XXXVIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XLI. THE METABOLIC PRODUCTS OF *PENICILLIUM CHARLESII* G. SMITH. II. THE MOLECULAR CONSTITUTION OF CAROLIC AND CAROLINIC ACIDS.

By PERCIVAL WALTER CLUTTERBUCK, HAROLD RAISTRICK and FRITZ REUTER.

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

(*Received December 21st, 1934.*)

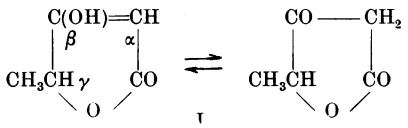
In a recent communication [Clutterbuck *et al.*, 1934] it was shown that *Penicillium Charlesii* G. Smith, a new species of *Penicillium* isolated by our late colleague Mr J. H. V. Charles from mouldy Italian maize, produced from glucose a polygalactose and a polymannose together with a number of hitherto undescribed organic acids. Of these acids carolic and carolinic acids were obtained in the largest yields. It is the purpose of this paper to describe the investigation of the molecular constitutions of these two acids.

Carolic acid gives an orange $FeCl_3$ colour but does not give an immediate violet colour with $NaNO_2$. In aqueous solution it is acid to Congo red and titrates in the cold as a monobasic acid, the titration not being materially altered by gently warming with excess $N/10$ NaOH. The acid does not however contain any active hydrogen as determined in dry anisole by the Zerewitinoff method, whilst it contains one active hydrogen when determined in pyridine (Roth). It appears therefore that carolic acid contains a non-lactonic ring which opens up almost completely during titration in the cold in aqueous solution, whilst in pyridine one ketonic group becomes enolised.

Carolinic acid gives with $FeCl_3$ an immediate precipitate, soluble in excess to a bright orange coloured solution, but does not give an immediate $NaNO_2$ colour. The acid turns Congo red blue, titrates sharply in the cold as a dibasic acid and contains 2 atoms of active hydrogen as determined in dry anisole or pyridine by the Zerewitinoff method (Roth).

Both carolic and carolinic acids on boiling with $2N$ H_2SO_4 undergo immediate hydrolysis, carolic acid giving quantitatively 1 molecule each of acetoin, CO_2 and butyrolactone, whilst carolinic acid gives 1 molecule each of acetoin, CO_2 and succinic acid.

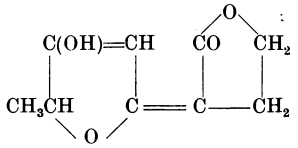
Both carolic and carolinic acids on brominating in dilute acetic acid solution give *d*-α-bromo-γ-methyltetronic acid ($C_5H_5O_3Br$) which on catalytic reduction with palladium and hydrogen very readily loses its bromine to give *l*-γ-methyltetronic acid (I).



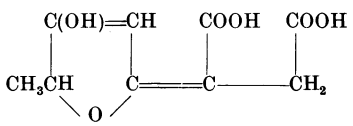
I

On hydrolysis with $2N$ H_2SO_4 (I) quantitatively gives 1 molecule each of acetoin and CO_2 and it is therefore reasonable to suppose that the acetoin and CO_2 obtained on hydrolysis of carolic and carolinic acids arise from the γ-methyltetronic acid nucleus present in these acids and separated from them on bromination. Carolic and carolinic acids may therefore be represented as arising from the condensation of 1 molecule of *l*-γ-methyltetronic acid with 1 molecule butyrolactone and 1 molecule succinic acid respectively, in each case with the elimination of 1 molecule of water.

Various methods of postulating these condensations might be suggested. It is improbable that an esterification occurs at the enolised β-carbonyl group of the γ-methyltetronic acid ring since both carolic and carolinic acids on reduction with Pd and hydrogen give products which on hydrolysis still decarboxylate (though much more slowly than the parent acids) but no longer break down into two C_4 compounds. Since both the acids themselves and the reduced acids give hydroxyketones on hydrolysis it seems unlikely that the β- and γ-carbons of the γ-methyltetronic acid ring are involved in the condensation. On the other hand condensation might take place at the lactonic carbonyl group of the γ-methyltetronic acid ring, when carolic and carolinic acids would have structures II and III respectively.



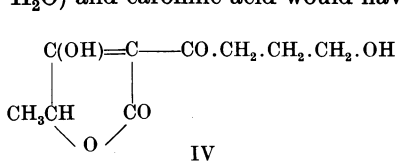
II



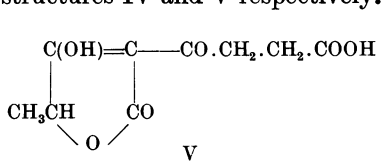
III

An example of this type of structure is given by Fittig and Ström's [1892] dibutyrolactone. It will be shown however that formulae of this type cannot explain a number of properties of these acids.

Alternatively linkage may occur at the α-CH_2 group, when carolic acid ($+H_2O$) and carolinic acid would have the structures IV and V respectively.

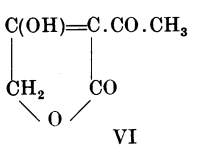


IV

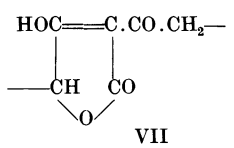


V

An example of this type of structure is given in Benary's [1910] α-acetyltetronic acid (VI) which has the grouping VII, in common with carolic and carolinic acids.



VI

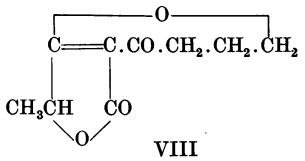


VII

As a result of the absolute analogy in properties and absorption spectra between α-acetyltetronic and carolic and carolinic acids and their derivatives, the ketonic formulae IV and V have now been adopted for the hydrated form of carolic acid ($C_9H_{10}O_4+H_2O$) and for carolinic acid respectively.

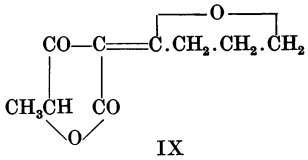
The structural formula of carolic acid ($C_9H_{10}O_4$) then requires the removal of

the elements of water from IV. This may be done in two ways giving formulae VIII and IX.



VIII

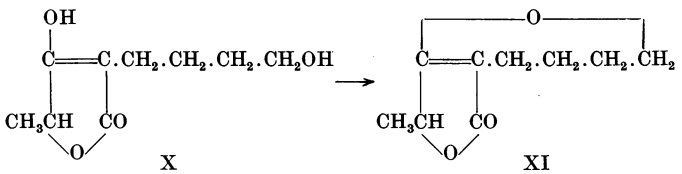
or



IX

No decisive chemical evidence can be advanced for these formulae, but VIII is to be preferred. Thus it seems probable that the 7-membered ring of VIII would be more labile than the 5-membered ring of IX and would therefore more readily explain the addition of H_2O by mere solution in water. Moreover some heterocyclic structures containing more than six members are known, and on solution in water do form equilibrated systems [*cf. e.g.*, Helferich and Schäfer, 1924].

Further, it should be noted that reduced carolic acid ($C_9H_{14}O_4$), which is a liquid titrating as a monobasic acid, and which is shown later to have the structure X, readily loses one molecule of H_2O on distillation *in vacuo* to give a crystalline neutral compound $C_9H_{12}O_3$ (XI).



X XI

This reaction cannot lead to the formation of a 5-membered ring but must give the same 7-membered ring as is postulated above in the formula VIII for carolic acid with the exception that the CO group immediately outside the tetronic acid ring in carolic acid is reduced to a CH_2 group. In order to account for the single active hydrogen in carolic acid when dissolved in pyridine it is postulated that the $COCH_2$ group of the 7-membered ring undergoes enolisation to $C(OH)=CH$. A number of similar enolisations in pyridine have recently been reported [Ishikawa and Kojima, 1934].

It remains now to be shown that these constitutional formulae are in full agreement with all the known properties of carolic, carolinic and α-acetyltetronic acids.

(*a*) *General properties of carolic, carolinic and α-acetyltetronic acids.*

The above formulae for carolic and carolinic acids would obviously permit optical activity. Carolic and carolinic acids give with excess of $FeCl_3$ characteristic orange colours indistinguishable from that obtained with α-acetyltetronic acid. In view of the substitution of the α-carbon of the tetronic acid ring we should not expect any of them to give an immediate $NaNO_2$ colour, as is indeed the case. The fact that all three acids can be recovered unchanged after boiling with acetyl chloride is explained by the fact that the only hydroxyl group is of the keto-enol type and is reconverted into the ketonic form in presence of this reagent. The acids also do not give benzoyl, *p*-nitro- or *p*-bromo-benzoyl derivatives, the material being recovered unchanged. Assuming the above configuration, carolic acid would not contain any active hydrogen in dry anisole whereas carolinic acid would contain two active hydrogens. Assuming also that carolic acid adds 1 molecule of water in aqueous solution, it would then become

a monobasic acid, the three carbonyl groups surrounding the hydrogen atom of the α-carbon of the tetronic acid ring imparting to it strongly acidic properties.

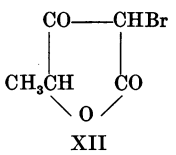
(b) Bromination of carolic, carolinic and α-acetyltetronic acids.

Considerable advance in our knowledge of the structure of these acids has been made by a detailed study of the products of bromination under varying conditions. Thus carolic acid in glacial acetic acid solution absorbs 1 molecule of bromine and gives 1 molecule of HBr and 1 molecule of monobromocarolic acid, $C_9H_9O_4Br$, containing its bromine atom in the butyrolactone half of the molecule since on hydrolysis it gives acetoin, CO_2 and bromobutyrolactone. In dilute acetic acid solution, on the other hand, carolic acid reacts in two ways:

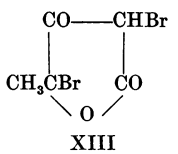
$$\text{I. } C_9H_{10}O_4 + 2Br_2 + H_2O = C_5H_4O_3Br_2 + C_4H_6O_2 + 2HBr$$
$$\text{II. } C_9H_{10}O_4 + 2Br_2 + H_2O = C_5H_5O_3Br + C_4H_5O_2Br + 2HBr$$

In reaction I the products are a C_5 dibromide and butyrolactone, and in reaction II a C_5 monobromide and a bromobutyrolactone. In aqueous solution reaction I occurs, and in 50 % acetic acid solution both reactions occur but reaction II predominates. If carolic acid is, on the other hand, allowed first to react with 1 molecule of bromine in glacial acetic acid solution, then diluted to about 50 % acetic acid and a further molecule of bromine added, the products are exclusively the C_5 monobromide, bromobutyrolactone and HBr.

