CV. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XLIII. THE METABOLIC PRODUCTS OF *PENICILLIUM CHARLESII* G. SMITH. III. THE MOLECULAR CONSTITUTION OF CARLIC AND CARLOSIC ACIDS.

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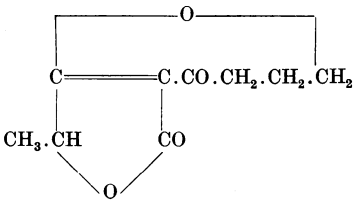
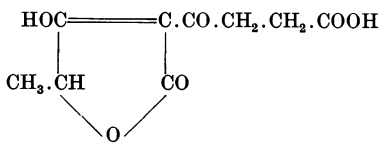
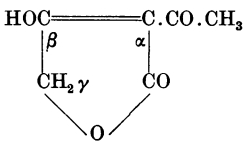
IT was recently shown [Clutterbuck *et al.*, 1934] that *Penicillium Charlesii* G. Smith, a new species of *Penicillium* isolated by our late colleague, Mr J. H. V. Charles, from mouldy Italian maize, produced from glucose a number of hitherto undescribed organic acids. Of the latter, carolic acid, $C_9H_{10}O_4$, and carolinic acid, $C_9H_{10}O_6$, which were obtained in the largest yields, have been shown to have the constitutions I and II respectively [Clutterbuck *et al.*, 1935]:



I. Carolic acid.



II. Carolinic acid.



III. α-Acetyltetronic acid.

The acids may be regarded, therefore, as condensation products of *l*-γ-methyltetronic acid with γ-hydroxybutyric (butyrolactone) and succinic acids respectively, condensation with elimination of water occurring between a carboxyl group of the latter acids and an α-hydrogen atom of the γ-methyltetronic acid ring.

Both acids showed somewhat unusual properties. They were broken down quantitatively with great ease on boiling with dilute mineral acids giving 1 mol. each of acetoin and CO_2, carolic acid giving also 1 mol. of butyrolactone, and carolinic acid 1 mol. of succinic acid. Both acids on bromination gave *d*-α-bromo-γ-methyltetronic acid, the α-side-chains being removed. Both were readily reduced with Pd and hydrogen, the CO group of the α-side-chain being converted

into a CH_2 group, and both gave with diazomethane methyl ethers which were attacked by NaOH with remarkable ease. They also showed rather characteristic behaviours with 2:4-dinitrophenylhydrazine. Thus carolic acid gave no visible immediate reaction with this reagent in 2*N* HCl but slowly over a period of 8 weeks a precipitate separated which at first consisted of a mixture of mono- and bis-dinitrophenylhydrazones of $C_9H_{10}O_4+H_2O$ and later became the pure bis-derivative. The monodinitrophenylhydrazone was soluble in $NaHCO_3$ and fairly soluble in water, the bis-derivative being insoluble in either reagent. Carolinic acid immediately gave a yellow monodinitrophenylhydrazone, insoluble in water but soluble in $NaHCO_3$, which was not converted into the bis-derivative on standing with excess of reagent.

It was found that α-acetyltetronic acid (III), which also contains the α-keto-substituted-tetronic acid nucleus, reacted in a manner showing an absolute analogy with the behaviour of carolic and carolinic acids.

In our first work [Clutterbuck *et al.*, 1934], in addition to carolic and carolinic acids, two further acids, each containing ten carbon atoms, *viz.* carlic acid, $C_{10}H_{10}O_6$, and carlosic acid, $C_{10}H_{12}O_6$, were also obtained in smaller yields. These acids have now been shown to be closely related in constitution to carolic and carolinic acids, and it is the purpose of this paper to describe the investigation of the molecular constitution of carlic and carlosic acids.

Carlic acid, $C_{10}H_{10}O_6$, M.P. 176°, $[\alpha]_{5461}-160°$ (in water), gives with $FeCl_3$ an immediate brownish yellow precipitate soluble in excess to an orange-yellow solution. It does not give a colour with $NaNO_2$. The acid blues Congo red and titrates in water as a dibasic acid. It does not however contain any active hydrogen atom, as determined in dry anisole by the Zerewitinoff method (Roth), whilst it contains two active hydrogen atoms when determined in pyridine. It appears therefore that carlic acid, like carolic acid, must contain a ring system which opens up in aqueous solution, whilst enolisation must occur in pyridine. A number of unusual enolisations of the CO groups of acid anhydrides (containing a $CH_2.CO$ group) in pyridine with activation of hydrogen have been recently reported [Ishikawa and Kojima, 1934].

