ustic acid rapidly reduces aqueous copper acetate in the cold as does ascorbic acid, which has an ene-diol structure as in (c), whereas acetoin gives only slight reduction. Fuson and co-workers (1939 *et seq.*), who have isolated both the interconvertible ene-diol and hydroxy-ketone forms of some highly substituted benzoinis, found the ene-diol forms to reduce cold copper acetate with the formation of the corresponding diketones analogous to (VIII) whereas the hydroxy-ketone forms did not.

(ii) Ustic acid, unlike ascorbic acid, does not reduce 2:6-dichlorophenolindophenol. Acetoin gives slight reduction on the alkaline side, but not on the acid side.

(iii) Neither ustic acid, acetoin nor ascorbic acid gives an immediate precipitate with Brady's reagent. All three compounds give flocculent precipitates on standing.

(iv) Ustic acid was recovered unchanged after prolonged treatment with ozone in either aqueous or ethyl acetate solution. Ascorbic acid, on the other hand, is oxidized rapidly in aqueous acetic acid by ozone to dehydro-ascorbic acid and finally to oxalic acid (Herbert, Hirst, Percival, Reynolds & Smith, 1933).

(v) Ustic acid, in pyridine solution, gave 3.84 active hydrogen atoms in a Zerewitinoff estimation. Structure (IX), with either of the hydroxy-ketone side chains (a) or (b) or either of the epoxy side chains (d) or (e), would be expected to give four active hydrogen atoms. If the side chain were the ene-diol form (c) five active hydrogen atoms would be expected.

(vi) Ustic acid is optically inactive. This fact might appear to favour the ene-diol form (c), which is the only form not containing an asymmetric carbon atom, were it not for the fact that the acetoin isolated from certain bacterial fermentations is also practically optically inactive.

We therefore propose structure (X) to illustrate many of the properties of ustic acid. This is done with the realization that the reactions of ustic acid can be equally well accounted for by postulating the side chain (b) $-\text{CO}.\text{CHOH}.\text{CH}_3$, and that some of them

are better represented by the ene-diol form (c) $-\text{C}(\text{OH})=\text{C}(\text{OH}).\text{CH}_3$. The epoxy side chains (d) and (e) can also not be excluded if the findings of Kling (1905), that acetoin may exist mainly in the form $\text{CH}_3-\text{C}(\text{OH})-\text{CH}.\text{CH}_3$, be taken to apply to

ustic acid.

It was mentioned earlier that ustic acid, when boiled with 2N-sulphuric acid, loses one molecule of water, with the formation of anhydro-ustic acid. Attempts to reconvert this substance into ustic acid, by treatment with cold sodium hydroxide, failed owing to deep-seated decomposition. Further, attempts to methylate or acetylate ustic acid, or to prepare its 2:4-dinitrophenylhydrazones led to the formation of the trimethyl or triacetyl derivatives of anhydro-ustic acid or to its mono-2:4-dinitrophenylhydrazones, all of which substances were also readily obtainable directly from anhydro-ustic acid itself. We believe that anhydro-ustic acid has either structure (XI) or (XII), with a slight preference for (XII), since the isocoumarin structure appears to be more stable than the phthalide structure (Bain, Perkin & Robinson, 1914).

Either (XI) or (XII) offers a logical explanation for the loss of one molecule of water in the formation of anhydro-ustic acid and for the marked differences in its properties from those of ustic acid. They also explain the formation of a neutral trimethyl ether or a neutral triacetate in their enol forms (XIb or XIIb) and the formation of a mono-2:4-dinitrophenyl-hydrazone in their ketone forms (XIa or XIIa).

Ustic acid is related structurally to certain other mould metabolic products and to a group of lichen acids. Two unnamed metabolic products of species in the *Penicillium brevi-compactum* Dierckx series have the empirical formulae $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$, and the structural formulae (XIII) and (XIV), respectively (Clutterbuck, Oxford, Raistrick & Smith, 1932; Oxford & Raistrick, 1933). It will be seen that ustic acid is the 3-methoxy derivative of

(XIII) and that dehydro-ustic acid is the 3-methoxy derivative of (XIV). The general properties of ustic acid and $C_{10}H_{10}O_6$ are strikingly similar, as are those of dehydro-ustic acid and $C_{10}H_{10}O_7$. So much is this the case that many of the difficulties encountered in elucidating the structures of ustic acid and dehydro-ustic acid had been previously experienced with the acids $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$.

Citromycetin, a yellow crystalline metabolite of species formerly included by Wehmer in his genus *Citromyces* but now regarded by Raper & Thom (1949) as belonging to the *Penicillium frequentans*

Westling series, was assigned by Hetherington & Raistrick (1931), who first described it, the partial structural formula (XV). When *O*-dimethylcitromycetin methyl ester is hydrolysed with ethanolic potassium hydroxide in nitrogen there is formed in good yield a hydroxydimethoxyphthalic acid, the cleavage being indicated by the dotted line in (XV). We have now shown that methylation of this hydrolytic product yields 3:4:6-trimethoxyphthalic acid identical with that obtained by permanganate oxidation of dehydro-ustic acid dimethyl ether.

Geodin, $C_{17}H_{12}O_7Cl_2$, and erdin, $C_{16}H_{10}O_7Cl_2$, are metabolic products of *Aspergillus terreus* Thom (Raistrick & Smith, 1936). On methylation with dimethyl sulphate and alkali there is formed from both geodin and erdin the same highly substituted benzoyl benzoic acid shown in structure (XVI) (Calam, Clutterbuck, Oxford & Raistrick, 1947).

This compound can clearly be derived from 3:4:6-trimethoxyphthalic acid and thus links geodin and erdin with ustic acid and citromycetin.

A group of four lichen acids has been investigated by Asahina and co-workers (1933 *et seq.*) who have shown that they all have the general structural formula (XVII), in which R and R' are carbon chains, and the detailed structures indicated below.

All four acids are built up from the two units (a) and (b), (a) being the skeleton present in the meta-

Physodic acid, $R = CH_2 \cdot CO \cdot C_6H_{11}$,
 α -Collatolic acid, $R = R' = CH_2 \cdot CO \cdot C_6H_{11}$,
 Aletronic acid, $R = R' = CH_2 \cdot CO \cdot C_6H_{11}$,
 Lobaric acid, $R = CO \cdot C_4H_9$,

$R' = C_6H_{11}$, $M = H$,
 $R' = C_6H_{11}$, $M = CH_3$,
 $R' = C_6H_{11}$, $M = H$,
 $R' = C_6H_{11}$, $M = CH_3$

bolites $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$ from *Penicillium brevicompactum*, and (b) the skeleton present in ustic acid.

A brief account of our work on ustic acid was presented by one of us to the Biochemical Society at its meeting on 18 February 1949 (Stickings, 1949).

EXPERIMENTAL

Cultures

Four different strains of *Aspergillus ustus* (Bainier) Thom & Church were examined in the course of this work. Strains S.A. 906, S.A. 1409 and S.A. 1658 were received from Dr A. L. James, Timber Research Laboratory, Transvaal Chamber of Mines, Exchange Yard, Johannesburg, South Africa. They were isolated from mouldy material occurring in South African mines. Strain BB 188 was received from our colleague Mr G. Smith. Their history is as follows. (1) Strain S.A. 906 received 13 June 1947, isolated from decayed flannel used in a mine. (2) Strain S.A. 1409 received 20 October 1947, isolated from decayed ventilation tubing in a mine. (3) Strain S.A. 1658 received 20 October 1947, isolated from a decayed treated flannel bag used in a mine. (4) Strain BB 188 isolated by Mr G. Smith 4 August 1943 from a mouldy American wireless set received from the Ministry of Supply.

All four strains were identified as *A. ustus* by Mr G. Smith. Strain S.A. 906 had been determined previously by Dr C. Thom as 'near to *Aspergillus minutus* Abbott'. The latter name is, however, no longer recognized by Thom & Raper (1945), being regarded by them as a synonym of *A. ustus*. Strain S.A. 1658 had been determined previously as *A. ustus* by the Centraalbureau voor Schimmelcultures, Baarn, Holland.