The C_5 monobromide has been shown to be *d*-α-bromo-γ-methyltetronic acid (XII) whilst the dibromide is very probably *d*-αγ-dibromo-γ-methyltetronic acid (XIII). Both of these bromides on catalytic reduction readily give the same *l*-γ-methyltetronic acid (I).



XII



XIII

With bromine in aqueous or 50 % acetic acid solution, carolinic acid reacts, like carolic acid, in two ways, the same *d*-α-bromo-γ-methyltetronic acid being obtained, whilst succinic and probably bromosuccinic acid replace butyrolactone and bromobutyrolactone. The identity of the two samples of *d*-α-bromo-γ-methyltetronic acid from carolic and carolinic acids respectively was proved by M.P., mixed M.P., titration and conversion into *l*-γ-methyltetronic acid and its dinitrophenylhydrazone.

It appears therefore that during bromination in dilute acetic acid solution the α-side chains are replaced by bromine. An exactly analogous behaviour was observed with synthetic α-acetyltetronic acid, which on bromination in dilute acetic acid gave α-bromotetronic acid.

(c) Hydrolysis of carolic, carolinic and α-acetyltetronic acids.

All three acids undergo hydrolysis with $2N$ H_2SO_4 at comparable rates, each giving 1 molecule of CO_2, carolic acid giving in addition 1 molecule acetoin and 1 molecule γ-hydroxybutyric acid (butyrolactone), carolinic acid giving 1 molecule acetoin and 1 molecule succinic acid, and α-acetyltetronic acid giving 1 molecule acetol and 1 molecule acetic acid. This rather striking property is undoubtedly due to the structure VII which they all have in common and which on opening the tetronic acid ring represents them as derivatives of diacetylacetic acid.

It will be noted, on the other hand, that formula II for carolic acid, obtained by analogy with dibutyrolactone, does not fit the ready hydrolysis of these compounds, since dibutyrolactone has been shown by Fittig and Ström [1892] on boiling with dilute mineral acid not to break down into its two C_4 compounds but to yield the extremely stable compound, oxetone:

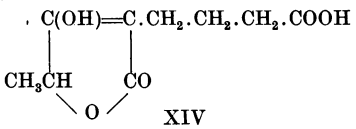
$$C_8H_{10}O_3+H_2O=C_7H_{12}O_2+CO_2.$$

(d) Reduction of carolic, carolinic and α-acetyltetronic acids.

Carolic acid, $C_9H_{10}O_4$, on reduction with palladium-charcoal-hydrogen, absorbs $2H_2$ and gives the reduced monobasic acid $C_9H_{14}O_4$ which on distillation loses H_2O and gives the neutral substance $C_9H_{12}O_3$. Carolinic acid, $C_9H_{10}O_6$, absorbs $2H_2$, giving the reduced acid $C_9H_{12}O_5$ whilst α-acetyltetronic acid $C_6H_6O_4$ also absorbs $2H_2$ at a comparable rate with carolic and carolinic acids, giving α-ethyltetronic acid. With α-acetyltetronic acid therefore the ketonic group of the side-chain is reduced to CH_2, and by analogy we might expect carolic and carolinic acids to behave similarly. Carolinic acid also gives the same general reaction, and the fact that reduced carolic acid appears to contain 1 molecule H_2O more than would be expected by analogy with reduced carolinic and α-acetyltetronic acids is explained by assuming that in reduced carolic acid the second ring becomes stabilised in the open form, and 1 molecule H_2O is lost only on distillation. All three acids before reduction give orange and after reduction red $FeCl_3$ colours, which are indistinguishable. The red colours given by the reduced acids are identical with those given by γ-methyltetronic and acetoacetic acids and are due to the keto-enol grouping in the tetronic acid ring.

Reduced carolic, carolinic and α-acetyltetronic acids (α-ethyltetronic acid) all give on hydrolysis substances which reduce Fehling's solution in the cold and slowly give bis-dinitrophenylhydrazones, and probably therefore contain the CHOH.CO grouping. This confirms the view that the tetronic acid ring itself is not affected by reduction under our conditions, and moreover γ-methyltetronic acid itself is not reduced with our catalyst.

Reduced carolic acid may therefore be represented by structure X and reduced carolinic acid by structure XIV.



XIV

It would be difficult to fit the results obtained on reduction with formula II for carolic acid and impossible with formula III for carolinic acid.

(e) Absorption spectra of carolic, carolinic, α-acetyltetronic, γ-methyltetronic acids and of their reduction products.

As will be shown in a subsequent publication, the structures assigned to the above compounds fit the requirements of their absorption spectra. These have been investigated by Dr E. L. Hirst of Birmingham University. Thus whilst carolic, carolinic and α-acetyltetronic acids all give a double-banded spectrum, one band characterising the tetronic acid ring and the second the ketone group immediately outside this ring, all the three corresponding reduced acids show only a single band corresponding to that of either γ-methyl- or α-ethyl-tetronic acid. Thus reduction results in all three cases in the loss of the ketonic absorption band.

(*f*) *Reactions of carolic, carolinic and α-acetyltetronic acids with 2:4-dinitrophenylhydrazine.*

The ketonic nature of all these compounds can be readily confirmed by means of 2:4-dinitrophenylhydrazine. Benary [1910], working with α-acetyltetronic acid and phenylhydrazine, found that a monophenylhydrazone was readily formed, and he showed that it was the carbonyl group of the side chain and not that of the tetronic acid ring which reacted. We have found that on treating α-acetyltetronic acid or carolinic acid with Brady's reagent (2:4-dinitrophenylhydrazine in 2*N* HCl) the yellow monodinitrophenylhydrazone is immediately precipitated. Carolic acid does not give any immediate precipitate. After standing 24–48 hours, a precipitate begins slowly to separate and continues to separate for 6–8 weeks. The precipitate initially contains a monodinitrophenylhydrazone which is fairly soluble in water, together with a bis-dinitrophenylhydrazone and after 8 weeks consists entirely of the pure bis-compound. Both compounds are dinitrophenylhydrazones of carolic acid + H_2O ($C_9H_{12}O_5$). The bis-dinitrophenylhydrazone tends to revert to the monoderivative on storing. It appears therefore that during this reaction, which is carried out in 2*N* HCl solution, H_2O is slowly added to open the second ring and that the monodinitrophenylhydrazone is then slowly precipitated and transformed into the bis-derivative.

All three monodinitrophenylhydrazones are soluble and the bis-derivative of carolic acid is insoluble in bicarbonate. It would be impossible to obtain a bis-derivative with formula II for carolic acid. Attempts to obtain the bis-derivatives with α-acetyltetronic and carolinic acids, on standing 6–8 weeks with Brady's reagent, were unsuccessful. Carolinic acid still gave only the monoderivative, while α-acetyltetronic acid suffered slow hydrolytic degradation under these conditions giving the bis-dinitrophenylhydrazone of methylglyoxal. The difference in behaviour of the monodinitrophenylhydrazones of carolic and carolinic acids towards an excess of Brady's reagent is probably due to the fact that the former dinitrophenylhydrazone is very soluble whilst the latter is insoluble in the reagent.

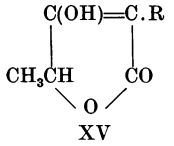
(*g*) *Reactions of carolic, carolinic, α-acetyltetronic and γ-methyltetronic acids with diazomethane.*

Carolic acid on treatment with diazomethane in incompletely dry ether, reacted very slowly, and after keeping overnight, removing the solvent and drying, a yellow oil was obtained which had a methoxyl content slightly greater (15·9 %) than that of a monomethyl derivative (14·5 %). This compound however slowly hydrolysed, even on shaking with water and readily on keeping overnight at room temperature with *N* NaOH, the methoxyl content of the material recovered from the alkaline solution being only 2·8 %. This behaviour was at first regarded as favouring the lactonic acid formula II for carolic acid, assuming that the lactonic ring opened to give the methyl ester. It has now been found however that the arrangement of ketonic groups in these acids renders the methoxyl group much more labile than in a normal methyl ether. Thus the methoxyl content of the monomethyl ether of γ-methyltetronic acid (I), which contains only one CO group in the β-position to the methoxyl group, fell on keeping with *N* NaOH for 24 hours from 24·5 to 14·7 %, and of the monomethyl ether of α-acetyltetronic acid (VI), which contains two CO groups in β-positions to the methoxyl group, fell from 20·7 to 3·6 %, the behaviour exactly resembling that of carolic acid itself. Carolinic acid gives a dimethyl derivative with diazomethane—a

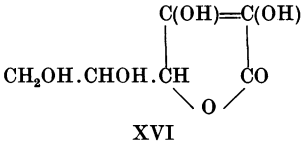
methyl ether methyl ester—which with *N* NaOH very readily loses one, and more slowly loses the second (presumably the ester), methyl group.

This close similarity in behaviour between α-acetyltetronic, carolic and carolinic acids, supported by the absorption spectra data, makes it very probable that in carolic and carolinic acids γ-hydroxybutyric and succinic acids are condensed respectively with γ-methyltetronic acid to give ketonic structures (IV and V) similar to α-acetyltetronic acid. These formulae have therefore been allotted to carolic ($+H_2O$) and carolinic acids respectively, which may therefore be formulated as α-[γ-hydroxybutyryl]-γ-methyltetronic and α-[β-carboxypropionyl]-γ-methyltetronic acids.