Carlosic acid, $C_{10}H_{12}O_6$, M.P. 181°, $[\alpha]_{5461}-160°$ (in water) gives with $FeCl_3$ an immediate brownish yellow precipitate insoluble in excess. It does not give a colour with $NaNO_2$. The acid blues Congo red and titrates in water as a dibasic acid. It gives an amount of methane corresponding to 2·2 (at 18°) to 2·5 (at 95°) active hydrogen atoms in dry anisole and 2·1 (at 18°) and 2·9 (at 95°) in pyridine as determined by the Zerewitinoff method (Roth).

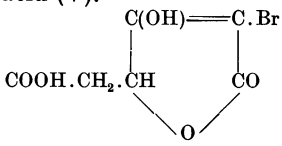
On hydrolysis by boiling with 2*N* H_2SO_4 carlic and carlosic acids, like carolic and carolinic acids, very quickly give 1 mol. of CO_2. Carlic and carlosic acids differ however from carolic and carolinic acids in that on continued hydrolysis they both slowly give a second molecule of CO_2 over a period of about 25 hours. Under these conditions of protracted hydrolysis, carlic acid gives 1 mol. each of acetoin and butyrolactone with 2 mols. of CO_2.

$$C_{10}H_{10}O_6+2H_2O=CH_3.CO.CHOH.CH_3+\underset{\llcorner\!\!\_\_O\_\_\!\!\lrcorner}{CH_2.CH_2.CH_2.CO}+2CO_2$$

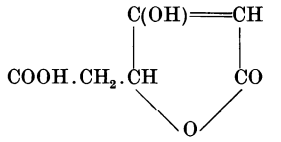
whilst carlosic acid gives 1 mol. each of acetoin and *n*-butyric acid with 2 mols. of CO_2. $C_{10}H_{12}O_6+2H_2O=CH_3.CO.CHOH.CH_3+CH_3.CH_2.CH_2.COOH+2CO_2$.

Carlic and carlosic acids on bromination in dilute acetic acid give the same product, $C_6H_5O_5Br$, M.P. 194°, which has been shown to be very probably *l*-α-bromo-γ-carboxymethyltetronic acid (IV) and which on catalytic reduction

with palladium and hydrogen is readily debrominated giving *l*-γ-carboxymethyltetronic acid (V).



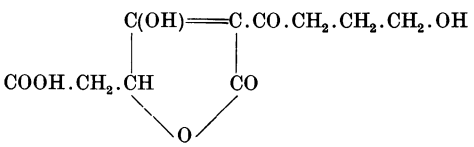
IV.



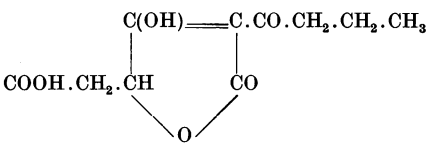
V.

This acid, on hydrolysis with $2N$ H_2SO_4, loses 1 mol. of CO_2 very readily as do carolic, carolinic and γ-methyltetronic acids, whilst a second molecule of CO_2 is lost more slowly, the behaviour being exactly parallel with that of carlic and carlosic acids. Protracted hydrolysis also results in the formation of 1 mol. of acetoin. It is therefore reasonable to suppose that the acetoin and 2 mols. of CO_2, obtained on hydrolysis of carlic and carlosic acids, arise from the *l*-γ-carboxymethyltetronic acid nuclei present in these acids and separated from them on bromination.

Carlic and carlosic acids therefore result from the condensation of 1 mol. of *l*-γ-carboxymethyltetronic acid with 1 mol. of γ-hydroxybutyric acid (butyrolactone) and 1 mol. of *n*-butyric acid respectively. Since synthetic α-acetyltetronic acid has been shown on bromination to lose the α-side-chain giving α-bromotetronic acid, and since carolic, carolinic, carlic and carlosic acids all lose their side-chains on bromination giving α-bromotetronic acid derivatives, it is reasonable to suppose that in the four metabolic acids the side-chains are attached to the α-carbon of the substituted tetronic acid ring as in α-acetyltetronic acid. This view is further supported by the complete analogy in properties between the four metabolic acids and α-acetyltetronic acid, and by their absorption spectra which have been investigated by Dr E. L. Hirst of Birmingham University, and will be reported in a subsequent publication. The following formulae have therefore been adopted for carlic acid ($+H_2O$) (VI) and carlosic acid (VII) respectively.