Mr Smith's notes on the four strains of *A. ustus* follow. BB 188 is a very typical strain. Colonies spore readily and abundantly, the conidial colour being smoky greyish-brown. Of the three South African strains S.A. 1409 and S.A. 1658 are both somewhat atypical in colony habit and are practically indistinguishable. Colonies are densely floccose, sporing tardily and somewhat sparsely. The colour of the conidial heads is, however, quite typical, smoky greyish-brown. S.A. 906 differs from the other two strains only in sporing even more sparsely. Morphologically all four strains fit well with the description given by Thom & Raper (1945) for *A. ustus*: foot cells very prominent; stalks smooth and brownish; heads at first more or less hemispherical, then irregular both in shape and size; vesicles bearing both metulae and phialides; conidia globose, echinulate, 3.5–4 μ . in diameter.

Cultural conditions and cultural characteristics of the different strains of *Aspergillus ustus*

Czapek-Dox solution. Glucose, 50 g.; $NaNO_3$, 2 g.; KH_2PO_4 , 1 g.; KCl , 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $FeSO_4 \cdot 7H_2O$, 0.01 g.; distilled water, 1 l.

Raulin-Thom solution. Glucose, 75 g.; tartaric acid, 4 g.; ammonium tartrate, 4 g.; $(NH_4)_2HPO_4$, 0.6 g.; $(NH_4)_2SO_4$, 0.25 g.; K_2CO_3 , 0.6 g.; $MgCO_3$, 0.4 g.; $FeSO_4 \cdot 7H_2O$, 0.07 g.; $ZnSO_4 \cdot 7H_2O$, 0.07 g.; distilled water, 1.5 l.

Portions of these solutions (350 ml.), contained in a series of 1 l. conical flasks plugged with cotton wool, were sterilized and inoculated with one or other of the four strains of *A. ustus* and were incubated in the dark at 24°. Single flasks were harvested after 7, 14 and 21 days' incubation and the culture filtrates from these were examined by Mrs S. Marcus with the following results.

None of them, in either medium and after either 7 or 14 days' incubation, showed any antibacterial activity at a dilution of 1:10 against either the Oxford H strain of *Staphylococcus aureus* or the N.C.T.C. strain 86 of *Escherichia coli*.

None of the Czapek-Dox filtrates of strains S.A. 906, S.A. 1409 or S.A. 1658 gave more than a pale pinkish-buff colour with $FeCl_3$, while those from strain BB 188 gave a moderate purplish-brown colour after 14 and 21 days' incubation.

The Raulin-Thom filtrates of all four strains gave purple to purplish-brown $FeCl_3$ colours varying in intensity from moderate with strain S.A. 1658 to very intense with S.A. 906. Br₂ water was rapidly decolorized by S.A. 906 and BB 188 after 21 days' incubation, but little precipitate was formed.

As a result of these findings the relative intensities of the maximum colours produced by the addition of aqueous $FeCl_3$ to 10 ml. of the 21-day Raulin-Thom culture filtrates were compared in a colorimeter. The results obtained and other estimations carried out are summarized in Table 1.

Table 1. *Cultural characteristics of culture filtrates of four strains of Aspergillus ustus*

Strain no.	Relative intensity of $FeCl_3$ colour	pH	Residual glucose by polarimeter (%)
S.A. 906	9	4.1	0.32
S.A. 1409	3	5.1	0.16
S.A. 1658	1	6.1	0.02
BB 188	3	4.1	0.27

Comparison of the relative intensity of $FeCl_3$ colours given by Raulin-Thom culture filtrates of *A. ustus* strain S.A. 906 after 16–39 days' incubation and harvested every 2–3 days showed clearly that the intensity of the $FeCl_3$ colour reaches a maximum after 18–21 days' incubation and thereafter slowly decreases. After 39 days' incubation the pH was 5.5, the glucose had almost completely disappeared and the $FeCl_3$ intensity was about 90% of that at its peak. Strain S.A. 906 was therefore used for the bulk preparation of ustic acid and the cultures were harvested after 21 days' incubation.

Isolation of crude ustic acid from culture filtrates of *Aspergillus ustus* strain S.A. 906

Batches of 100-flask cultures of *A. ustus* strain S.A. 906, grown on Raulin-Thom medium, were harvested after 21 days' incubation at 24°. At the end of this time the upper surface of the mycelium was white and the reverse yellow. The culture fluid was also yellow and each flask contained a small amount of yellow amorphous sediment. The culture fluid was decanted from the mycelium and clarified by

filtration through a pad of kieselguhr on a large filter. The clear yellow filtrate (31 l.) was acidified to congo red with conc. HCl (3 ml./l.). Crude ustic acid was isolated from the acidified filtrate by one of the following methods:

(a) It was extracted with ethyl acetate four times, using 1/4 vol. of ethyl acetate for the first extraction and 1/10 vol. for each of the other three extractions. This treatment removed about 85% of the ustic acid, and the residual aqueous layer which was discarded gave a brown FeCl_3 reaction. The combined ethyl acetate extracts were evaporated under reduced pressure at 40° leaving a brown viscous gum (47 g.). This was treated with ether (400 ml.), when an amorphous buff-coloured solid separated and was filtered off (7 g.). It gave no FeCl_3 reaction, and has not been investigated further. On removal of the ether there remained a pale-brown viscous gum (40 g.), a portion of which, dissolved in water, gave only a slight immediate precipitate with Brady's reagent (2:4-dinitrophenylhydrazine in 2N-HCl).

(b) Charcoal (8 g./l.), previously activated by heating to 135° for 2 hr., was added to the acidified filtrate. The suspension was stirred for some hours and then allowed to settle. The charcoal was separated by filtration, washed with water and dried in a desiccator. The filtrate, which gave little colour with FeCl_3 , was discarded. The dried charcoal was extracted with dry ether in a long wide glass column, by percolation under gravity, during several days. On removal of the ether from the extract there remained a pale-brown gum (22 g.), a portion of which gave an immediate copious precipitate with Brady's reagent.

Purification of crude ustic acid

The gums obtained by either of the methods (a) or (b) proved difficult to crystallize and were finally purified by taking advantage of the fact that ustic acid forms a K salt which is only slightly soluble in methanol. The gums obtained from each batch of 100 flasks either by method (a) or (b) were separately dissolved in cold methanol (2 ml./g.), and a 25% solution of anhydrous potassium acetate in methanol (10 ml./g.) was added. Crystals began to separate at once from the gums prepared by either method. After holding for 2 days in a refrigerator, the crystals were collected by filtration, washed with methanol and ether, and dried. Average yield of K salt from method (a) 21–24 g. of buff-coloured prisms giving only a slight immediate precipitate with Brady's reagent; by method (b) 11–12 g. of yellow crystals giving an immediate copious precipitate with Brady's reagent.

The crude K salt (24 g.) from method (a) was dissolved in a minimum of water, acidified to congo red with HCl, and the solution was extracted twice with an equal volume of ethyl acetate. The combined extracts, after washing with a little water and re-extraction of the wash water with ethyl acetate, were evaporated under reduced pressure to about

100 ml., and boiling benzene (500 ml.) was added. On cooling and allowing to stand in the refrigerator for 2 days, 15.0 g. of pale-brown crystals of ustic acid, m.p. $166\text{--}167^\circ$ (decomp.), were obtained. A further 2.5 g. of less pure crystalline material was obtained by evaporating the filtrate and treating with light petroleum. The recovery yield from the K salt was 85%. The final mother liquors, on evaporation, yielded a gum (1.3 g.) which could not be crystallized.

The crude K salt (11–12 g.) from method (b), when treated similarly, gave a 60% recovery yield of ustic acid, m.p. $166\text{--}167^\circ$ (decomp.). From the mother liquors a small amount of dehydro-ustic acid, m.p. 124° (see p. 62), separated on standing. This substance, which is doubtless an artifact arising by oxidation of ustic acid in the charcoal extraction method (b), accounts for the formation of an immediate precipitate with Brady's reagent from the K salt.

The crystalline ustic acid prepared by either method (a) or (b) was recrystallized by dissolving in boiling ethyl acetate (5 ml./g.) and adding boiling benzene (25 ml./g.) with the addition of charcoal. On cooling the filtrate, ustic acid separated in rosettes of colourless rhombic prisms, m.p. 167° (decomp.). The colour of the crude ustic acid can also be removed by passing an ethyl acetate solution of it through a short alumina column. By repeated recrystallization and purification the melting point of ustic acid was raised to $169\text{--}170^\circ$ (decomp.).

The average overall yield of crystalline ustic acid obtained by method (a) from a number of batches of cultures of *A. ustus* strain S.A. 906 was about 0.5 g./l. of culture filtrate. By method (b) the yield was 0.2 g./l. of culture filtrate.