Finally the close analogy between the tetronic acid half of the molecule of carolic and carolinic acids (XV) and the structure of ascorbic acid (XVI) (vitamin C) should be noted.



XV



XVI

This analogy in structure is strongly borne out by the absorption spectra. As will be shown in a later publication, γ-methyltetronic acid gives a similar spectrum to that of ascorbic acid, and the band moves in acid and alkaline solution in the same way as does the band of ascorbic acid.

Experimental.

A. *General properties of carolic, carolinic and α-acetyltetronic acids.*

(i) *Carolic acid.*

Carolic acid, $C_9H_{10}O_4$, M.P. 132°, $[\alpha]_{5461}+84°$, gives a bright orange $FeCl_3$ colour and a negative nitro-chromic acid reaction for the primary alcohol grouping, but the latter test becomes positive on standing, due probably to ring opening. The immediate violet colour with $NaNO_2$, which is given by tetronic acids having the α-CH_2 group either unsubstituted or substituted by an easily removable substituent, *e.g.* bromine [Wolff, 1896], is not given immediately by carolic acid. On warming with concentrated H_2SO_4, carolic acid dissolves, giving a colourless solution which on heating to 140° becomes a deep bluish red.

An aqueous solution of the acid blues Congo red. The acid in dry anisole solution has however no active hydrogen as indicated by negative Zerewitinoff determinations by Roth's method. In pyridine, on the other hand, the acid possesses one active hydrogen. Thus 7·52 mg. carolic acid at 28° in dry anisole gave 0·02 ml. CH_4, and 9·75 mg. in pyridine gave 1·18 ml. CH_4, the latter corresponding to 0·98 active hydrogen atom. Carolic acid titrates in cold aqueous solution as a monobasic acid; thus 0·2322 g. required 12·80 ml. *N*/10 NaOH for titration to a permanent pink colour with phenolphthalein (the first pink flush was obtained with 12·6 ml. *N*/10 NaOH), corresponding to an equivalent of 181·4 ($C_9H_{10}O_4$, assumed monobasic, requires 182·1). This titration was not materially altered on heating with excess NaOH. Thus heating for ½ hour at 55°, and subsequently for a further ¾ hour at 80–85° only increased the titre from 12·8 to 13·3 and 13·7 ml. respectively.

The acid also shows a somewhat anomalous behaviour in respect to melting-point. A sample of the acid crystallised from absolute alcohol melted at 132°. Not only did the melting-point tend to fall on storage, but on melting and resetting it fell and became constant at 112°. Moreover there was no loss of water

or CO_2 on heating in dry nitrogen at the melting-point although the melting-point again fell from 132 to 112°. The material melting at 112° on recrystallisation from alcohol again melted at 132°. This change may be related to the keto-enolic type of structure.

The acid gives a very soluble silver salt. Thus 0·3073 g. of carolic acid was neutralised with 17·1 ml. *N*/10 NaOH and $AgNO_3$ added. The silver salt on concentration *in vacuo* at room temperature to 5 ml. showed no signs of crystallising.

Attempted acetylation of carolic acid. Acetylation of carolic acid both by the acetic anhydride-pyridine and by the acetic anhydride-sodium acetate methods failed owing to complete decomposition and blackening. Darkening did not occur however when carolic acid was heated either with acetic anhydride or with acetyl chloride, and acetylation was therefore attempted as follows. 1 g. carolic acid was heated with 15 ml. acetyl chloride for 1 hour in a boiling water-bath, the acetyl chloride mostly distilled off and light petroleum added. The product (0·95 g.) separated as a colourless crystalline solid, was rubbed up with successive amounts of light petroleum and melted at 112°. After recrystallisation from ethyl alcohol, it melted at 132° (*cf.* previous section). The melting-point was not depressed on mixing with carolic acid. Also 0·0264 g. required 1·48 ml. *N*/10 NaOH corresponding to an equivalent of 178·3. ($C_9H_{10}O_4$, assumed monobasic, requires 182). It appears that carolic acid does not form an acetyl derivative and that it is unlikely therefore that it contains a free OH grouping.

Action of alkaline iodine on carolic acid. Although carolic acid with strong reagents (10 % NaOH and *N*/10 I) readily gives CHI_3, no CHI_3 is formed nor is iodine absorbed when the acid is titrated with alkaline iodine in very dilute solution by the method used for determination of kojic acid [Birkinshaw and Raistrick, 1931]. Thus 5 ml. of an aqueous solution containing 10·86 mg. carolic acid, on standing at room temperature for 90 minutes with 40 ml. *N*/10 iodine and 50 ml. *N*/10 NaOH showed an absorption of only 0·3 ml. *N*/10 iodine.

Action of Wijs's reagent on carolic acid. 0·3947 g. carolic acid in 10 ml. CCl_4 was treated with 25 ml. Wijs's solution. The difference in titration of the blank and the reaction fluid was only 0·8 ml. *N*/10 thiosulphate. (Theoretical for absorption of 1 molecule I_2=43·4 ml.). The absorption may therefore be regarded as negligible.

(ii) *Carolinic acid.*

Carolinic acid, $C_9H_{10}O_6$, M.P. 129° (monohydrate, M.P. 123°), $[\alpha]_{5461}+60°$ (in water) gives with $FeCl_3$ an immediate orange precipitate soluble in excess to a bright orange coloured solution. It does not give a colour with $NaNO_2$. An aqueous solution blues Congo red. The acid titrates sharply in the cold as a dibasic acid, and determinations in dry anisole by the Zerewitinoff method for active hydrogen give values which are slightly high for two active hydrogen atoms (Roth). Thus 11·015 mg. carolinic acid gave 2·39 ml. CH_4 (at 18°) and 2·46 ml. CH_4 (at 95°). ($C_9H_{10}O_6$ requires for one active hydrogen the production of 1·05 ml. CH_4 at N.T.P.) Similar values were obtained in pyridine.

Carolinic acid like carolic acid does not react with Wijs's reagent nor does it acetylate on boiling with acetyl chloride. It does not give a bluish red colour with hot concentrated H_2SO_4 as does carolic acid.

(iii) *α-Acetyltetronic acid, $C_6H_6O_4$.*

This was synthesised by Benary's [1909] method, by condensation of ethyl β-aminocrotonate and chloroacetylchloride with subsequent hydrolysis of the

condensation product. After recrystallisation from benzene it melted at 83·5°. (Found: C, 50·65; H, 4·25 %. $C_6H_6O_4$ requires C, 50·69; H, 4·26 %.) It gives an orange $FeCl_3$ colour identical with that of carolic acid but no colour with $NaNO_2$. On titration to phenolphthalein 53·1 mg. required 3·695 ml. $N/10$ NaOH corresponding to an equivalent of 143·7 ($C_6H_6O_4$ titrating as a monobasic acid requires 142).

B. *Bromination of carolic, carolinic and α-acetyltetronic acids.*

Considerable advance in our knowledge of the structure of these acids has been made by a detailed study of the products obtained by bromination under varying conditions.

(I) *Bromination of carolic acid in glacial acetic acid solution: preparation of* d-*monobromocarolic acid.* 2 g. carolic acid were dissolved in 20 ml. glacial acetic acid and a solution containing 2·5 ml. Br in 100 ml. glacial acetic acid was added 1 ml. at a time at room temperature. The reaction was slow at first but became very much more rapid, and an amount of bromine was decolorised corresponding to 2 atoms Br. Hydrobromic acid was given off. The solution was placed in a desiccator over solid KOH and the hydrobromic and acetic acids were quickly removed at room temperature at very low pressure. A thick yellow oil remained which crystallised only after standing for several weeks. The dried material from a second experiment was therefore immediately rubbed up with a little methyl alcohol and allowed to stand in the cold room, when 1·37 g. of crystals separated. The mother-liquor was boiled with a little charcoal, filtered and again placed to crystallise in the cold room, giving a second crop of crystals, weight 0·65 g. The total crude yield represents 70 % of the theory for the production of monobromocarolic acid. The crystals melted at 149–151°. Bromocarolic acid is best purified by recrystallisation (1·2 g.) from boiling methyl alcohol (14 ml.) from which solvent it separates in hard, very large homogeneous plates (0·8 g.), M.P. 158°. (Found (Schoeller): C, 41·57, 41·59; H, 3·47, 3·42; Br, 31·15, 31·29 %. $C_9H_9O_4Br$ requires C, 41·38; H, 3·48; Br, 30·61 %.)

0·1435 g. in 20 ml. absolute alcohol required 5·53 ml. $N/10$ NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 260 ($C_9H_9O_4Br$ titrating as a monobasic acid requires 261).

Monobromocarolic acid in water ($c=1·06$) has $[\alpha]_{5461}=+39°$.

In a control experiment it was shown that 50 % of the bromine absorbed had been eliminated as HBr and was precipitated and estimated as AgBr.

Hydrolysis of d-*monobromocarolic acid.* This hydrolysis was carried out in order to determine whether the bromine atom had entered the butyrolactone or the methyltetronic acid half of the molecule. Thus if it had entered the methyltetronic acid ring we should expect on hydrolysis to obtain diacetyl, butyrolactone and CO_2, whereas if it had entered the butyrolactone half of the molecule we should expect acetoin, CO_2 and either bromo- or hydroxy-butyrolactone.

0·818 g. bromocarolic acid was hydrolysed by boiling for 3 hours with 20 ml. $2N$ H_2SO_4 in a stream of CO_2-free nitrogen, the gases evolved being passed up a reflux condenser and then first through a bubbler containing Brady's reagent (2 : 4-dinitrophenylhydrazine in $2N$ HCl) and finally through bubblers containing $N/10$ baryta. The baryta bubblers showed an evolution of 58·9 ml. $N/10$ CO_2 corresponding to 94 % of the theoretical for 1 molecule CO_2. The bubbler containing Brady's reagent, on standing, contained only a very slight precipitate as is usual when acetoin is formed. Diacetyl could not have been formed since this substance is so very volatile that a heavy precipitate would have been obtained under our conditions.