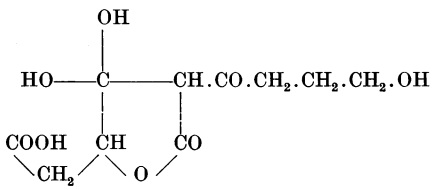


VI. Carlic acid ($+H_2O$).

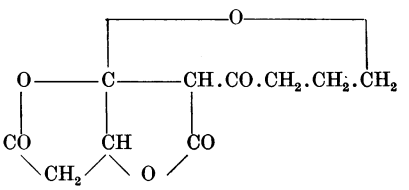


VII. Carlosic acid.

The structural formula of carlic acid then requires the removal of 1 mol. of H_2O from VI. Carlic acid titrates as a dibasic acid in water but contains almost no active hydrogen (in anisole) and two active hydrogen atoms in pyridine. The most probable formula for carlic acid, in view of these facts, is then given by IX, which is derived by hydration of the β-keto group of the tetronic acid ring (VIII) followed by double ring closure.



VIII.



IX. Carlic acid.

This structure would give practically no active hydrogen in anisole, but by analogy with the compounds investigated by Ishikawa and Kojima [1934] would show two active hydrogen atoms in pyridine due to enolisation of the two CO groups having CH_2 groups adjacent to them.

It now remains to be shown that these constitutional formulae are in full agreement with all the known properties of these acids.

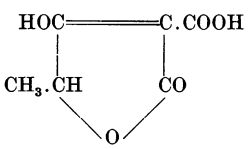
(*a*) *General properties of carlic and carlosic acids.* The above formulae for carlic and carlosic acids would obviously permit optical activity, and in view of the stable substitution of the α-carbon of the tetronic acid ring, we should not expect a colour with $NaNO_2$. The fact that both acids can be recovered unchanged after boiling with acetyl chloride is explained by the fact that all the hydroxyl groups are of the keto-enol type and revert to the ketonic form in presence of this reagent. With carlic acid we should not expect any active hydrogen in dry anisole, but there is the possibility of two enolic groups being formed in pyridine, thus accounting for the two active hydrogen atoms. With carlosic acid, two active hydrogens would be expected with certainty and a third might arise by enolisation in the side chain. The value found in anisole was 2·5. Both acids however on titration with aqueous sodium hydroxide would react as dibasic acids, if it be assumed that with carlic acid two rings open at the β-carbon of the tetronic acid ring.

(*b*) *Bromination of carlic and carlosic acids.* On bromination in approximately 50 % acetic acid under the conditions used for carolic and carolinic acids, carlic and carlosic acids gave the same bromo-derivative, $C_6H_5O_5Br$. This compound gave a red colour with $FeCl_3$ and a violet colour with $NaNO_2$, the latter reaction being characteristic of tetronic acids having the α-CH_2 either unsubstituted or substituted by an easily removable substituent, *e.g.* bromine. It titrated as a dibasic acid and on reduction with palladium-charcoal-hydrogen it readily gave the corresponding dibasic acid $C_6H_6O_5$. This acid also gave the same $FeCl_3$ and $NaNO_2$ colour reactions as did the brominated acid, and on hydrolysis with 2*N* H_2SO_4 very quickly gave 1 mol. of CO_2 and then much more slowly a second molecule of CO_2, whilst after complete hydrolysis, 1 mol. of acetoin remained. The hydrolysis therefore follows the equation

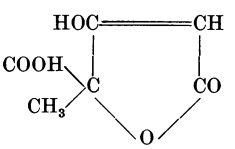
$$C_6H_6O_5 + H_2O = CH_3.CHOH.CO.CH_3 + 2CO_2.$$

The acid $C_6H_6O_5$ differs from γ-methyltetronic acid, $C_5H_6O_3$, by CO_2 and by titrating as a dibasic acid, whereas the latter acid is monobasic. The absorption spectrum of the acid $C_6H_6O_5$, as will be shown in the later publication previously referred to is almost identical with that of γ-methyltetronic acid.

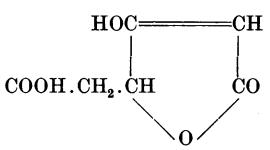
It seems reasonable to suppose therefore that the acid $C_6H_6O_5$ is a carboxy-derivative of γ-methyltetronic acid. Three formulae are conceivable, X, XI, XII.



X.



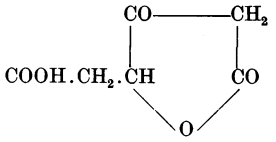
XI.



XII.