As will be shown later, ustic acid contains a methoxyl group so that there is a possibility that this group might have been introduced by the use of methanol in the purification procedure described above. This possibility was disproved as follows. In one of the isolation experiments using the charcoal method (b) a portion of the dried charcoal was eluted with CHCl_3 instead of ether. The CHCl_3 eluate was evaporated to dryness, extracted with ether and filtered. On removal of the solvent from the ether filtrate, a gum (1.75 g.) remained. This was dissolved in acetone (2 ml.) and treated with boiling CHCl_3 (100 ml.). Crystalline ustic acid (0.39 g.) separated, m.p. $166\text{--}167^\circ$ (decomp.) not depressed on admixture with ustic acid, m.p. $167\text{--}168^\circ$ (decomp.) prepared via the K salt from methanol solution.

Isolation of ustic acid from culture filtrates of Aspergillus ustus strains S.A. 1409, S.A. 1658 and BB 188

Ustic acid was also obtained, in much smaller amounts, from culture filtrates of the strains S.A. 1409, S.A. 1658 and BB188. Growth on Raulin-Thom medium followed a similar course to that of strain S.A. 906, the maximum intensity of the FeCl_3 reaction being attained after 21, 28 and 21 days, respectively. Flasks were harvested and treated

Table 2. *The isolation of ustic acid from four strains of Aspergillus ustus*

Strain	Incubation period (days)	Residual glucose by polarimeter (%)	pH	Metabolism solution (g./l.)				M.p. of ustic acid ($^\circ$)
				Crude extract	Ether-insoluble	K salt	Crystalline ustic acid	
S.A. 906	21	0.32	4.1	1.5	0.4	0.75	0.5	166–167
S.A. 1409	24	0.08	5.3	0.56	0.15	0.12	0.05	160–165
S.A. 1658	24	0.00	6.9	0.21	0.04	0.01	0.001	164–166
BB188	21	0.43	4.5	0.46	0.12	0.10	0.04	167–168

by method (a), with the results shown in Table 2. The K salts from these three strains all gave an immediate precipitate with Brady's reagent, suggesting the presence of dehydro-ustic acid. The ustic acid obtained was identified by mixed melting point and by comparison of properties with the material from S.A. 906.

Although strain BB188 gave a purplish FeCl_3 reaction when grown on Czapek-Dox medium, no ustic acid seemed to be formed. Growth followed a different course, the most striking characteristic being the formation of orange pigments, which are being further investigated. The gummy residue from ethyl acetate extraction of the metabolism solution (after removal of the pigment) gave a red colour with FeCl_3 , but did not reduce cold Fehling's solution, and treatment with 25% methanolic potassium acetate failed to give any K salt.

General properties of ustic acid

Ustic acid crystallizes in colourless rhombic prisms, m.p. 169–170° with gentle decomposition, a gas being evolved and a straw-coloured liquid remaining which does not recrystallize on cooling. It does not contain N, S, or Cl. (Found: C, 51.8, 51.5; H, 4.7, 4.8; OCH_3 , 12.0; $\text{C}-\text{CH}_3$, 8.7% = 1.47 $\text{C}-\text{CH}_3$ groups*; active H atoms (in pyridine), 3.8. $\text{C}_{11}\text{H}_{12}\text{O}_6$ requires: C, 51.6; H, 4.7; 10CH_3 , 12.1; $1\text{C}-\text{CH}_3$, 5.9%.) Ustic acid has no optical activity. It dissolves readily in water, ethanol, methanol, acetone and dioxan, less readily in ethyl acetate (about 10 vol./g.) and with difficulty in ether. It is almost insoluble in benzene, CHCl_3 , CCl_4 or light petroleum. An aqueous solution of ustic acid has the following properties: (1) It is colourless and acid in reaction (pH of 0.05N-solution, 3.4), and becomes only faintly coloured on the addition of alkali. (2) Titration with 0.1N-NaOH, using phenolphthalein as indicator, gives a very indefinite end point. The equivalent lies between 128 and 256, suggesting one strong and one or more weak acid groups. (3) It shows a pale blue fluorescence in ultraviolet light. An acetone solution gives a pale greenish-blue fluorescence. (4) Aqueous FeCl_3 gives an intense purple colour which is stable for many hours. In ethanolic solution the colour is much bluer. (5) On gradual addition of Br_2 water the latter is decolorized. No colour develops until excess of Br_2 is present and no turbidity or precipitate appears. (6) With Brady's reagent the solution remains clear for 2 hr. Over a period of 5 days fine yellow needles are slowly deposited (see p. 61). (7) The addition, at room temperature, of Fehling's solution, or aqueous CuSO_4 in neutral solution, or copper acetate solution, results in rapid reduction to cuprous oxide and the formation of dehydro-ustic acid (see p. 62).

Potassium ustate. Pure ustic acid (1.0 g.) dissolved in methanol (2 ml.) was treated with 25% methanolic potassium acetate solution (10 ml.). After standing in the refrigerator overnight, the slightly yellow crystals were collected (1.14 g., theoretical yield) and recrystallized from boiling water, from which colourless hexagonal plates of potassium ustate separated (0.43 g.). An aqueous solution of these crystals had a pH of 7.0. Potassium ustate (0.0485 g.), after ashing with conc. H_2SO_4 , yields 0.0144 g. of anhydrous K_2SO_4 ,

* Ustic acid and a number of its derivatives and breakdown products give abnormally high $\text{C}-\text{CH}_3$ values, cf. the two acetates of anhydro-ustic acid (p. 61); dehydro-ustic acid (p. 62); and, particularly, 4:6-dihydroxy-3-methoxyphthalic acid (p. 63) and 3:4:6-trimethoxyphthalic acid (p. 64) neither of which contains a $\text{C}-\text{CH}_3$ group.

corresponding to an equivalent of 255 for ustic acid; $\text{C}_{11}\text{H}_{11}\text{O}_6\text{K}$ requires equiv. 256.

Anhydro-ustic acid

A solution of ustic acid (0.4842 g.) in 2N- H_2SO_4 (20 ml.) was boiled under reflux, and a slow stream of N_2 was passed through the reaction mixture. The issuing gas was scrubbed, first through a bubbler containing Brady's reagent, and then through one containing 0.2N-aqueous $\text{Ba}(\text{OH})_2$. The reaction was complete in 1 hr. No precipitate appeared in the Brady's reagent, and only 8% of the theoretical amount for 1 mol. of CO_2 was absorbed in the baryta.

The straw-coloured reaction mixture was diluted to 100 ml., partly neutralized with 2N-NaOH to pH 2 and then extracted twice with an equal volume of ethyl acetate. The combined extracts were washed once with water (10 ml.) and evaporated to dryness. The residual, almost colourless, crystalline solid (0.41 g.) was recrystallized first from ethyl acetate (8 ml.) and then from benzene (25 ml.). *Anhydro-ustic acid* was thus obtained as colourless needles, m.p. 145–159°. The melting point is very indefinite, the crystals melting to a pale-yellow liquid which does not readily solidify on cooling. (Found: C, 55.3, H, 4.4; OCH_3 , 12.9, 12.9. $\text{C}_{11}\text{H}_{10}\text{O}_6$ requires: C, 55.5; H, 4.2; 10CH_3 , 13.0%.)

Anhydro-ustic acid is somewhat soluble in water from which it crystallizes in colourless rods. Its aqueous solution gives a number of reactions which are markedly different from those given by the parent substance. (1) It becomes yellow on the addition of dil. NaOH. (2) Aqueous FeCl_3 gives a purple colour which is much fainter than that given by ustic acid and which, on standing, becomes deeper and redder. In ethanolic solution the colour is pale green, changing to yellow on dilution with water. (3) Brady's reagent gives an immediate yellow precipitate. (4) It does not reduce Fehling's solution in the cold. (5) It can be extracted by solvents from a solution in aqueous NaHCO_3 .