The hydrolysate was next cooled and titrated. On subtracting from the total titration figure the titre of the H_2SO_4 used for hydrolysis, it was found that 3·4 ml. *N* acid still remained. This corresponds closely to one equivalent of acid (theoretical 3·13 ml.) and it appears therefore that the bromo- (or hydroxy-)butyrolactone is almost entirely present as the opened-up acid. This was later confirmed by titration of the isolated acid. The neutralised solution was made up to a standard volume and aliquot portions used in the following experiments.

(*a*) A portion of the neutralised hydrolysate was distilled *in vacuo* and the distillate shown to contain acetoin by its reduction of Fehling's solution in the cold, by its positive Voges-Proskauer reaction and by the slow production, in the way characteristic of acetoin, of the bis-dinitrophenylhydrazone of diacetyl, M.P. 318° decomp., unaltered by admixture with an authentic specimen.

(*b*) The acetoin content was determined in an aliquot by the Wood-Ost method, the reducing power of the whole hydrolysate corresponding to 0·2656 g. acetoin, representing 96 % of the theoretical from 0·818 g. of bromocarolic acid.

(*c*) The ionised Br of a portion of the hydrolysate was determined by precipitation as AgBr and corresponded to 31 % of the bromine actually present. It appears therefore that only one-third of the bromine has been separated by hydrolysis and that two-thirds still remain attached to one of the hydrolysis products, *viz.* to the butyrolactone.

(*d*) The residual neutral hydrolysate was extracted continuously with ether until acetoin was completely removed, the solution then acidified and again extracted continuously with ether. On removing the ether, a brown oil remained which was dried overnight over H_2SO_4 *in vacuo*. 0·0972 g. of this oil required, on neutralisation in the cold to phenolphthalein, 5·4 ml. *N*/10 NaOH corresponding to an equivalent of 180 ($C_4H_7O_3Br$ titrating as a monobasic acid requires 183), and contained 40·3 % Br (theoretical for $C_4H_7O_3Br = 43·7$ %). The material did not give a crystalline phenylhydrazide as does butyrolactone under the same conditions, and on heating with NaOH lost its bromine, giving presumably a dihydroxybutyric acid which was not, however, further identified.

From these experiments it appears certain that bromination of carolic acid in glacial acetic acid leads to the absorption of 1 molecule Br and to the formation of monobromocarolic and hydrobromic acids, the former of which has its Br atom in the butyrolactone half of the molecule.

(II) *Bromination of carolic acid in aqueous solution and in 50 % acetic acid solution.* Carolic acid reacts with bromine in aqueous and in 50 % acetic acid solution in two ways:

$$\text{(i)} \qquad C_9H_{10}O_4 + 2Br_2 + H_2O = C_5H_4O_3Br_2 + C_4H_6O_2 + 2HBr$$

thus giving rise to $\alpha\gamma$-dibromo-γ-methyltetronic acid and butyrolactone;

$$\text{(ii)} \qquad C_9H_{10}O_4 + 2Br_2 + H_2O = C_5H_5O_3Br + C_4H_5O_2Br + 2HBr$$

thus forming α-bromo-γ-methyltetronic acid and bromobutyrolactone. In aqueous solution reaction (i), and in 50 % acetic acid solution reaction (ii) predominates.

Thus when 1 g. carolic acid is dissolved in 40 ml. water or 50 % acetic acid and a standardised solution of bromine in water or 50 % acetic acid respectively is added, immediate decoloration of bromine occurs and a sharp end-point is obtained after the equivalent of 2 molecules of bromine has been added. If the ionised bromine of an aliquot portion of these two solutions be then determined by precipitation as AgBr, it is found that 2 molecules of HBr have been formed in both reactions.

(*a*) *Isolation of* d-α-*bromo-γ-methyltetronic acid*, $C_5H_5O_3Br$. 1 g. of carolic acid was dissolved in 20 ml. 50 % acetic acid and the theoretical amount ($2Br_2$) of a solution containing 2·5 ml. bromine in 100 ml. 50 % acetic acid slowly added. The solution was then rapidly evaporated to dryness by standing over sticks of KOH in a high vacuum at room temperature. Under these conditions, acetic and hydrobromic acids, butyrolactone and bromobutyrolactone pass over to the KOH, together with any dibromomethyltetronic acid which may have been formed, since this is decomposed under these conditions. 0·85 g. of crude material remained (the theoretical amount for the production of 1 molecule of $C_5H_5O_3Br$ is 1·06 g.). The residual crude α-bromo-γ-methyltetronic acid was well washed with cold benzene and light petroleum and then weighed 0·60 g. M.P. 165°. After several recrystallisations from benzene it separated in beautiful long needles and the M.P. became constant at 172°. It gave a red $FeCl_3$ and a violet $NaNO_2$ colour. (Found (Schoeller): C, 31·17; H, 2·83; Br, 41·49 %. $C_5H_5O_3Br$ requires C, 31·10; H, 2·61; Br, 41·40 %.)

0·0604 g. required 6·30 ml. *N*/20 NaOH for neutralisation to phenolphthalein corresponding to an equivalent of 192 ($C_5H_5O_3Br$ titrating as a monobasic acid requires 193).

It had in water ($c=2·7$ g.) $[\alpha]_{5461}=+9·47°$.

(*b*) *Reduction of* d-α-*bromo-γ-methyltetronic acid* $C_5H_5O_3Br$ *to an acid* $C_5H_6O_3$, *shown to be* l-γ-*methyltetronic acid*. *d*-α-Bromo-γ-methyltetronic acid (2 g.) was next reduced catalytically by palladium-charcoal-hydrogen. The sodium salt of the acid hydrogenated with great ease and was therefore used in order to minimise loss by adsorption on the charcoal.

The reduction, which was carried out as described later, was complete in about 5 minutes and corresponded to an uptake of 1 molecule H_2. The catalyst was filtered off and washed with 2*N* NaOH and then with water. The filtrate and washings were combined, acidified and extracted three times with an equal volume of ether. The ethereal extracts were dried over $MgSO_4$ and the ether removed *in vacuo*. In this way was obtained 0·71 g. of almost pure crystalline acid corresponding to 60 % of the theoretical yield, M.P. 112°. The acid recrystallised from benzene-light petroleum in curved feathery needles, M.P. 115°. It also readily sublimed *in vacuo* and the sublimate melted at 115°. (Found (Schoeller): C, 52·72, 52·75; H, 5·33, 5·30 %. $C_5H_6O_3$ requires C, 52·61; H, 5·30 %.)

0·1329 g. required 11·63 ml. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 114 ($C_5H_6O_3$ titrating as a monobasic acid requires 114).

The free acid in water ($c=1·426$) had $[\alpha]_{5461}=-21°$.

The acid gave strong red $FeCl_3$ and violet $NaNO_2$ colours, and with Brady's reagent readily gave a crystalline monodinitrophenylhydrazone, M.P. 235°, unchanged by recrystallisation from nitrobenzene-toluene. (Found (Schoeller): C, 44·95; H, 3·31; N, 18·71 %. $C_{11}H_{10}O_6N_4$ requires C, 44·90; H, 3·43; N, 19·05 %.)

The products of hydrolysis of the acid were next investigated.

The titrated solution referred to above (0·1329 g.) was washed into a hydrolysis flask, the total volume with washings being 50 ml. 50 ml. 2*N* H_2SO_4 were added and the solution heated to boiling in a stream of CO_2-free nitrogen, the resultant gases being passed through baryta bubblers. After 3 hours' hydrolysis, 23·4 ml. *N*/10 CO_2 had been evolved (theory for 1 molecule $CO_2=23·3$ ml.). The hydrolysate was cooled, the H_2SO_4 neutralised with concentrated KOH, made up to 150 ml. and aliquot portions used for the following tests. The solution gave a positive Voges-Proskauer reaction, reduced Fehling's solution in the cold, slowly in the manner characteristic of acetoin gave the bis-dinitrophenyl-

hydrazone of diacetyl, M.P. 318° (not depressed by mixing with an authentic specimen), and on determination of the acetoin content by the Wood-Ost method was shown to contain 0·1004 g. of carbinol (theory for 1 molecule acetoin 0·1026 g. carbinol).

The acid melted at 115°, was laevorotatory, titrated as a monobasic acid, contained one CO group and gave on hydrolysis 1 molecule each of CO_2 and acetoin. It seemed certain therefore that it was *l*-γ-methyltetronic acid the *dl*-form of which had previously been synthesised by Benary [1911], who gives the M.P. as 117°. The *dl*-variety of γ-methyltetronic acid was accordingly synthesised by Benary's method by condensation of ethyl sodiomalonate with chloroacetyl chloride and subsequent hydrolytic decarboxylation. It melted at 115°. The synthetic acid gave the same red $FeCl_3$ and violet $NaNO_2$ colours, and on hydrolysis under the conditions quoted above gave quantitatively 1 molecule each of CO_2 and acetoin. Although the melting-points of the two samples of methyltetronic acid were the same, they showed a slight depression on mixing due, undoubtedly, to the fact that the synthetic acid was the *dl*-variety, whereas the acid from the monobromide was the *l*-variety. Both samples of acid, however, gave the same dinitrophenylhydrazone, M.P. 235°, and the two hydrazones on admixture showed no depression of melting-point.

The acid was brominated in dry chloroform under conditions used by Wolff [1896] for bromination of tetronic acid, *i.e.* conditions leading with certainty to the formation of *d*-α-bromo-γ-methyltetronic acid. An acid was thus readily obtained which after recrystallisation from benzene had M.P. 172°. The acid gave a red $FeCl_3$ and violet $NaNO_2$ colour and showed no depression of M.P. on mixing with the *d*-α-bromo-γ-methyltetronic acid, $C_5H_5O_3Br$, obtained by bromination of carolic acid in 50 % acetic acid solution. It is certain therefore that the acid split off from carolic acid on bromination in 50 % acetic acid solution is *d*-α-bromo-γ-methyltetronic acid.