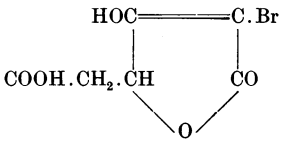
Formula X is however excluded since this acid was an intermediate substance in the synthesis of γ-methyltetronic acid and was shown to exist only as the ester or as salts, CO_2 being lost immediately on acidification [Benary, 1911; Clutterbuck *et al.*, 1935]. Our acid however is quite stable in acid solution at room temperature and is stable to heat up to its M.P. of 182°. Formula XI

appears to be impossible also for a similar reason, since opening the tetronic acid ring would lead to a β-ketodicarboxylic acid. We have seen however that on hydrolysis the acid loses 1 mol. of CO_2 very quickly in the same way as γ-methyltetronic acid, whilst the second molecule of CO_2 is only lost slowly. This is best explained in terms of formula XII when the reaction becomes:



$$+H_2O = COOH.CH_2.CHOH.CO.CH_2.COOH$$
$$= COOH.CH_2.CHOH.CO.CH_3 + CO_2$$
$$= CO_2 + CH_3.CHOH.CO.CH_3 + CO_2.$$

Here 1 mol. of CO_2 is lost at the same rate as with γ-methyltetronic acid in each case from the COOH group in the β-position to the CO group, whilst the second molecule of CO_2 is lost more slowly from the COOH group in the γ-position to the CO group in the acid $C_5H_8O_4$. Compounds with formulae X and XI also would hardly be expected to be stable to heat up to a M.P. of 182°. The acid $C_6H_6O_5$ is therefore regarded as γ-carboxymethyltetronic acid XII, and its bromo-derivative will therefore have the structure XIII.



XIII.

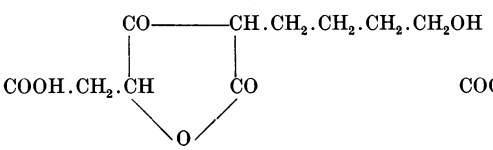
It appears therefore that during bromination of carlic and carlosic acids in dilute acetic acid the α-side-chains are removed and replaced with bromine in a way entirely analogous to that with synthetic α-acetyltetronic acid and with carolic and carolinic acids.

(*c*) *Hydrolysis of carlic and carlosic acids.* The ready hydrolysis of these acids with boiling $2N$ H_2SO_4 has already been discussed and brings them into line with carolic, carolinic and α-acetyltetronic acids. This property is undoubtedly due to the α-ketotetronic acid structure which is common to all five acids, and which on opening the tetronic acid ring represents them as derivatives of diacetylacetic acid.

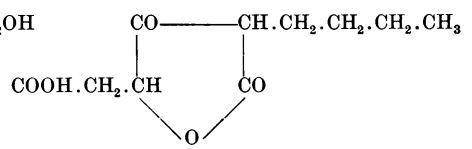
(*d*) *Reduction of carlic and carlosic acids.* Carlic acid, $C_{10}H_{10}O_6$, on reduction with palladium-charcoal-hydrogen, absorbs $2H_2$ and gives the reduced dibasic acid $C_{10}H_{14}O_6$. This result is explained, as with carolic acid, by assuming that on dissolving in water 1 mol. of H_2O is added, and that the CO group of the side-chain is reduced to a CH_2 group.

Carlosic acid, $C_{10}H_{12}O_6$, on reduction absorbs $2H_2$, giving the reduced dibasic acid $C_{10}H_{14}O_5$, and behaves exactly as do carolinic and α-acetyltetronic acids. Here again therefore the reaction is merely the reduction of the CO group of the side-chain to a CH_2 group.

Reduced carlic and carlosic acids may therefore be represented by structures XIV and XV.



XIV.



XV.

Like reduced carolic, carolinic and α-acetyltetronic acids, reduced carlic and carlosic acids give red $FeCl_3$ colours identical with the colour given by γ-methyltetronic, α-ethyltetronic and acetoacetic acids. The reduced acids, as also reduced carolic and carolinic acids, hydrolyse very slowly with boiling $2N$ H_2SO_4 and give substances which reduce Fehling's solution in the cold, slowly give bis-dinitrophenylhydrazones, and probably therefore contain the CHOH.CO grouping. This confirms the view that the tetronic acid ring itself is not reduced under our conditions and moreover, γ-methyltetronic acid itself does not reduce with our catalyst.

(*e*) *Absorption spectra of carlic and carlosic acids and of the corresponding reduced acids.* As will be shown in the subsequent publication previously referred to, the structures assigned to these acids fit the requirements of their absorption spectra. Thus carlic and carlosic acids, like carolic, carolinic and α-acetyltetronic acids, all give a double-banded spectrum in aqueous solution. On the other hand, reduced carlic and carlosic acids, like reduced carolic and carolinic acids, give a single band corresponding to that of either γ-methyl- or α-ethyl-tetronic acid. Thus reduction has resulted in the loss of the ketonic absorption band.