Alkaline hydrolysis of anhydro-ustic acid. A solution of anhydro-ustic acid (0.1 g.) in 2N-NaOH (10 ml.) was held at room temperature for 3 days. The solution, initially yellow, turned green after 1 hr. and remained that colour. It no longer gave a precipitate with Brady's reagent, nor did it reduce cold Fehling's solution. The solution was acidified to congo red and extracted with ethyl acetate (20 ml.). The extract was evaporated leaving a red gum (0.075 g.) which was dissolved in ethyl acetate (2 ml.), and benzene (20 ml.) was added. Tiny pink nodules separated (0.03 g.), m.p. 191° (decomp.) with the formation of a crystalline sublimate, m.p. 174–177°. The melting point of the substance was not depressed on admixture with 4:6-dihydroxy-3-methoxyphthalic acid (see p. 63). It was recrystallized from ethyl acetate (1 ml.) and benzene (5 ml.) with charcoal, the filtrate being diluted with an equal volume of light petroleum b.p. 60–80°. The product separated as colourless prisms (0.01 g.), m.p. 194–195° (decomp.). (Found: C, 47.5; H, 3.8. Calc. for $\text{C}_6\text{H}_5\text{O}_7$: C, 47.4; H, 3.5%.) An aqueous solution of the compound gave a pure purple colour with aqueous FeCl_3 , and did not give a precipitate with Brady's reagent.

Ustic acid itself, hydrolysed in the same way, gave a deep-red solution no longer reducing Fehling's solution, but giving an immediate precipitate with Brady's reagent. Extraction with ethyl acetate, after acidification, yielded a gum (0.085 g.) an aqueous solution of which gave an intense purple colour with aqueous FeCl_3 . No pure substance was, however, isolated from the gum.

DERIVATIVES OF USTIC ACID AND ANHYDRO-USTIC ACID

Products of methylation

(a) *With diazomethane.* Ustic acid, treated in ethereal solution with ethereal diazomethane for 2 days, yielded a gum from which no pure substance could be isolated. An aqueous solution of the gum no longer reduced cold Fehling's solution, nor did it give any colour with FeCl_3 . On the other hand, Brady's reagent yielded an immediate precipitate.

(b) *With dimethyl sulphate and aqueous NaOH.* Ustic acid gave very similar results to those obtained in method (a).

(c) *With dimethyl sulphate and K_2CO_3 in acetone.* Ustic acid (0.20 g.), anhydrous K_2CO_3 (1 g.), dimethyl sulphate (1 ml.) and dry acetone (20 ml.) were heated under reflux for 1.5 hr. Volatile solvents were removed on the water pump and the residue was vigorously shaken with water (20 ml.). A solid (0.075 g.) separated. It was twice crystallized from methanol yielding colourless rhombic plates of *trimethylanhydro-ustic acid*, m.p. 171–172.5°. (Found: C, 60.3; H, 6.0; OCH_3 , 43.5. $\text{C}_{11}\text{H}_{16}\text{O}_6$ requires: C, 60.0; H, 5.8; 4OCH_3 , 44.3%.) Trimethylanhydro-ustic acid is neutral in reaction and insoluble in cold NaOH.

From the aqueous filtrate from the crude trimethylanhydro-ustic acid there was isolated a small quantity of 3:4:6-trimethoxyphthalic anhydride. In repeat experiments this product was not obtained, while the yield of trimethylanhydro-ustic acid was low and variable and purification difficult. In one case a small amount of dimethyldehydro-ustic acid (see p. 62) was isolated. The nature of these two by-products suggests that oxidation is liable to occur at some stage in the reaction.

Anhydro-ustic acid (0.10 g.), methylated as described above for ustic acid, gave, in about the same yield, trimethylanhydro-ustic acid, m.p. 170–172°; it did not depress the melting point of the corresponding product from ustic acid itself.

(d) *With dimethyl sulphate and K_2CO_3 in methanol.* Ustic acid (0.20 g.), anhydrous K_2CO_3 (1 g.), dimethyl sulphate (1 ml.) and dry methanol (20 ml.) were heated under reflux for 1.5 hr., yielding finally a yellow solution. The methanol was removed and water (20 ml.) was added. The resulting clear yellow solution was extracted twice with an equal volume of ethyl acetate which, on evaporation, left a negligible residue. The aqueous solution was acidified to congo red and re-extracted twice with an equal volume of ethyl acetate. On removal of the solvent, a colourless gum (0.19 g.) remained and crystallized partially on standing. Treatment of the gum with dry ether yielded a crystalline powder (0.075 g.), m.p. 155–162° (slight decomp.). After three crystallizations from water, *ustic acid 4-methyl ether* separated as large, almost colourless, rhombic prisms (0.01 g.), m.p. 181–182° with slight decomposition to a yellow liquid. (Found: C, 52.9; H, 5.1. $\text{C}_{12}\text{H}_{14}\text{O}_7$ requires: C, 53.3; H, 5.2%.) An aqueous solution of ustic acid 4-methyl ether gives a deep-purple colour with FeCl_3 , reduces cold aqueous copper acetate and gives a precipitate with Brady's reagent only after some hours.

Products of acetylation

(a) *With acetic anhydride and sodium acetate.* Ustic acid (0.1 g.), anhydrous sodium acetate (0.1 g.) and acetic anhydride (1 ml.) were mixed and boiled for 1 min. The pale-

yellow solution was cooled and poured into water. A gum, which was insoluble in cold NaOH, separated, but no pure substance could be isolated from it.

(b) *With acetic anhydride and pyridine.* Ustic acid (0.5 g.) was dissolved in pyridine (6 ml.), and acetic anhydride (5 ml.) was added. The solution was held at room temperature for 6 days and then poured into ice water (100 ml.) when a gum separated. After acidification to congo red the mixture was extracted with benzene. The benzene solution was washed twice with 2N-HCl, then with water, dried over Na_2SO_4 and evaporated. The residual gum (0.71 g.) was redissolved in benzene (50 ml.) and extracted twice with fresh, 5% aqueous NaHCO_3 . The NaHCO_3 extract was rejected. The residual gum (0.63 g.) from the benzene layer was treated with dry ether (20 ml.). It dissolved completely, but crystals separated in the refrigerator, wt. 0.14 g., m.p. 155–158°. These were crystallized three times from methanol yielding *triacetylanhydro-ustic acid* as colourless rhombic prisms, m.p. 159–160°. (Found: C, 56.7; H, 4.4; OCH_3 , 8.5; $\text{C}-\text{CH}_3$, 18.5. $\text{C}_{11}\text{H}_7\text{O}_3(\text{OCOCH}_3)_3$ requires: C, 56.0; H, 4.4; 10CH_3 , 8.5; $4\text{C}-\text{CH}_3$, 16.5 or 18.5% if allowance is made for the high value obtained with ustic acid.) Triacetylanhydro-ustic acid is neutral in reaction and insoluble in water.

The ether filtrate from triacetylanhydro-ustic acid (0.14 g.) was treated with an equal volume of light petroleum and held at 0° for some days. The crystals so obtained (0.18 g.), m.p. 120–124°, were recrystallized from aqueous methanol and then from ethanol. The compound, the nature of which is not clear, was thus obtained as colourless rhombic prisms, m.p. 125–126°. (Found: C, 53.7; H, 4.8; OCH_3 , 7.6, 7.5; $\text{C}-\text{CH}_3$, 22.1. $\text{C}_{11}\text{H}_8\text{O}_3(\text{OCOCH}_3)_4$ requires: C, 53.8; H, 4.8; 10CH_3 , 7.3; $5\text{C}-\text{CH}_3$, 17.7 or 19.4% if allowance is made for the high value obtained with ustic acid.) This compound, which is neutral in reaction and insoluble in water, is very similar in appearance to, though differing greatly in melting point from, triacetylanhydro-ustic acid.

Anhydro-ustic acid (0.2 g.) was dissolved in pyridine (2.5 ml.), and acetic anhydride (2 ml.) was added. The solution was held for several days at room temperature and was then poured into water (50 ml.). The resulting gum soon solidified and was separated by filtration (weight 0.15 g., m.p. 137–150°). Two crystallizations from ethanol yielded triacetylanhydro-ustic acid (0.08 g.), m.p. 160–161.5°, not depressing the melting point of triacetylanhydro-ustic acid prepared from ustic acid itself.