(*c*) *Isolation of the dibromide $C_5H_4O_3Br_2$ and its identification as* d-αγ-*dibromo-γ-methyltetronic acid.* 1 g. of carolic acid was dissolved in 40 ml. water and the theoretical amount of bromine water added. Using the silver method it was shown that 50 % of the bromine absorbed was eliminated as HBr. The solution was then extracted three times with an equal volume of ether, the ethereal extracts dried with anhydrous $MgSO_4$, filtered and the ether removed *in vacuo*. In this way 1·55 g. of a product were obtained consisting chiefly of beautifully crystalline material together with a little oil, presumably butyrolactone. The product was dissolved in dry ether and two volumes of light petroleum were added. On standing 0·38 g. of large crystals, consisting of a mixture of long colourless prisms and flat hard plates was obtained, M.P. 114–5°. It was found impracticable to separate this mixture by fractional crystallisation but a satisfactory separation was effected by fractional sublimation into the anhydrous dibromide $C_5H_4O_3Br_2$, prisms, M.P. 88°, and its monohydrate $C_5H_4O_3Br_2$, H_2O, plates, M.P. 135°. The hydrate loses water at the melting-point and, after resetting, melts constantly at 88°. (Found for anhydrous substance (Schoeller): C, 21·82; H, 1·48 %. $C_5H_4O_3Br_2$ requires C, 22·08; H, 1·49 %: Found for hydrate: C, 20·90; H, 2·05 %. $C_5H_4O_3Br_2$, H_2O requires C, 20·70; H, 2·09 %.)

Bromine determinations on these two compounds were unsatisfactory, probably owing to the large amount of bromine in the molecule. Thus a duplicate specimen of each of the anhydrous and hydrated materials was submitted to two independent analysts (*a*) and (*b*) with the following results: *Anhydrous dibromide*, Found: (*a*) 57·4 %; (*b*) 55·9 %. ($C_5H_4O_3Br_2$ requires 58·8 %.) *Monohydrate*, Found: (*a*) 53·4 %; (*b*) 52·7 %. ($C_5H_4O_3Br_2$, H_2O requires 55·1 %.)

Specific rotation of monohydrate in water ($c=2{\cdot}04$) $[\alpha]_{5461}=+36{\cdot}2°$.

Both dibromides on titration to an initial end-point, followed by keeping overnight at room temperature with excess $N/10$ NaOH, showed the presence of one acidic and one lactonic grouping, from which it follows that the bromide M.P. 135°, $C_5H_4O_3Br$, H_2O is a true hydrate and not the opened-up acid. Accurate titration values could not be obtained owing to the partial hydrolysis (about 8 %) of the bromine atoms on standing with $N/10$ NaOH at room temperature overnight.

Both the hydrated and anhydrous forms of the dibromide on hydrogenation with Pd-charcoal and hydrogen readily give a monobasic acid, $C_5H_6O_3$, M.P. 115°, giving a red $FeCl_3$ colour and a violet $NaNO_2$ colour, which shows no depression of melting-point when mixed with the corresponding acid obtained by hydrogenation of *d*-α-bromo-γ-methyltetronic acid. This acid $C_5H_6O_3$ has been shown to be *l*-γ-methyltetronic acid. The dibromide is therefore almost certainly αγ-dibromo-γ-methyltetronic acid, since with this formulation the substance would behave as a mono-acid monolactone, and moreover, as we have shown, αα′-dibromo-γ-methyltetronic acid, like αα′-dibromotetronic acid [Wolff, 1896] is a very unstable neutral substance and in presence of water readily decomposes.

(III) *Bromination of carolic acid first in glacial acetic acid, followed by dilution to 50 % acetic acid and further bromination.* The best yields of *d*-α-bromo-γ-methyltetronic acid were obtained by adding bromine (1 molecule) in glacial acetic acid solution to a solution of carolic acid (1 molecule) in the same solvent, diluting to 50 % acetic acid solution and then adding a second molecule of bromine in 50 % acetic acid. The bromide was then isolated as in the previous experiments and the yield proved to be almost theoretical. The bromide did not depress the M.P. of the specimen prepared as previously described.

(IV) *Bromination of carolinic acid in 50 % acetic acid solution.* Carolinic acid (1 g.) was brominated in 50 % acetic acid solution exactly as in the experiment with carolic acid; 2 molecules bromine were absorbed giving rise as was shown by precipitation as AgBr to 2 molecules HBr. The solution was evaporated in high vacuum over KOH to dryness. A crystalline residue remained which was extracted with boiling benzene. The insoluble portion was filtered off and the filtrate, on cooling, deposited crystals of *d*-α-bromo-γ-methyltetronic acid which were shown to be identical with the bromide from carolic acid. On bromination, carolinic acid gives, therefore, the same *d*-α-bromo-γ-methyltetronic acid as does carolic acid.

The benzene-insoluble residue was carefully sublimed and the sublimate resublimed. A white powder was obtained which melted at 188° and did not depress the M.P. of an authentic specimen of succinic acid. On titration to phenolphthalein, 0·0641 g. required 10·80 ml. $N/10$ NaOH corresponding to an equivalent of 59·4 ($C_4H_6O_4$ titrating as a dibasic acid requires 59).

(V) *Bromination of carolinic acid in aqueous solution.* 1 g. of carolinic acid was brominated in aqueous solution exactly as with carolic acid, in order to discover whether the same αγ-dibromo-γ-methyltetronic acid was also produced. Here on evaporation to dryness a crystalline residue remained which was shown to consist of this dibromide and succinic acid. With carolic acid the products were the dibromide and the volatile oily butyrolactone and these were readily separated. Greater difficulty was experienced with the products from carolinic acid. The readiest separation was obtained by fractional sublimation. On subliming, succinic acid first formed the usual compact sublimate whilst below this the dibromide formed very characteristic large well-shaped prisms. These were easily

detached and after resublimation had M.P. 88°, not depressed by mixing with a sample of the dibromide $C_5H_4O_3Br_2$ from carolic acid. The succinic acid was also resublimed and characterised by M.P., mixed M.P. with an authentic specimen, and by titration.

It appears, therefore, that carolinic acid on bromination gives the same *d*-α-bromo- and *d*-αγ-dibromo-γ-methyltetronic acids as does carolic acid, and this was confirmed by reduction of the two bromides, when the same *l*-γ-methyltetronic acid resulted, which gave the same monodinitrophenylhydrazone as was obtained in the experiments with carolic acid.

(VI) *Bromination of α-acetyltetronic acid.* 1·19 g. of α-acetyltetronic acid were dissolved in 15 ml. 50 % acetic acid and a standard solution of bromine in 50 % acetic acid was added from a burette. The solution, which absorbed 2 molecules bromine, was evaporated as before over KOH in a high vacuum and gave a colourless crystalline solid. After recrystallisation from benzene it melted at 183°. (Found: C, 26·95; H, 1·94; Br, 44·30 %. $C_4H_3O_3Br$ requires C, 26·83; H, 1·69; Br, 44·66 %.) It did not depress the M.P. of a synthetic sample of α-bromotetronic acid [Wolff, 1896]. Like the latter, it gave a violet colour with $NaNO_2$, bromine being displaced from the α-position. These facts show conclusively that during the bromination of α-acetyltetronic acid, the acetyl group is removed and replaced by a bromine atom in the same way as are the side chains of carolic and carolinic acids.

C. *Hydrolysis of carolic, carolinic and α-acetyltetronic acids.*

One of the most characteristic properties of these acids is their very ready hydrolysis by boiling dilute acids and alkalis.

(I) *Hydrolysis of carolic acid.* Carolic acid (5 g.) was hydrolysed by boiling under a reflux condenser for 3 hours with 250 ml. $2N H_2SO_4$ in a current of CO_2-free nitrogen, the gases evolved being passed through bubblers containing standard baryta. CO_2 equivalent to 524 ml. $N/10$ baryta was produced. The evolution of 1 molecule CO_2 by 1 molecule of carolic acid requires 549 ml. baryta.

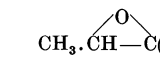
The hot hydrolysis solution was neutralised with N NaOH to a permanent end-point, the solution titrating in the manner of a lactone. The neutral solution was then distilled *in vacuo* to dryness several times and the distillate and residue were examined separately.

(*a*) *Distillate.* This was made up to 1250 ml. and aliquot portions were examined.

(1) A portion readily reduced Fehling's solution in the cold and gave a strong Voges-Proskauer reaction usually regarded as characteristic of acetoin and diacetyl.

(2) On adding excess 2 : 4-dinitrophenylhydrazine in $2N$ HCl, an orange turbidity was produced and a crystalline bis-dinitrophenylhydrazone slowly separated on standing 6 weeks, which after recrystallisation from nitrobenzene-toluene melted at 318°. Samples of diacetyl and acetoin were similarly treated with Brady's reagent. Diacetyl immediately gave a heavy precipitate of the yellow monodinitrophenylhydrazone which on standing overnight with excess of reagent was gradually converted into the orange bis-dinitrophenylhydrazone. This, on crystallisation from nitrobenzene-toluene, melted at 318°. With acetoin, however, an orange turbidity was first obtained and an orange bis-dinitrophenylhydrazone, M.P. 318°, gradually separated over a period of 6 weeks. It did not depress the melting-point of the bis-dinitrophenylhydrazone of diacetyl.

Acetoin, therefore, which according to Kling [1905, 1, 2; 1906] is not a hydroxyketone but has the structure



does not give an immediate precipitate with 2:4-dinitrophenylhydrazine, but is slowly oxidised to diacetyl.

The bis-dinitrophenylhydrazone obtained from the distillate gave the following figures on micro-analysis (Schoeller): C, 43·44, 43·35; H, 3·25, 3·28; N, 24·99, 24·87 %. $C_{16}H_{14}O_8N_8$ requires C, 43·04; H, 3·16; N, 25·11 %.