(*f*) *Reactions of carlic and carlosic acids with 2:4-dinitrophenylhydrazine.* In our earlier paper it was shown that α-acetyltetronic and carolinic acids immediately gave yellow monodinitrophenylhydrazones. Carolic acid, on the other hand, gave no immediate precipitate, probably owing to the solubility of the monodinitrophenylhydrazone. After 48 hours a precipitate slowly began to separate which at first consisted of a mixture of mono- and bis-derivatives and later became pure bis-dinitrophenylhydrazone.

Carlic acid gave no precipitate even after 2 weeks and only after 8 weeks showed a small separation of crystals which however proved to be the bis-dinitrophenylhydrazone of diacetyl. Carlic acid must therefore very slowly have been degraded by acid hydrolysis. A similar behaviour has been observed with γ-carboxymethyltetronic acid.

Carlosic acid, like carolinic and α-acetyltetronic acids, gave an immediate precipitate of the monodinitrophenylhydrazone. This derivative was also the only substance isolated after long standing with the reagent. Small amounts of other substances were formed, probably by degradation of carlosic acid, but were not characterised.

(*g*) *Reactions of carlic and carlosic acids with diazomethane.* Carlic acid, like carolic acid, on treatment with diazomethane in incompletely dry ether, very slowly reacted and, after standing overnight, gave a yellow oil which had a methoxyl content rather greater than that of a dimethyl derivative. The elements of water must therefore have been added, and a methyl ether methyl ester have been formed.

Carlosic acid under the same conditions reacted immediately, and gave an oil which had a methoxyl content also high for that of a dimethyl compound, presumably a methyl ether methyl ester.

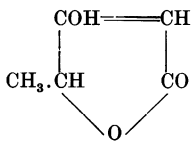
As with the methyl derivatives of α-acetyltetronic, carolic and carolinic acids, both these oils were readily hydrolysed by N NaOH at the ordinary temperature, the methyl ether grouping again being attacked.

The fact that all these acids gave methyl derivatives having high methoxyl values suggests that the α-CO group of the side-chain undergoes enolisation and subsequent methylation to a slight extent.

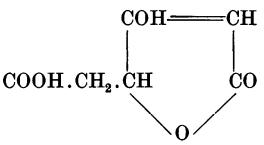
This close similarity in behaviour between carlic and carlosic acids on the one hand and α-acetyltetronic, carolic and carolinic acids on the other hand,

supported by the absorption spectra data makes it very probable that in carlic ($+H_2O$) and carlosic acids, γ-hydroxybutyric and n-butyric acids respectively are condensed with l-γ-carboxymethyltetronic acid to give the ketonic structures VI and VII similar to α-acetyltetronic acid. These formulae have therefore been allotted to carlic ($+H_2O$) and carlosic acids respectively, which may therefore be formulated as α-[γ-hydroxybutyryl]-γ-carboxymethyltetronic acid and α-butyryl-γ-carboxymethyltetronic acid.

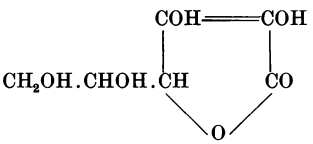
A further point of interest is the close relationship of these products to ascorbic acid (vitamin C). Thus carolic and carolinic acids both contain the γ-methyltetronic acid nucleus (XVI), carlic and carlosic acids the γ-carboxymethyltetronic acid nucleus (XVII), both of which show close analogy with the structure of ascorbic acid (XVIII).



XVI.



XVII.



XVIII.

All these acids have similar absorption spectra and show similar shifts of the absorption band in acid and alkaline solution.

Experimental.

Carlic acid, $C_{10}H_{10}O_6$, M.P. 176°, $[\alpha]_{5461}-160°$ (in water) gives with $FeCl_3$ an immediate brownish yellow precipitate soluble in excess to an orange-yellow solution. It does not give a colour with $NaNO_2$ but blues Congo red and titrates sharply in water as a dibasic acid. Thus 0·1214 g. required 10·70 ml. N/10 NaOH corresponding to an equivalent of 113·4. ($C_{10}H_{10}O_6$ titrating as a dibasic acid requires 113.) It does not contain any active hydrogen as determined in dry anisole by the Zerewitinoff method (Roth), 6·78 mg. at 28° giving only 0·04 ml. CH_4. In dry pyridine however it contains two active hydrogen atoms, 8·955 mg. at 28° giving 1·71 ml. CH_4, corresponding to 1·93 active hydrogen atoms. Carlic acid, like carolic acid, must therefore have a ring system which opens on dissolving in water and titrates, and in pyridine two carbonyl groups must enolise.