Reaction product with 2:4-dinitrophenylhydrazine

Ustic acid (0.20 g.) was dissolved in water (5 ml.), and Brady's reagent, 0.32% 2:4-dinitrophenylhydrazine in 2N-HCl, (150 ml.) was added. The initially clear solution began to cloud after 2 hr., and after 3 days there was copious separation of fluffy balls of yellow needles (0.14 g.). A further crop (0.15 g.) separated during the next 2 days. After two crystallizations from ethyl acetate *anhydro-ustic acid mono-2:4-dinitrophenylhydrazone* was obtained as characteristic hexagonal yellow prisms. (Found: C, 49.1; H, 3.9; N, 12.3; OCH_3 , 7.3. $\text{C}_{17}\text{H}_{14}\text{O}_9\text{N}_4$ requires: C, 48.8; H, 3.4; N, 13.4; 10CH_3 , 7.4%.) The substance has no definite melting point. It softens at 140°, partly melts at 150°, partly resets at 160° and finally remelts at 180°; when heated very rapidly it softens at 143° and melts at 152–160° with decomposition. The compound gives a red colour when dissolved in ethanol and treated with a drop of aqueous NaOH (Neuberg's test)

indicating a mono-derivative. It is insoluble in NaHCO_3 , but dissolves in Na_2CO_3 to give a red colour.

Anhydro-ustic acid (0.10 g.) was dissolved in water (6 ml.), and Brady's reagent (80 ml.) was added. An immediate non-crystalline precipitate was formed. It was separated by filtration, washed, dried (weight 0.185 g.) and crystallized from ethyl acetate. Anhydro-ustic acid mono-2,4-dinitrophenylhydrazone, identical in properties and appearance—characteristic hexagonal yellow prisms—with the product from ustic acid described above, was thus obtained. The dinitrophenylhydrazone from ustic acid and the dinitrophenylhydrazone from anhydro-ustic acid and a mixture of the two, when heated in the same bath, behaved in the same way.

Action of ozone on ustic acid

(a) *In water.* Ustic acid (0.22 g.) was dissolved in water (20 ml.) and cooled in ice water. Ozonized oxygen was passed through the solution for 4 hr. At the end of this time the solution still reduced cold Fehling's solution and gave only a slight cloudiness with Brady's reagent. Evaporation of the solution under reduced pressure and titration of the distillate yielded only 5% of the theoretical for the production of 1 mol. of volatile acid. The residue after evaporation was crystallized from ethyl acetate (3 ml.) and benzene (30 ml.) and yielded unchanged ustic acid, m.p. 166° (decomp.), weight 0.155 g. = 70% recovery.

(b) *In ethyl acetate.* A solution of ustic acid (0.25 g.) in ethyl acetate (20 ml.) was similarly ozonized for 6 hr. Water (50 ml.) was added and the whole was evaporated to dryness *in vacuo*. Crystallization of the residue yielded unchanged ustic acid, m.p. 167° (decomp.), weight 0.17 g. = 68% recovery.

DEHYDRO-USTIC ACID

Ustic acid (0.255 g., 0.0010 g. mol.) and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.6 g., 0.0024 g. mol.) were dissolved in cold water (10 ml.). A green solution resulted, but no Cu_2O separated. 0.1 N-NaOH was added from a burette until the solution became permanently neutral (pH 7). A copious precipitate of Cu_2O separated. The amount of NaOH required (50 ml.) is that necessary to neutralize the products of the reaction:

The Cu_2O was separated by filtration and the clear filtrate was acidified to congo red and extracted twice with an equal volume of ethyl acetate. The gum (0.24 g.) remaining after removal of the solvent was crystallized from water (1.5 ml.) using charcoal, when *dehydro-ustic acid* separated in colourless diamond-shaped prisms (0.16 g.); a further quantity (0.03 g.) of less pure material was obtained from the liquors; total yield 70%. A second crystallization from water gave the pure acid, m.p. $124\text{--}125^\circ$ with loss of water, the melt not resetting on cooling. The melting point is somewhat dependent on the rate of heating since water is lost slowly even below the melting point. (Found, on material dried *in vacuo* at room temperature: C, 48.3; H, 4.7; OCH_3 , 11.4; C-CH_3 , 6.2; loss at $100^\circ/16\text{ mm.}$, 6.9. $\text{C}_{11}\text{H}_{12}\text{O}_8(\text{C}_{11}\text{H}_{10}\text{O}_7 \cdot \text{H}_2\text{O})$ requires: C, 48.5; H, 4.4; IOCH_3 , 11.4; IC-CH_3 , 5.5; loss of H_2O , 6.6%.) The clear yellow gum which results from the loss of water on heating dehydro-ustic acid does not crystallize on standing. However, when dissolved in boiling undried benzene, crystals of dehydro-ustic acid separate, m.p. $123\text{--}125^\circ$ with loss of water, the melting point being unchanged on admixture with hydrated dehydro-ustic acid.

Dehydro-ustic acid dissolves fairly readily in ethyl acetate and water. It is slightly soluble in hot benzene from which it can be crystallized, and is almost insoluble in light petroleum. An aqueous solution of dehydro-ustic acid has the following properties: (1) At neutral or alkaline pH values it is yellow, the colour being discharged on acidification. (2) It shows no fluorescence in ultraviolet light, nor does an acetone solution of dehydro-ustic acid. (3) Aqueous FeCl_3 gives an intense purple colour indistinguishable from that given by ustic acid. (4) On gradual addition of bromine water the latter is decolorized, but a transient cloudiness is produced, disappearing after a few seconds. At no time is a permanent precipitate observed. (5) With Brady's reagent an immediate cloudiness is produced, followed shortly by the formation of a copious flocculent precipitate. (6) It does not reduce Fehling's solution at room temperature or aqueous CuSO_4 or copper acetate solution.

Derivatives of dehydro-ustic acid

Methyl derivatives. A mixture of hydrated dehydro-ustic acid (0.71 g.), dimethyl sulphate (3 ml.), anhydrous K_2CO_3 (3 g.) and dry acetone (50 ml.) was refluxed for 1.5 hr. Volatile solvents were removed on the water pump, water (50 ml.) was added to the residue, and the alkaline mixture was shaken vigorously for 20 min. when the oil which had separated crystallized to a yellow solid which was separated by filtration (wt. 0.52 g., m.p. $118\text{--}120^\circ$). This *methyl dimethyldehydro-ustate* crystallized from benzene-light petroleum in pale-yellow rectangular prisms. For analysis the substance was sublimed at $110\text{--}115^\circ$ in a high vacuum, the lemon-yellow sublimate melting at $119\text{--}120^\circ$ without decomposition and recrystallizing on cooling. (Found: C, 57.1; H, 5.5; OCH_3 , 41.6. $\text{C}_{14}\text{H}_{16}\text{O}_8$ requires: C, 56.8; H, 5.4; 4OCH_3 , 41.9%.) Methyl dimethyldehydro-ustate gives no colour with ethanolic FeCl_3 , but gives an immediate yellow precipitate with Brady's reagent in aqueous ethanol.

The alkaline aqueous filtrate from the ester, after standing at room temperature for 24 hr., was extracted twice with an equal volume of ether, the extract being rejected. The aqueous layer was then acidified to congo red and extracted three times with an equal volume of ethyl acetate. On removal of the solvent a solid (0.18 g.), m.p. $148\text{--}151^\circ$, remained. This was crystallized three times from water from which *dimethyldehydro-ustic acid* separated as long colourless rhombic prisms or needles, m.p. $152\text{--}153^\circ$ without decomposition and resetting on cooling. (Found: C, 54.8; H, 5.3; OCH_3 , 32.6. $\text{C}_{13}\text{H}_{14}\text{O}_7$ requires: C, 55.3; H, 5.0; 3OCH_3 , 33.0%.) Dimethyldehydro-ustic acid is soluble in cold aqueous NaHCO_3 . It gives no colour with aqueous FeCl_3 and gives an immediate precipitate with Brady's reagent. Alkaline hydrolysis of methyl dimethyldehydro-ustic acid leads to breakdown. Acid hydrolysis was more successful. A mixture of the ester (0.05 g.) and 2N-HCl (1 ml.) was boiled for 5 min. after the solid had dissolved. On cooling, colourless crystals (0.045 g.) separated, m.p. $150\text{--}152^\circ$ unchanged on admixture with dimethyldehydro-ustic acid.

Mono-2,4-dinitrophenylhydrazone. Dehydro-ustic acid (0.2 g.) was dissolved in water (5 ml.), and Brady's reagent (150 ml.) was added. The mixture was held at room temperature for 3 days when the initial flocculent precipitate had settled to a crystalline solid which was collected (0.32 g.). It was recrystallized by dissolving in hot ethyl acetate (2 ml.) and adding benzene (10 ml.). *Dehydro-ustic acid mono-2,4-dinitrophenylhydrazone* separated as yellow prisms (0.26 g.).