It did not depress the melting-point of the bis-dinitrophenylhydrazone of diacetyl, but the manner of its separation showed conclusively that acetoin and not diacetyl was the actual product of hydrolysis.

(3) The amount of acetoin present was estimated by the reducing power as determined by the Wood-Ost method. 10 ml. contained 0·016 g. acetoin. Hence the total reducing power corresponded to that of 2 g. of the carbinol (theory 2·42 g. assuming that 1 molecule carolic acid gives 1 molecule acetoin). As a trace of acetoin is lost in the stream of nitrogen during hydrolysis this result may be regarded as sufficiently accurate.

(4) The amount of acetoin present was also estimated by weighing the bis-dinitrophenylhydrazone. 250 ml. with excess of Brady's reagent gave, after standing 6 weeks, 2·39 g. dinitrophenylhydrazone. The theoretical amount of hydrazone assuming $C_9H_{10}O_4 \rightarrow C_{16}H_{14}O_8N_8$ is 2·45 g.

(*b*) *Residue.* The residue was dissolved in a little water, acidified with 2*N* H_2SO_4 and the solution exhaustively extracted with ether. After removal of the ether, a very mobile sweet-smelling light brown oil remained and was dried for a short time *in vacuo* over H_2SO_4. This was shown to be butyrolactone as follows.

On distillation at the ordinary pressure, it almost completely passed over at 203°. It was redistilled and a sample on micro-analysis gave the following figures (Schoeller): C, 55·48, 55·58; H, 7·03, 7·06 %. $C_4H_6O_2$ requires C, 55·77; H, 7·03 %. 0·0930 g. required on titration in the cold less than 0·1 ml. *N*/10 NaOH, and after heating for ½ hour with excess NaOH and back-titrating, 10·75 ml. corresponding to an equivalent of 86·5 ($C_4H_6O_2$ titrating as a monobasic acid requires 86).

The oil on oxidation with HNO_3 (1:1) readily gave succinic acid. On heating with a slight excess of phenylhydrazine in a few ml. alcohol for 2 hours on a boiling water-bath, it gave a phenylhydrazide which crystallised in large shining plates from ethyl acetate, M.P. 94°. (Found: C, 61·88, 61·87; H, 7·09, 7·26; N, 14·68, 14·45 %. $C_{10}H_{14}O_2N_2$ requires C, 61·83; H, 7·27; N, 14·43 %.)

The phenylhydrazide did not depress the melting-point of a specimen prepared from butyrolactone which had been synthesised by the method of Fittig and Chanlaroff [1884]. Similar production of a hydrazide by valerolactone was early recorded by Wislicenus [1887].

The amount of butyrolactone produced on hydrolysis was estimated in a separate experiment as follows. 2·1521 g. carolic acid were hydrolysed as above, washed into a 700 ml. Erlenmeyer flask and titrated with *N* NaOH, first in the cold to neutralise the mineral acid present, and then after heating to estimate the butyrolactone. The total amount of *N* NaOH used by the butyrolactone was 11·90 ml. Assuming that 1 molecule carolic acid gives 1 molecule butyrolactone and no other acid products, 11·82 ml. *N* alkali would be required.

It is thus evident that hydrolysis of carolic acid gives rise to CO_2, acetoin and butyrolactone according to the equation

$$C_9H_{10}O_4 + 2H_2O = CO_2 + CH_3.CO.CHOH.CH_3 + CH_2.CH_2.CH_2.CO \quad (\text{O bridge})$$

(II) *Hydrolysis of carolinic acid.* 1·004 g. carolinic acid (the hydrated form $C_9H_{10}O_6$, H_2O, M.P. 122° was used) were hydrolysed by boiling with 40 ml. dilute H_2SO_4 (equivalent to 95·2 ml. *N* acid) in a stream of CO_2-free nitrogen, the gases evolved being passed through standard baryta as with carolic acid. After 3 hours the equivalent of 84·0, after 4 hours of 85·9 ml. *N*/10 alkali had been used. The reaction $C_9H_{10}O_6, H_2O \rightarrow CO_2$ requires the production of 86·5 ml. *N*/10 CO_2. 1 molecule of CO_2 had therefore been produced.

The residual fluid and washings after cooling required 103·8 ml. *N* alkali for neutralisation in the cold, corresponding to a residual acidity of $103{\cdot}8 - 95{\cdot}2 = 8{\cdot}6$ ml. *N* acid. This figure was not increased on heating with excess NaOH. Assuming that a dibasic acid remained after hydrolysis, the residual acidity should be equivalent to 8·65 ml. *N* acid. The neutralised fluid was then evaporated *in vacuo* to dryness, water added and the process repeated five times. The combined distillates were made up to a known volume. This solution reduced Fehling's solution readily in the cold, gave a positive Voges-Proskauer reaction, and a portion of it with dinitrophenylhydrazine in 2*N* HCl slowly, after the manner of acetoin, gave the bis-dinitrophenylhydrazone of diacetyl, M.P. 318°, the M.P. not being depressed by admixture with an authentic specimen. The amount of acetoin in an aliquot portion of the distillate was determined as with carolic acid by the Wood-Ost method. The solution contained 0·3639 g. carbinol. The reaction $C_9H_{12}O_7 \rightarrow C_4H_8O_2$ requires from 1·004 g. carolinic acid, the production of 0·3808 g. carbinol. The amount recovered represents therefore 96 % of the theoretical for 1 molecule acetoin.

The residue remaining after distillation was dissolved in a little water, acidified and extracted continuously with ether. After removal of ether and drying, 0·528 g. of very pale brown crystalline material remained, which was shown to be succinic acid. The theoretical amount of succinic acid assuming that 1 molecule carolinic acid gave 1 molecule succinic acid is 0·51 g.

Carolinic acid therefore is hydrolysed with the same ease as carolic acid, giving 1 molecule each of CO_2, acetoin and succinic acid, according to the equation:

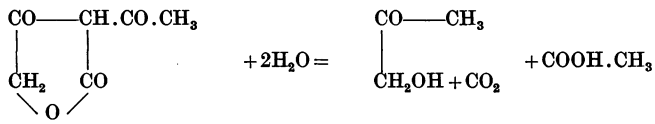
$$C_9H_{10}O_6 + 2H_2O = CO_2 + CH_3.CO.CHOH.CH_3 + COOH.CH_2.CH_2.COOH.$$

(III) *Hydrolysis of α-acetyltetronic acid.* 1·0551 g. of the acid were dissolved in 40 ml. dilute H_2SO_4 ($\equiv$ 82·6 ml. *N* H_2SO_4) and hydrolysed as with carolic acid. Immediately the fluid began to boil, CO_2 was evolved rapidly, as with carolic, carolinic and γ-methyltetronic acids. The total amount of CO_2 evolved corresponded to 146 ml. *N*/10 baryta. (The theoretical amount, assuming that 1 molecule $C_6H_6O_4$ gives 1 molecule CO_2 is 148·6 ml.) The hydrolysis fluid required 90·0 ml. *N* NaOH for neutralisation and there was, therefore, in addition to mineral acid 7·4 ml. *N* acid remaining after hydrolysis. Assuming that 1 molecule of a monobasic acid had been formed, the residual acidity would correspond to 7·43 ml. *N* acid. The neutralised fluid was distilled to dryness several times *in vacuo* and the combined distillates made up to a known volume. This liquid immediately reduced Fehling's solution in the cold, and with 2:4-dinitrophenylhydrazine gave slowly, over a period of 6 weeks, after the manner of acetoin, an orange-red precipitate which after recrystallisation from nitrobenzene-toluene melted at 304°. It did not depress the M.P. of an authentic

specimen of the bis-dinitrophenylhydrazone of methylglyoxal. From the slow formation, oxidation appeared to occur and the actual hydrolysis product was very probably acetol. (Found: C, 41·78; H, 2·97; N, 25·97 %. $C_{15}H_{12}O_8N_8$ requires C, 41·66; H, 2·80; N, 25·92 %.)

The residual salts, on treatment with *p*-phenylphenacyl bromide [Drake and Bronitsky, 1930], gave an ester of M.P. 111°, which was not depressed by mixing with an authentic specimen of the phenylphenacyl ester of acetic acid. (Found: C, 75·67; H, 5·53 %. $C_{16}H_{14}O_3$ requires C, 75·56; H, 5·55 %.)

α-Acetyltetronic acid therefore breaks down on acid hydrolysis in the same way and with the same ease as do carolic and carolinic acids, giving 1 molecule each of acetol, CO_2 and acetic acid.



D. *Reduction of carolic, carolinic and α-acetyltetronic acids.*

In these and all subsequent reductions a palladium-charcoal catalyst was used. This was prepared as follows: 0·2 g. palladium chloride was dissolved in 25 ml. 2*N* HCl, shaken with 1 g. norite and saturated with hydrogen. The catalyst was filtered off and washed thoroughly with water. The activity of the catalyst appeared to be even greater after such short exposure to air, agreeing with the results of Willstätter and Waldschmidt-Leitz [1921]. The catalyst was now returned to the shaking apparatus and resaturated with hydrogen. The substance to be reduced was dissolved in the appropriate solvent, added to the catalyst and shaken, the rate of absorption of hydrogen being measured.

(I) *Reduction of carolic and carolinic acids.* With carolic acid (1 g. in 50 ml. water) and carolinic acid (1 g. in 50 ml. alcohol) the reduction was complete in 3 hours and hydrogen corresponding to $2H_2$ was absorbed in each case.

With carolic acid the reduction product, after filtering off the catalyst, was isolated by continuous extraction with ether, the ethereal extract being dried with $MgSO_4$ and the solvent removed. A colourless oil remained which did not crystallise even on long standing. It only became constant in weight when dried in high vacuum at 60–70° for 10 hours. (Found: C, 58·11, 58·31; H, 7·85, 7·81 %. $C_9H_{14}O_4$ requires C, 58·03; H, 7·58 %.)