In regard to the yield of carlic acid, it was noted in our first paper that the acidified metabolism solutions after extraction 10–12 times with an equal volume of ether, and subsequently 5–6 times with chloroform, still had a negative rotation, and it was suggested that this was probably due to residual carlic acid. It has now been found that by adding still larger amounts of HCl for acidification and extracting with chloroform up to 30 times that the yield of carlic acid can be more than doubled.

Carlosic acid, $C_{10}H_{12}O_6$, M.P. 181°, $[\alpha]_{5461}-160°$ (in water) gives with $FeCl_3$ an immediate brownish yellow precipitate insoluble in excess. It does not give a colour with $NaNO_2$. It blues Congo red and titrates as a dibasic acid. Thus 0·1040 g. required 9·20 ml. N/10 NaOH corresponding to an equivalent of 113·1. ($C_{10}H_{12}O_6$ titrating as a dibasic acid requires 114.) In a determination of active hydrogen in dry anisole by the Zerewitinoff method (Roth) 8·46 mg. gave 1·86 ml. CH_4 at 18° and 2·10 ml. CH_4 at 95°, corresponding to 2·2 and 2·5 active hydrogen atoms respectively, while in pyridine 7·515 mg. gave 1·55 ml. CH_4 at 18° and 2·12 ml. CH_4 at 95° corresponding to 2·1 and 2·9 active hydrogen atoms respectively.

Both of these acids fail to give benzoyl-, *p*-nitro- or *p*-bromo-benzoyl-derivatives and can be recovered unchanged after boiling for 1 hour with acetyl chloride. They probably do not contain therefore under these conditions a free hydroxyl group.

It has been confirmed that carlosic acid, which is the most highly reduced of these acids, is only obtained when Czapek-Dox medium is used and not with Raulin-Thom solution. It is interesting to note that the metabolism on the latter medium proceeds twice as quickly as on the former medium.

A. *Bromination of carlic and carlosic acids. Preparation of l-α-bromo-γ-carboxymethyltetronic acid.*

1 g. of carlic or carlosic acid was dissolved in 15 ml. 50 % aqueous acetic acid and a standardised solution of bromine in the same solvent added. Immediate decoloration of the bromine occurred and a sharp end-point was obtained after the equivalent of 2 mols. of bromine had been added. The solution was then rapidly evaporated to dryness over sticks of KOH in a high vacuum at room temperature. A crystalline mass remained, weighing in each case 1 g. After recrystallisation from boiling ether-light petroleum the product in both cases melted at 194° (decomp.) and on mixing the two samples there was no depression of M.P. The acid gave a red $FeCl_3$ colour and a violet $NaNO_2$ colour. (Found (Schoeller): C, 30·44; H, 2·23; Br, 33·61 %. $C_6H_5O_5Br$ requires C, 30·39; H, 2·13; Br, 33·72 %.)

0·1045 g. on neutralisation in aqueous solution to phenolphthalein required 8·80 ml. *N*/10 NaOH corresponding to an equivalent of 119 ($C_6H_5O_5Br$ titrating as a dibasic acid requires 118·5). Rotation in water ($c=0·562$) $[\alpha]_{5461}-117°$.

Reduction of l-α-bromo-γ-carboxymethyltetronic acid, $C_6H_5O_5Br$, to an acid $C_6H_6O_5$ shown to be l-γ-carboxymethyltetronic acid. *l*-α-Bromo-γ-carboxymethyltetronic acid, 0·25 g., was reduced catalytically using palladium-charcoal-hydrogen. The reduction, which was carried out as with α-bromo-γ-methyltetronic acid was complete in about 5 minutes and corresponded to an uptake of 1 mol. of hydrogen per mol. of substance reduced. The catalyst was filtered off and washed with 2*N* NaOH and then with water. The filtrate and washings were combined, acidified and extracted five times with an equal volume of ether, the ethereal extracts dried over anhydrous $MgSO_4$ and the ether removed *in vacuo*. A crystalline product remained which after recrystallisation from boiling ether-light petroleum melted at 182°. The acid gave a red colour with $FeCl_3$ and a violet one with $NaNO_2$. (Found (Schoeller): C, 45·61; H, 3·90 %. $C_6H_6O_5$ requires C, 45·55; H, 3·83 %.) 0·0129 g. on neutralisation in aqueous solution to phenolphthalein required 1·60 ml. *N*/10 NaOH corresponding to an equivalent of 80·6 ($C_6H_6O_5$ titrating as a dibasic acid requires 79). Rotation in water ($c=0·483$) $[\alpha]_{5461}-52°$.

l-γ-Carboxymethyltetronic acid does not give an immediate precipitate with Brady's reagent, but during 6–8 weeks a precipitate slowly separated which proved however to be the bis-dinitrophenylhydrazone of diacetyl, M.P. 318°, not depressed by mixing with an authentic specimen. Complete hydrolytic decarboxylation had therefore occurred.