When heated slowly it softens at 155° and decomposes slightly without discoloration then resets to a yellow solid and remelts at 222–227° with vigorous decomposition and darkening; if inserted in a bath at 200° and heated, it decomposes vigorously and sets at once, then decomposes at 230° with darkening. Further crystallization from ethyl acetate-benzene or from ethyl acetate-light petroleum did not change this behaviour. (Found, on material crystallized from ethyl acetate-light petroleum and dried at 100°/16 mm.: C, 47.7; H, 3.8; N, 13.2; OCH_3 , 7.1; loss in weight on drying, 4.4. $\text{C}_{17}\text{H}_{14}\text{O}_{10}\text{N}_4$ requires: C, 46.9; H, 3.2; N, 12.9; IOCH_3 , 7.1; loss of $1\text{H}_2\text{O}$ from $\text{C}_{17}\text{H}_{14}\text{O}_{10}\text{N}_4 \cdot \text{H}_2\text{O}$, 4.0%.) The compound gives a red colour with Neuberg's reagent, indicating a monoderivative. It is soluble in NaHCO_3 to give a yellow solution, and in Na_2CO_3 to give a red solution.

Quinoxaline. Dehydro-ustic acid (0.27 g.) and *o*-phenylenediamine hydrochloride (0.20 g., 10% excess) in ethanol (10 ml.) was refluxed for 30 min. The green solution was cooled and poured into water (50 ml.). The almost colourless crystalline solid which separated (weight 0.30 g., m.p. 216° (decomp.)) was recrystallized three times from 30 to 40% aqueous ethanol, using charcoal. The quinoxaline forms slightly yellow needles or plates, m.p. 226–227° with vigorous gas evolution. The air-dried material contains water and ethanol. (Found, on material dried at 100°/16 mm.: C, 62.3; H, 4.3; N, 8.8; OCH_3 , 9.6. $\text{C}_{17}\text{H}_{14}\text{O}_8\text{N}_4$, the quinoxaline from $\text{C}_{11}\text{H}_{10}\text{O}_7$, requires: C, 62.6; H, 4.3; N, 8.6; IOCH_3 , 9.5%.)

The quinoxaline is only slightly soluble in boiling water, but dissolves in cold NaHCO_3 and in 2*N*-HCl. In ethanolic solution it gives a bluish-grey colour with FeCl_3 , which changes to purple on dilution with water. Solutions in ethanol or benzene do not fluoresce in ultraviolet light. The brown solution in cold conc. H_2SO_4 turns to deep blue on warming.

Oxidation of dimethyldehydro-ustic acid by alkaline KMnO_4

Dimethyldehydro-ustic acid (0.24 g.) was dissolved in a small excess of aqueous Na_2CO_3 and boiled under reflux with 0.05*M*- KMnO_4 (100 ml.), further 50 ml. portions being added until the red colour persisted for 30 min. (total volume 350 ml.). The mixture was cooled, decolorized with NaHSO_3 , acidified and extracted twice with an equal volume of ethyl acetate. The extracts were washed with water (20 ml.) and evaporated to give a crude solid residue (0.13 g.). After two recrystallizations from water, 3:4:6-trimethoxyphthalic acid (87.5 mg.) separated as colourless rhombic tablets, m.p. 210–211° after softening at 180°. If placed in a bath heated above 180° the crystals melt at once evolving water, then reset, and remelt at 210–211°. (Found, on air-dried material: loss of weight at 100°/16 mm., 6.8. Calc. for $\text{C}_{11}\text{H}_{12}\text{O}_7 \cdot \text{H}_2\text{O}$: loss of $1\text{H}_2\text{O}$, 6.6%. Found, on material dried at 100°/16 mm. C, 51.7, 51.6; H, 4.7, 4.6; OCH_3 , 37.0, 36.3. Calc. for $\text{C}_{11}\text{H}_{12}\text{O}_7$: C, 51.6; H, 4.7; 3OCH_3 , 36.3%.) The acid gives no colour with FeCl_3 .

The above 3:4:6-trimethoxyphthalic acid (27 mg.) was heated in an open test tube in an oil bath until the bath temperature reached 220°. At this point yellowish leaflets began to appear as a sublimate. The tube was cooled and the whole contents were crystallized from benzene (5 ml.). The solution, which showed a strong blue-purple fluorescence, deposited short stout prisms of 3:4:6-trimethoxyphthalic anhydride (weight 18 mg., m.p. 212–213°). (Found: C, 55.5,

55.4; H, 4.2, 4.1; OCH_3 , 38.8. Calc. for $\text{C}_{11}\text{H}_{10}\text{O}_6$: C, 55.5; H, 4.2; 3OCH_3 , 39.1%.)

Synthetic 3:4:6-trimethoxyphthalic acid and 3:4:6-trimethoxyphthalic anhydride were prepared according to the method of Bargellini (1914). They were identical in appearance and properties with the oxidation products recorded above. Synthetic 3:4:6-trimethoxyphthalic acid melted at 209–210° and did not depress the melting point of the oxidation acid. The synthetic anhydride melted at 209–210°; mixed with the oxidation anhydride it melted at 209–211°.

Oxidation of dehydro-ustic acid by alkaline H_2O_2

Dehydro-ustic acid (0.2582 g.) was dissolved in cold *N*-NaOH (10.0 ml.), and H_2O_2 ('100-vol.', 5 ml.) was added. After standing 35 min. when the yellow colour had faded and a precipitate was no longer given with Brady's reagent, the solution was acidified with *N*- H_2SO_4 (11.0 ml.), and steam-distilled exhaustively on the water pump at 40–45° for the estimation of volatile acid. The distillate required 9.9 ml. of 0.1*N*-NaOH for neutralization corresponding to 104% of the theoretical for 1 mol. volatile acid/mol. of dehydro-ustic acid. The titrated distillate was evaporated to dryness and converted into the *p*-bromophenacyl derivative in the usual way. The crude *p*-bromophenacyl derivative was purified by repeated recrystallization from light petroleum, b.p. 60–80°, and finally by sublimation in a high vacuum at 80–90°. It was obtained as colourless crystals, m.p. 87–89° not depressed on admixture with authentic *p*-bromophenacyl acetate, m.p. 86.5–88.5°, prepared from sodium acetate. (Found: C, 46.6; H, 3.4; Br, 30.9. Calc. for $\text{C}_{10}\text{H}_8\text{O}_3\text{Br}$: C, 46.7; H, 3.5; Br, 31.1%.) The volatile acid obtained in the oxidation thus consisted essentially of acetic acid together with small amounts of some other volatile acid.

The acidified steam-distillation residue was extracted three times with an equal volume of ethyl acetate, and the extracts were evaporated to give a dry solid (m.p. 188–190°, decomp., wt. 0.18 g., 83% yield). This was crystallized twice from ethyl acetate-benzene, once with charcoal, then from acetone-benzene giving colourless, flat, hexagonal prisms and needles, m.p. 192–193° with decomposition to a yellow liquid which partially resets on cooling, remelts at 170–176° with the formation of a sublimate, probably of the anhydride, of nearly colourless crystalline rods, m.p. 174–178°. (Found: C, 47.6; H, 3.4; OCH_3 , 13.6, 13.5; C-CH_3 , 1.2. $\text{C}_9\text{H}_8\text{O}_7$ requires: C, 47.4; H, 3.5; IOCH_3 , 13.6%; C-CH_3 , nil.) This acid, 4:6-dihydroxy-3-methoxyphthalic acid, gives with aqueous FeCl_3 an intense purple colour which is indistinguishable from that given by ustic acid and dehydro-ustic acid.

In a larger-scale preparation using dehydro-ustic acid (2.0 g.) dissolved in water (250 ml.) and with the addition of H_2O_2 ('100-vol.', 50 ml.) and 2*N*-NaOH (50 ml.), 0.95 g. of pure 4:6-dihydroxy-3-methoxyphthalic acid, m.p. 192° (decomp.) was obtained.