0·1736 g. dissolved in water required 9·50 ml. *N*/10 NaOH for neutralisation in the cold to phenolphthalein corresponding to an equivalent of 183 ($C_9H_{14}O_4$ titrating as a monobasic acid requires 186). It appears therefore that in the reduction of carolic acid, $C_9H_{10}O_4$, $2H_2$ are absorbed and the reduced acid $C_9H_{14}O_4$ contains $2H_2$ more than carolic acid.

The reduced acid, on distillation in a high vacuum, loses 1 molecule H_2O, giving a neutral product which after recrystallisation from ether-light petroleum melted at 45°. (Found (Schoeller): C, 64·19, 64·10; H, 7·11, 7·19 %. $C_9H_{12}O_3$ requires C, 64·24; H, 7·19 %.) This compound does not titrate in the cold with dilute NaOH.

These results are explained with our formula for carolic acid (VIII) by assuming that on dissolving carolic acid in water before reduction, 1 molecule H_2O is added, thus opening the 7-membered ring (IV), that the CO group of the side chain so formed is reduced to CH_2 and the molecule becomes stabilised in the open form (X), but that on distillation 1 molecule H_2O is again split off with reformation of the 7-membered ring (XI).

With carolinic acid, the reduction product was isolated, after filtering off the catalyst, by evaporating the alcoholic fluid *in vacuo* to a small volume, when the reduced acid crystallised. After recrystallising from water it melted at 143°. (Found: C, 53·95, 54·15; H, 6·05, 6·05 %. $C_9H_{12}O_5$ requires C, 53·98; H, 6·05 %.)

0·0703 g. required 6·95 ml. *N*/10 NaOH for neutralisation to phenolphthalein corresponding to an equivalent of 101 ($C_9H_{12}O_5$ titrating as a dibasic acid requires 100). It appears therefore that in the reduction of carolinic acid, $C_9H_{10}O_6$, $2H_2$ are absorbed and that the product has the empirical formula $C_9H_{12}O_5$. This is readily explained by assuming that the CO group immediately outside the tetronic acid ring in (V) is reduced to CH_2.

(II) *Reduction of α-acetyltetronic acid.* 0·7 g. of acetyltetronic acid was dissolved in 25 ml. of water and reduced with palladium-charcoal-hydrogen as with carolic acid. 2 molecules of hydrogen were absorbed. The reduction was complete in about 3 hours, the rate being identical with those for carolic and carolinic acids. The reduced acid, after filtering off the catalyst, was recovered by continuous ethereal extraction. On removing the ether a colourless crystalline residue was obtained which after recrystallisation from benzene melted at 127–28°. (Found: C, 56·14; H, 6·28 %. $C_6H_8O_3$ requires C, 56·22; H, 6·30 %.) The acid did not depress the M.P. of a sample of α-ethyltetronic acid which was synthesised by bromination of ethyl α-ethylacetoacetate in ether with subsequent ring closure by removal of ethyl bromide on heating [Wedel, 1883]. The reduction reaction is therefore $C_6H_6O_4 + 2H_2 = C_6H_8O_3 + H_2O$, and the product is undoubtedly α-ethyltetronic acid having the CO group immediately outside the tetronic acid ring in (VI) reduced to CH_2.

The close similarity in structure between the three acids is further seen from the fact that whereas carolic, carolinic and α-acetyltetronic acids give orange $FeCl_3$ colours, the corresponding reduced acids all give red $FeCl_3$ colours indistinguishable from those of γ-methyltetronic or acetoacetic acids. None of them gives a colour with $NaNO_2$.

(E) *Hydrolysis of reduced carolic and carolinic acids.*

(*a*) *Reduced carolic acid.* 1·079 g. of dried reduced carolic acid were hydrolysed by boiling with 50 ml. dilute H_2SO_4 in a stream of CO_2-free nitrogen, the CO_2 evolved being absorbed in the usual way in baryta bubblers. The hydrolysis was effected very much more slowly than with carolic acid itself, hydrolysis of which was complete in less than 3 hours. Thus the amounts of CO_2 evolved in 7, 15, 26, 34 and 41 hours' boiling respectively corresponded to 42·1, 72·6, 90·8, 98·3 and 101·3 ml. *N*/10 baryta. The theoretical amount of CO_2, assuming that 1 molecule $C_9H_{14}O_4$ gives 1 molecule CO_2, corresponds to 116 ml. *N*/10 baryta. The hydrolysis was stopped after 42 hours, the flask cooled and the solution titrated. Whereas the H_2SO_4 originally used required 102·2 ml. *N* NaOH for neutralisation, the hydrolysis fluid required 102·8 ml. *N* NaOH. It appears therefore, since hydrolysis was not absolutely complete, that no acid other than CO_2 had been formed during hydrolysis.

The neutralised solution was made up to 250 ml. It no longer gave any $FeCl_3$ colour. It reduced Fehling's solution readily in the cold, and on distillation of an aliquot portion to dryness *in vacuo* at 45° the substance causing reduction of Fehling's solution did not pass over into the distillate but could be extracted by means of ether from the salts remaining. The reducing substance was not therefore acetoin but a neutral non-volatile substance. The solution gave with 2:4-dinitrophenylhydrazine in 2*N* HCl an immediate orange turbidity, and a crystalline product slowly separated during 2 weeks which after recrystallisation from

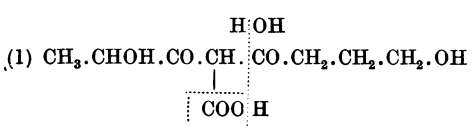
nitrobenzene-toluene melted at 225° and gave the following figures on micro-analysis: C, 46·72, 46·69; H, 4·31, 4·47; N, 21·39, 21·58 %. $C_{20}H_{22}O_9N_8$ requires C, 46·31; H, 4·28; N, 21·61 %. This is obviously the bis-dinitrophenylhydrazone of a diketone $C_8H_{14}O_3$ and, since from the slow formation of the dinitrophenylhydrazone oxidation has occurred, as previously noted with acetoin, the product of hydrolysis is most probably the corresponding ketone-alcohol $C_8H_{16}O_3$. Such a compound would arise from reduced carolic acid by hydrolytic decarboxylation. $C_9H_{14}O_4 + H_2O = C_8H_{16}O_3 + CO_2$.

Ethereal extraction of the residual salts gave a colourless oil which did not crystallise even on long standing. No method was found of isolating this ketone-alcohol in a state of complete purity. On distillation in a high vacuum condensation occurred, a change which we have frequently encountered with other ketone-alcohols.

(*b*) *Reduced carolinic acid.* The hydrolysis was carried out as before. 1·1896 g. reduced carolinic acid gave amounts of CO_2 corresponding to 73·0, 105·3, 109·9, 113·9 ml. *N*/10 baryta in 6, 20, 27 and 34 hours respectively. The theoretical amount of CO_2, assuming that 1 molecule $C_9H_{12}O_5$ gives 1 molecule CO_2 is 119 ml. *N*/10 baryta. In this experiment, unlike that with reduced carolic acid, an amount of residual acid resulted which corresponded to 6·5 ml. *N* acid. Assuming that 1 molecule of a monobasic acid had been formed, the residual acid should correspond to 5·95 ml. *N* acid. The neutral solution was then evaporated to a small volume, acidified with H_2SO_4 and extracted continuously with ether. After removal of ether and drying, a pale yellow oil remained which did not crystallise and could not be obtained in a state of purity. On neutralisation in the cold to phenolphthalein, 92·2 mg. of this oil required 5·12 ml. *N*/10 NaOH corresponding to an equivalent of 180 ($C_8H_{14}O_4$ requires 174). The oil reduced Fehling's solution readily in the cold and was characterised by the fact that with dinitrophenylhydrazine in 2*N* HCl it slowly gave, in the manner of acetoin, a bis-dinitrophenylhydrazone, which after recrystallisation from nitrobenzene-toluene melted at 248° and was soluble in sodium bicarbonate. (Found: C, 45·34; H, 3·93; N, 20·23 %. $C_{20}H_{20}O_{10}N_8$ requires C, 45·09; H, 3·79; N, 21·04 %.) This would correspond to a bis-dinitrophenylhydrazone of a diketone $C_8H_{12}O_4$ and since its formation was slow and oxidation probably occurred as with acetoin the hydrolysis product was probably $C_8H_{14}O_4$ as required by the equation $C_9H_{12}O_5 + H_2O = C_8H_{14}O_4 + CO_2$.

It has been shown that acid hydrolysis of reduced carolic and carolinic acids proceeds much more slowly than that of the parent acids. Further, while the parent acids each give rise to two C_4 compounds on hydrolysis—acetoin and butyrolactone in the case of carolic acid and acetoin and succinic acid from carolinic acid—each of the two reduced acids gives rise to a C_8 hydroxyketone. The results also indicate that the tetronic acid ring in carolic and carolinic acids has not been attacked during reduction, a conclusion which is supported by the fact that γ-methyl- and α-ethyl-tetronic acids are not reduced under the conditions of our reduction experiment.

These facts are readily explained from the formulae proposed for carolic acid (IV) and carolinic acid (V). Writing these structural formulae with the tetronic acid ring opened, as occurs during hydrolysis, we find



Carolic acid and its hydrolysis products; acetoin, CO_2 and γ-hydroxybutyric acid (butyrolactone).

(2) $CH_3.CHOH.CO.CH(COOH).CH_2.CH_2.CH_2.CH_2OH$

Reduced carolic acid and its hydrolysis product $C_8H_{16}O_3$, *i.e.* octan-6-one-1:7-diol.

(3) $CH_3.CHOH.CO.CH(COOH).CO.CH_2.CH_2.COOH$

Carolinic acid and its hydrolysis products, acetoin, CO_2 and succinic acid.