Hydrolysis of l-γ-carboxymethyltetronic acid, $C_6H_6O_5$. 0·205 g. of the acid was heated with 20 ml. 2*N* H_2SO_4 in a stream of CO_2-free nitrogen exactly as in the hydrolysis of carolic acid, the resultant gases being passed through a dinitrophenylhydrazine bubbler and then through baryta bubblers. The amounts of CO_2 collected in 3, 9 and 17 hours respectively corresponded to 27·8, 40·8 and 51·8 ml. *N*/10 baryta. The theoretical amount of CO_2 assuming that 1 mol. of

$C_6H_6O_5$ gives 1 mol. of CO_2 requires 25·95 ml. $N/10$ acid. It is obvious therefore that 1 mol. of CO_2 is very readily liberated, the time taken corresponding exactly with those for carolic, carolinic, γ-methyltetronic and α-acetyltetronic acids, whilst a second molecule of CO_2 is liberated much more slowly. The hydrolysate was cooled and after neutralisation with concentrated KOH made up to 100 ml.; aliquot portions were 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-dinitrophenylhydrazone 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 0·098 g. acetoin (theory for 1 mol. acetoin, 0·1142 g.).

As happens in all the hydrolysis experiments in which acetoin is formed, a little of the acetoin passed through the condenser in the stream of nitrogen, and the dinitrophenylhydrazine bubbler on standing gave slowly a very small amount of precipitate which proved to be the bis-dinitrophenylhydrazone of diacetyl, M.P. 318°. If, however, the hydrolysis product had been diacetyl and not acetoin a very copious precipitate would have been formed in the dinitrophenylhydrazine bubbler, as control experiments have proved.

The acid $C_6H_6O_5$ behaves therefore like γ-methyltetronic acid in giving a red colour with $FeCl_3$, a violet one with $NaNO_2$ and on hydrolysis giving readily 1 mol. of acetoin and 1 mol. of CO_2, but differing in titrating as a dibasic (instead of monobasic) acid and in slowly giving on hydrolysis a second molecule of CO_2. As shown in the theoretical part of this paper, these facts fit best the view that the structure of this acid is γ-carboxymethyltetronic acid (XII), whilst the bromide is α-bromo-γ-carboxymethyltetronic acid (XIII).

B. *Hydrolysis of carlic acid.*

0·8114 g. carlic acid, M.P. 176°, was hydrolysed as with carolic and carolinic acids, by boiling with 50 ml. dilute H_2SO_4 ($\equiv$ 118·7 ml. N) in a stream of CO_2-free nitrogen, the gases evolved being passed up a reflux condenser and through a bubbler containing Brady's dinitrophenylhydrazine reagent, and then through a series of bubblers containing standardised baryta. After 3, 7, 14, 21 hours the amounts of CO_2 liberated corresponded to 73·0, 95·45, 129·45 and 138·85 ml. $N/10$ baryta respectively. Assuming $C_{10}H_{10}O_6 \rightarrow 2CO_2$, the theoretical amount of CO_2 is equivalent to 143·5 ml. $N/10$ baryta. One molecule of CO_2 was therefore readily eliminated but the second molecule only slowly. The dinitrophenylhydrazine bubbler showed a slight precipitate which increased on standing due to a little acetoin passing the condenser. The residual fluid after cooling required 119·2 ml. N alkali for neutralisation, corresponding to a residual acidity of 0·5 ml. N acid. On warming the neutral solution with excess N NaOH, a further amount of alkali was absorbed, owing to the hydrolysis and neutralisation of butyrolactone, making the total residual acidity up to 3·8 ml. N acid. Assuming that 1 mol. of carlic acid gives 1 mol. of butyrolactone, the theoretical amount of NaOH required is 3·6 ml. N. The neutralised solution was next evaporated *in vacuo* to dryness several times, the combined distillates made up to 500 ml. and investigated qualitatively and quantitatively (Wood-Ost method) for the presence of acetoin, whilst the salts remaining were acidified and the butyrolactone so obtained was isolated by continuous extraction with ether.