Methylation of the acid $\text{C}_9\text{H}_8\text{O}_7$

Formation of 3:4:6-trimethoxyphthalic acid

A mixture of the above acid, $\text{C}_9\text{H}_8\text{O}_7$ (0.2 g.), dimethyl sulphate (1.0 ml.), anhydrous K_2CO_3 (1.0 g.) and dry acetone (20 ml.) was refluxed for 1.5 hr. Volatile solvents were removed on the water pump and the residue was refluxed for 20 min. with *N*-NaOH (20 ml.). The clear colourless solution was cooled, acidified with 2*N*-HCl and extracted with ethyl

acetate. Removal of the solvents from the extract left a solid (0.20 g.) which was crystallized from water (4 ml.) (charcoal). 3:4:6-trimethoxyphthalic acid (0.18 g.) separated, m.p. 209–211° not depressed on admixture with synthetic material. (Found, on air-dried material: C, 48.1; H, 5.0; $C-CH_3$, 2.7. Calc. for $C_{11}H_{12}O_7$, H_2O : C, 48.2; H, 5.2%; $C-CH_3$, nil.) It was also converted into the anhydride (see p. 63), m.p. 210–211°, unchanged on mixing with the synthetic anhydride. (Found: C, 55.5; H, 3.9. Calc. for $C_{11}H_{10}O_5$: C, 55.5; H, 4.2%.)

Decarboxylation of dehydro-ustic acid

(a) *In hot dilute acid.* A solution of hydrated dehydro-ustic acid (0.5470 g.) in 2N- H_2SO_4 (20 ml.) was boiled under reflux and a slow stream of CO_2 -free N_2 was passed through the solution. The issuing gas was scrubbed, first through Brady's reagent and then through 0.2N-aqueous $Ba(OH)_2$. After 6.7 hr. the Brady's reagent was still clear and 48.4% of 1 mol. equiv. CO_2 was absorbed in the $Ba(OH)_2$. The olive-green reaction solution was diluted with water to 40 ml. and extracted three times with an equal volume of ethyl acetate. The combined extracts were separated into acidic and phenolic fractions by extraction with fresh aqueous 1% $NaHCO_3$. The acidic fraction (0.30 g.) consisted of unchanged dehydro-ustic acid. The phenolic fraction, a viscous olive-brown oil (0.22 g.), was hardened with petrol to a greyish-white powder (0.19 g.) which was crystallized from benzene (15 ml.) (charcoal) to yield yellow prisms, m.p. 98–100°. Further recrystallization from benzene raised the m.p. to 100–101° without decomposition. (Found: C, 57.3, 57.1; H, 4.9; 4.8; OCH_3 , 14.8. $C_{10}H_{10}O_5$ requires: C, 57.1; H, 4.8; OCH_3 , 14.8%.) Decarboxydehydro-ustic acid (3:5-dihydroxy-2-methoxyphenyl methyl diketone) is very soluble in water. Its aqueous solution (pH ~4.5) is yellow and turns orange when made alkaline. It gives an immediate yellow precipitate with Brady's reagent. With aqueous $FeCl_3$ a dirty blue colour is observed changing rapidly to brown and then to green, the green colour slowly deepening on standing. No $FeCl_3$ colour is observed in ethanol.

(b) *In quinoline-copper chromite.* A mixture of hydrated dehydro-ustic acid (0.4916 g.) in purified quinoline (15 ml.) and copper chromite (0.25 g.) was heated, in an atmosphere of N_2 , as in method (a), at 120–150° for 70 min. CO_2 equivalent to 102% theoretical for 1 mol. CO_2 was evolved. The reaction mixture was acidified and extracted with ethyl acetate. The extract, which was very dark in colour, was purified as described in method (a). The crude crystalline phenolic fraction (0.26 g.), m.p. 96–99°, was recrystallized from benzene (charcoal) yielding decarboxydehydro-ustic acid (0.145 g), m.p. 98–99.5°, unchanged on admixture with material prepared by method (a).

The quinoxaline derivative was prepared by boiling a solution of the diketone (0.10 g.) and *o*-phenylenediamine hydrochloride (0.10 g.) in ethanol (10 ml.). After 30 min. the solution was poured into water to give a clear yellow solution. It was made alkaline and extracted three times with ether to remove unchanged amine, then acidified to congo red and extracted again three times with an equal volume of ether. The yellow solid (0.10 g.) remaining after removal of the solvent melted at 264°. After crystallizing twice from a mixture of ethanol (7 ml.) and water (40 ml.), once with charcoal, the quinoxaline derivative separated in yellow rhombic prisms, m.p. 264–266°, with darkening and sublimation. (Found: C, 68.2, 68.1; H, 5.0, 4.9; N, 9.6; OCH_3 , 11.7, 11.6. $C_{16}H_{14}O_3N_2$ requires: C, 68.1; H, 5.0; N, 9.9; $10CH_3$,

11.0%.) The compound does not fluoresce in acetone or benzene solution. In conc. H_2SO_4 it gives a brown solution unchanged on warming.

Oxidation of decarboxydehydro-ustic acid with alkaline H_2O_2

The method described for the oxidation of dehydro-ustic acid, using excess H_2O_2 (see p. 63) gave only a low yield and was therefore modified. Decarboxydehydro-ustic acid (0.258 g.) was dissolved in water (50 ml.), and N-NaOH (10.0 ml.) was added yielding an orange-red solution. H_2O_2 ('100 vol.') was added 6 drops at a time, with shaking, at intervals of 3 min., testing with Brady's reagent before each addition. After addition of 0.4 ml. H_2O_2 Brady's reagent gave only a slight precipitate. The brown solution was now treated with N- H_2SO_4 (11.0 ml.), diluted to about 100 ml. and distilled exhaustively on the water pump at 35–40° for the estimation of the volatile acid. The distillate required 11.66 ml. 0.1N-NaOH for neutralization, corresponding to 95% of the theoretical for 1 mol. volatile acid/mol. of decarboxydehydro-ustic acid. The titrated distillate was converted in good yield into the *p*-bromophenacyl derivative which was purified as described previously, m.p. 85–86° not depressed on admixture with synthetic *p*-bromophenacyl acetate. No other *p*-bromophenacyl derivative was detected.

The distillation residue, which had become browner during the exhaustive evaporation, was extracted three times with ethyl acetate (50 ml.) and the extracts were evaporated to give a dark gummy solid (0.20 g.). By crystallization from ethyl acetate-benzene-petrol and by sublimation of the residues, a yield of 0.12 g. (53% of the theoretical) of material melting from 197° was obtained.

A purer product resulted from a repeat experiment in which the distillation was omitted. The crude product (0.37 g. from 0.50 g. decarboxydehydro-ustic acid) was crystallized from acetone (4 ml.) and benzene (40 ml.). Colourless crystals (weight 0.26 g., m.p. 205–207° without decomposition) were obtained and were prepared for analysis by sublimation in a high vacuum at 120–130°; m.p. 205–207°. (Found: C, 52.3; H, 4.4; OCH_3 , 16.7. $C_8H_8O_5$ requires: C, 52.2; H, 4.4; $10CH_3$, 16.9%.) The oxidation product, 3:5-dihydroxy-2-methoxybenzoic acid, gives with aqueous $FeCl_3$ first a greyish-brown colour quickly deepening to a true brown and becoming deeper and redder on standing. In ethanol solution $FeCl_3$ gives no colour reaction.

The oxidation acid (0.17 g.) was methylated by the dimethyl sulphate- K_2CO_3 -acetone method described for the methylation of the acid $C_6H_5O_7$ (see p. 63). The crude methylation product (0.15 g.) was crystallized from water (15 ml.) with charcoal. 2:3:5-Trimethoxybenzoic acid was thus obtained as colourless needles, m.p. 102–103°, unchanged after sublimation in a high vacuum at 90–95°. The melting point was not depressed on admixture with authentic synthetic 2:3:5-trimethoxybenzoic acid, m.p. 101–103° (see p. 65). (Found: C, 56.7; H, 6.1; OCH_3 , 43.7. Calc. for $C_{10}H_{12}O_5$: C, 56.6; H, 5.7; $3OCH_3$, 43.7%.)

The oxidation acid (0.10 g.) was also ethylated by the same method using diethyl sulphate in place of dimethyl sulphate. On acidification of the alkaline hydrolysis solution the ethylation product separated as an oil which slowly crystallized (weight 0.045 g., m.p. 79–81°). It was sublimed in a high vacuum at 80–90°. The colourless sublimate of 3:5-diethoxy-2-methoxybenzoic acid melted at 81–83° not de-

pressed on admixture with an authentic synthetic specimen, m.p. 82–83° (see below). The mixture reset on cooling and remelted at the same temperature. (Found: C, 60.0; H, 6.7; alkoxy calc. as OCH_3 , 38.3. $\text{C}_{12}\text{H}_{16}\text{O}_5$ requires: C, 60.0; H, 6.7; 3 alkoxy groups calc. as OCH_3 , 38.7%.)