(4) $CH_3.CHOH.CO.CH(COOH).CH_2.CH_2.CH_2.COOH$

Reduced carolinic acid and its hydrolysis product $C_8H_{14}O_4$, *i.e.* ζ-hydroxy-ϵ-keto-*n*-octanoic acid.

F. *Reactions of carolic, carolinic and α-acetyltetronic acids with 2:4-dinitrophenylhydrazine.*

(I) *Carolic acid.* Carolic acid does not give an immediate precipitate with Brady's reagent (2:4-dinitrophenylhydrazine in 2*N* HCl) but, on standing with excess of reagent, a voluminous crystalline precipitate begins to separate after 48 hours and continues to increase over a period of several weeks. Thus, using 1·0024 g. carolic acid and a large excess of reagent, the dry weight of hydrazone was after 1 week 1·70 g.; after 2 weeks 2·06 g.; after 3 weeks 2·45 g.; and after 8 weeks 3·12 g. (Theory, assuming total conversion into $C_{21}H_{20}O_{11}N_8$, 3·08 g.) This material appeared homogeneous and melted at 219°. After repeated crystallisation from nitrobenzene-toluene it separated in orange needles melting at 225° (decomp.). A sample was dried and analysed. (Found (Schoeller): C, 45·02, 45·15; H, 3·61, 3·66; N, 20·03, 20·10 %. $C_{21}H_{20}O_{11}N_8$ requires C, 44·98; H, 3·60; N, 19·99 %.) The substance is insoluble in bicarbonate and is probably the bis-dinitrophenylhydrazone of the opened-up form of carolic acid $C_9H_{12}O_5$ (IV).

When the above reaction was stopped after 8–10 days, the product contained in addition to the above bis-dinitrophenylhydrazone, the monodinitrophenylhydrazone of carolic acid. This was separated from the bis-dinitrophenylhydrazone by fractional recrystallisation from absolute alcohol in yellow needles melting at 176° (decomp.). (Found (Schoeller): C, 47·23; H, 4·19; N, 14·82 %. $C_{15}H_{16}O_8N_4$ requires C, 47·36; H, 4·24; N, 14·73 %.)

The substance is readily soluble in bicarbonate and is therefore the monodinitrophenylhydrazone of the opened-up form of carolic acid $C_9H_{12}O_5$.

It is fairly soluble in water and this probably explains the fact that the hydrazone does not appear immediately on treating carolic acid with Brady's reagent. The slow formation of these precipitates may also be due to the slow opening in 2*N* HCl solution of the 7-membered ring of carolic acid, with the slow unmasking of the second carbonyl group.

The same products can be obtained more quickly by allowing carolic acid and dinitrophenylhydrazine to react in alcoholic solution, as in Brady's more recent method [1931].

(II) *Carolinic acid.* Carolinic acid gives an immediate yellow precipitate with dinitrophenylhydrazine in 2*N* HCl. This material after recrystallisation from nitrobenzene-toluene separated in orange needles melting at 228°. A sample was dried and analysed. (Found (Schoeller): C, 46·06, 46·06; H, 3·81, 3·78; N, 14·14, 14·19 %. $C_{15}H_{14}O_9N_4$ requires C, 45·67; H, 3·58; N, 14·21 %.) The substance is soluble in bicarbonate and is therefore the monodinitrophenylhydrazone of carolinic acid.

When this derivative was allowed to stand for 8 weeks in contact with a large excess of Brady's reagent there was no further reaction.

(III) *α-Acetyltetronic acid.* According to Benary [1910] this acid with phenylhydrazine gives a monophenylhydrazone, the CO group of the side chain, and not of the tetronic acid ring, reacting. With 2:4-dinitrophenylhydrazine, also, it gives an immediate yellow precipitate, which after recrystallisation from nitrobenzene-toluene was obtained as yellow needles, melting at 226°, and was soluble in $NaHCO_3$. (Found (Schoeller): C, 44·92, 44·81; H, 3·19, 3·25; N, 17·17, 17·15 %. $C_{12}H_{10}O_7N_4$ requires C, 44·71; H, 3·13; N, 17·39 %.)

It was found however that if this monodinitrophenylhydrazone were kept with excess of the reagent for 6–8 weeks, it was slowly degraded by acid hydrolysis, giving the bis-dinitrophenylhydrazone of methylglyoxal.

G. *Reactions of carolic, carolinic, γ-methyltetronic and α-acetyltetronic acids with diazomethane.*

The acids were suspended in commercial dry (incompletely dry) ether and a solution of diazomethane in ether was added. With carolinic, γ-methyltetronic and α-acetyltetronic acids the reaction was vigorous, but with carolic acid it proceeded slowly for several hours. After standing overnight with excess of reagent, the ethereal solutions were evaporated and dried over H_2SO_4 *in vacuo* for several days. In all cases yellow oils resulted. With γ-methyltetronic acid, the oil contained 24·5 % OMe ($C_5H_5O_2OMe$ requires 24·2 % OMe), with carolic acid 15·9 % OMe ($C_9H_9O_3OMe + H_2O$ requires 14·5 % OMe), with carolinic acid 28·4 % OMe ($C_9H_8O_4(OMe)_2$ requires 25·6 % OMe), and with α-acetyltetronic acid 20·7 % OMe ($C_6H_5O_3OMe$ requires 19·9 % OMe).

Valuable information as to the nature of these methyl derivatives, particularly in relation to the molecular constitutions of carolic and carolinic acids, was obtained by examining their behaviour towards cold dilute NaOH.

The four methylation products (1 g. in each case) were kept at room temperature for 24 hours with 20 ml. *N*/NaOH.

The monomethyl derivative of γ-methyltetronic acid almost completely dissolved, and extraction of the alkaline solution with ether led to the recovery of only a trace of material. After acidification and ethereal extraction, removal of solvent and drying, a yellow oil was obtained, the methoxyl content of which had fallen from 24·5 % to 14·7 %.

The monomethyl derivative of carolic acid did not go completely into solution. The oil recovered by extraction of the alkaline solution still contained 14·6 % OMe (originally 15·9 %), but the oil recovered after acidification and constituting the larger portion contained only 2·8 % OMe. The portion reacting had therefore been almost completely demethylated. It was found also that shaking the neutral methyl compound with water quickly rendered it acidic, some demethylation occurring.

The dimethyl derivative of carolinic acid almost completely dissolved and the oil recovered from the Na salts after acidification contained 10·0 % OMe (originally 28·4 %). After a further period of hydrolysis the methoxyl content

fell to 8·1 %. One methyl group appears therefore to be lost readily and the second group much more slowly.

The monomethyl derivative of α-acetyltetronic acid, which, like the methyl derivatives of carolic and carolinic acids, contains two CO groups in β-positions to the methoxyl group, was hydrolysed with great ease and the product recovered from the Na salts after acidification contained only 3·6 % OMe.

It appears therefore that the methyl derivative of γ-methyltetronic acid, which contains only one CO group in the β-position to the methyl group, behaves much more like a true methyl ether in that it is not readily attacked by NaOH, whereas the methyl groups of the derivatives of α-acetyltetronic and carolic acids and one of the methyl groups of carolinic acid all react like readily hydrolysable methyl ester groupings. The methyl group of α-acetyltetronic acid is however a true methyl ether grouping, is in the β-position of the tetronic acid ring and has two CO groups in β-positions to it, this juxtaposition of potentially acidic groups accounting for the ease with which it is hydrolysed. It seems likely therefore that the readily hydrolysable methyl groups of carolic and carolinic acids are true methyl ether groupings and have the same relative positions. The second methyl group of carolinic acid, which hydrolyses more slowly, must therefore be the true methyl ester grouping.

Summary.

Penicillium Charlesii G. Smith produces from glucose carolic acid ($C_9H_{10}O_4$) and carolinic acid ($C_9H_{10}O_6$). The molecular constitutions of these two acids have been investigated and it has been shown that they are closely related derivatives of γ-methyltetronic acid.

Carolic acid hydrate has been shown to be α-[γ-hydroxybutyryl]-γ-methyltetronic acid and carolinic acid to be α-[β-carboxypropionyl]-γ-methyltetronic acid. Both acids are therefore closely related structurally to ascorbic acid (vitamin C).

A number of derivatives and breakdown products are described.

We gratefully acknowledge much assistance in the preparation of large quantities of carolic and carolinic acids given by Messrs G. Smith and W. K. Anslow.

REFERENCES.

Benary (1909). *Ber. deutsch. chem. Ges.* **42**, 3912.
—— (1910). *Ber. deutsch. chem. Ges.* **43**, 1065.
—— (1911). *Ber. deutsch. chem. Ges.* **44**, 1763.
Birkinshaw and Raistrick (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 139.
Brady (1931). *J. Chem. Soc.* 756.
Clutterbuck, Haworth, Raistrick, Smith and Stacey (1934). *Biochem. J.* **28**, 94.
Drake and Bronitsky (1930). *J. Amer. Chem. Soc.* **52**, 3715.
Fittig and Chanlaroff (1884). *Liebig's Ann.* **226**, 331.
—— and Ström (1892). *Liebig's Ann.* **267**, 192.
Helferich and Schäfer (1924). *Ber. deutsch. chem. Ges.* **57**, 1911.
Ishikawa and Kojima (1934). *Sci. Rep. Tokyo Bunrika Daigaku* Sect. A, **1**, 43–50. (*Chem. Zentr.* 1934, **1**, 2411).
Kling (1905, 1). *Compt. Rend. Acad. Sci.* **140**, 1457.
—— (1905, 2). *Ann. Chim.* [8], **5**, 554, 556.
—— (1906). *Bull. Soc. Chim.* [3], **35**, 215.
Wedel (1883). *Liebig's Ann.* **219**, 104.
Willstätter and Waldschmidt-Leitz (1921). *Ber. deutsch. chem. Ges.* **54**, 113.
Wislicenus (1887). *Ber. deutsch. chem. Ges.* **20**, 401.
Wolff (1896). *Liebig's Ann.* **291**, 238.