SYNTHESES

(a) *2:3:5-Trihydroxybenzoic acid*. The method used followed the general directions given by Baker & Brown (1948). 3:5-Dihydroxybenzoic acid (0.77 g.) (*Organic Syntheses*, 21, 27) was dissolved in 2N-NaOH (12.5 ml.), and $\text{K}_2\text{S}_2\text{O}_8$ solution (1.35 g. in 35 ml. water) was added during 3–5 hr. on stirring at 10–15°. After stirring for a further 0.5 hr. the deep-red solution was held at room temperature overnight. It was then acidified with HCl to congo red and was extracted four times with half its volume of ether. This extract, on evaporation, left a residue of impure starting material. The extracted aqueous layer was treated with conc. HCl (5 ml.), hydrolysed for 30 min. on the steam bath, cooled and extracted three times with an equal volume of ether. On removal of the solvent there remained a brown gummy solid (0.30 g.) which was dissolved in ethyl acetate (10 ml.), and benzene (60 ml.) was added. A dark flocculent precipitate separated and was removed by filtration. The filtrate, on evaporation, left a dry brown solid (0.20 g.) which was crystallized from water (3 ml.), but the product (0.13 g.) contained some green impurity which was removed by sublimation. The buff-coloured sublimate (0.06 g.) was crystallized twice from water with charcoal and then once more from water. 2:3:5-Trihydroxybenzoic acid separated as pale-yellow silky needles (0.03 g.), m.p. 226–227° with decomposition and incompletely, darkening above this temperature and melting completely at 263–280°. (Found, on air-dried material: C, 40.8; H, 5.0; loss at 100°/16 mm., 17.4. $\text{C}_7\text{H}_8\text{O}_5 \cdot 2\text{H}_2\text{O}$ requires: C, 40.8; H, 4.9; loss of $2\text{H}_2\text{O}$, 17.5%. On material dried at 100°/16 mm.: C, 49.2; H, 3.8. $\text{C}_7\text{H}_8\text{O}_5$ requires: C, 49.4; H, 3.6%.) Aqueous solutions of 2:3:5-trihydroxybenzoic acid are not very stable and when exposed to air at room temperature turn brown after some hours. With aqueous FeCl_3 they give a transient green colour changing rapidly to brown. In ethanol a more stable bluish green colour is observed, but this also fades to brown after 1 hr. Since this work was completed the synthesis of 2:3:5-trihydroxybenzoic acid has been described by Corbett, Hassall, Johnson & Todd (1950), who record m.p. 223° (decomp.).

(b) *2:3:5-Trimethoxybenzoic acid*. Hydrated 2:3:5-trihydroxybenzoic acid (0.10 g.), prepared as above, was methylated by the dimethylsulphate- K_2CO_3 -acetone method. The crude product (0.09 g.), m.p. 97–100°, was crystallized from water (10 ml.), m.p. 101–102° without decomposition, wt. 0.065 g. Sublimation in a high vacuum at 90–95° raised the m.p. to 101–103°. Smith & Laforge (1931) record the m.p. as 105°; Baker, Brown & Scott (1939) give 104°. (Found: C, 56.1; H, 5.5; OCH_3 , 43.7. Calc. for $\text{C}_{10}\text{H}_{12}\text{O}_5$: C, 56.6; H, 5.7; 3OCH_3 , 43.7%.)

(c) *3:5-Diethoxy-2-hydroxybenzoic acid*. 3:5-Dihydroxybenzoic acid (0.77 g.) was oxidized with $\text{K}_2\text{S}_2\text{O}_8$, and the unchanged starting material was removed as described in section (a) above. The solution was then treated gradually with diethyl sulphate (5 ml.) and 2N-NaOH with stirring at 45–55° during 2 hr. The mixture was acidified and extracted with ether, the extract being rejected. The aqueous layer was hydrolysed with conc. HCl (5 ml.) for 30 min. on the water

bath when some tarry matter separated. The whole was extracted with ether (3 × 50 ml.) and the ether was re-extracted with 0.5N- NaHCO_3 and then with water. The NaHCO_3 extract and wash water were acidified, re-extracted with ether, and the ether removed leaving a partly crystalline residue (0.20 g.). This residue was extracted with boiling benzene, the benzene was removed by evaporation and the resulting solid (0.155 g.) was crystallized from water with charcoal. 3:5-Diethoxy-2-hydroxybenzoic acid separated as nearly colourless plates, m.p. 125–127° (0.05 g.). (Found: C, 58.3; H, 6.4; OC_2H_5 , 39.5. $\text{C}_{11}\text{H}_{14}\text{O}_5$ requires: C, 58.4; H, 6.2; $2\text{OC}_2\text{H}_5$, 39.8%.) The acid gives a blue colour with FeCl_3 in aqueous solution and a greenish blue in ethanol.

(d) *3:5-Diethoxy-2-methoxybenzoic acid*. The above 3:5-diethoxy-2-hydroxybenzoic acid (0.04 g.) was methylated by the dimethyl sulphate- K_2CO_3 -acetone method. The crude product (0.035 g.), m.p. 78–80°, was sublimed at 80–90° in a high vacuum, recrystallized from light petroleum b.p. 40–60° (3 ml.), then resublimed. 3:5-Diethoxy-2-methoxybenzoic acid sublimed as a colourless solid, m.p. 82–83°. (Found: C, 60.1; H, 6.7. $\text{C}_{12}\text{H}_{16}\text{O}_5$ requires: C, 60.0; H, 6.7%.)

Methylation of a degradation product of citromycesin

The methyl ester of *O*-dimethylcitromycesin was hydrolysed with ethanolic KOH and the resulting dimethoxyhydroxyphthalic acid was isolated as described by Hetherington & Raistrick (1931). It melted at 180° with loss of water, reset on cooling, and remelted at 197–201°. Its aqueous solution gave a pure purple colour with FeCl_3 . This compound (0.15 g.) was methylated by the dimethyl sulphate- K_2CO_3 -acetone method described on p. 63. After acidification colourless rhombic prisms, (0.07 g.) separated, m.p. 210.5–212°, after softening at 180° with loss of water. When mixed with 3:4:6-trimethoxyphthalic acid, m.p. 211–212°, obtained by degradation of ustic acid (see p. 63), it melted at 211.5–212.5°, resetting on cooling and remelting at the same temperature.

The above 3:4:6-trimethoxyphthalic acid (0.05 g.) was converted into the anhydride by heating in an open tube in an oil bath to 220° when it melted and began to sublime. The whole melt was now sublimed *in vacuo* at 100–150°. The sublimate, very pale yellow in colour, melted at 211–212° and did not depress the melting point of synthetic 3:4:6-trimethoxyphthalic anhydride (see p. 63). The mixture reset on cooling and remelted at the same temperature. (Found: C, 55.8; H, 4.3. $\text{C}_{11}\text{H}_{10}\text{O}_6$ requires: C, 55.5; H, 4.2%.)

SUMMARY

1. A new, colourless, crystalline mould metabolic product, *ustic acid*, $\text{C}_{11}\text{H}_{12}\text{O}_7$, m.p. 169–170° (decomp.), has been isolated from culture filtrates of four different strains of *Aspergillus ustus* (Bainier) Thom & Church which were grown on Raulin-Thom solution.

2. A number of derivatives and breakdown products of ustic acid are described.

3. Ustic acid has been shown to be 2:4-dihydroxy-5-methoxybenzoic acid with an unbranched side chain $-\text{C}_3\text{H}_5\text{O}_2$ in position 6.

4. The most favoured structure for this side chain is $-\text{CHOH}.\text{CO}.\text{CH}_3$, though many of the pro-

perties of ustic acid are as well illustrated by the side chains $-\text{CO}.\text{CHOH}.\text{CH}_3$, $-\text{C}(\text{OH})=\text{C}(\text{OH}).\text{CH}_3$, $-\text{CH}.\text{C}(\text{OH}).\text{CH}_3$, $-\text{C}(\text{OH}).\text{CH}.\text{CH}_3$ or by equivalent structures.

librium mixtures of two or more of these five tautomers, depending on the experimental conditions obtaining when any particular test is carried out.

5. The close structural relationship existing between ustic acid and a number of other mould metabolic products and lichen acids is pointed out.

6. The synthesis of 3:5-diethoxy-2-hydroxybenzoic acid and of 3:5-diethoxy-2-methoxybenzoic acid is described.

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