The distillates reduced Fehling's solution in the cold, gave a positive Voges-Proskauer reaction, gave slowly the bis-dinitrophenylhydrazone of diacetyl, M.P. 318° (not depressed by mixing with an authentic specimen) and gave a

copper reduction by the Wood-Ost method corresponding to a total of 0·2767 g. acetoin (the theoretical amount assuming that 1 mol. of carlic acid gives 1 mol. of acetoin is 0·316 g.). The result is a little low, owing to the loss during the long period of bubbling, a loss detected in the dinitrophenylhydrazine bubbler.

The ether-extracted salts gave a sweet-smelling mobile oil which was identified as butyrolactone since it readily gave the beautifully crystalline phenylhydrazide of γ-hydroxybutyric acid, which crystallised in large plates from ethyl acetate, M.P. 94° (not depressed by mixing with an authentic specimen), prepared as described in our previous paper [Clutterbuck *et al.*, 1935].

Carlic acid (1 mol.) on hydrolysis with dilute mineral acid gives therefore 1 mol. each of acetoin and butyrolactone and 2 mols. of CO_2, one readily as with carolic acid, the second more slowly.

C. *Hydrolysis of carlosic acid.*

1·045 g. carlosic acid, M.P. 181°, were hydrolysed under the same conditions as with carlic acid by boiling with 40 ml. dilute H_2SO_4 ($\equiv$93·50 ml. N). After 3, 6, 13, 20 and 27 hours, the amounts of CO_2 liberated corresponded to 98·6, 126·0, 149·1, 165·5 and 171·7 ml. $N/10$ baryta respectively. Assuming $C_{10}H_{12}O_6 \rightarrow 2CO_2$, the theoretical amount of CO_2 is equivalent to 183·2 ml. $N/10$ baryta. One molecule of CO_2 was therefore readily eliminated and a second molecule more slowly. The dinitrophenylhydrazine bubbler again showed a precipitate due to a small amount of acetoin passing the condenser. The residual fluid after cooling required 97·90 ml. N NaOH for neutralisation, corresponding to a residual acidity of $97·9 - 93·5 = 4·40$ ml. N acid. Assuming that 1 mol. of carlosic acid gives 1 mol. of a monobasic acid, the theoretical amount of residual acidity would be 4·58 ml. N. The neutralised solution was next evaporated *in vacuo* to dryness several times, the combined distillates made up to 500 ml. and investigated as before for the presence of acetoin, whilst the salts were acidified with $2N$ H_2SO_4, the hydrolysis acid separated by distillation in steam, neutralised and isolated as the *p*-phenylphenacyl ester by the method of Drake and Bronitsky [1930].

The distillates reduced Fehling's solution in the cold, gave a positive Voges-Proskauer reaction, gave slowly with Brady's reagent the bis-dinitrophenylhydrazone of diacetyl, M.P. 318° (not depressed on admixture with an authentic specimen), and gave a copper reduction by the Wood-Ost method corresponding to 0·3572 g. acetoin (the theoretical amount assuming that 1 mol. of carlosic acid gives 1 mol. of acetoin is 0·4035 g.). The *p*-phenylphenacyl ester of the volatile hydrolysis acid melted after recrystallisation from 95 % alcohol at 82°, and after fractional sublimation still melted at 82°. (Found: C, 76·39, 76·42; H, 6·30, 6·41 %. $C_{18}H_{18}O_3$ requires C, 76·58; H, 6·43 %.) A sample of the *p*-phenylphenacyl ester of *n*-butyric acid was synthesised and melted at 82° and did not depress the M.P. of the ester from the hydrolysis acid. It appears that Drake and Bronitsky's melting-point of 97° for the *p*-phenylphenacyl ester of *n*-butyric acid is incorrect. The *p*-phenylphenacyl ester of *iso*butyric acid was also prepared for comparison and melted both before and after sublimation at 89°. A mixture of this ester and that of the volatile acid from carlosic acid melted at 75–76°.

Carlosic acid (1 mol.) on hydrolysis with dilute mineral acid gives, therefore, 1 mol. each of acetoin and *n*-butyric acid and 2 mols. of CO_2, one readily as with carolic acid, the second more slowly.

D. *Reduction of carlic and carlosic acids.*

Carlic and carlosic acids are reduced readily under the same conditions as with carolic and carolinic acids.

Carlic acid (1 g.) was dissolved in 25 ml. of water, the catalyst prepared from 1 g. charcoal and 0·2 g. palladium chloride, as described for carolic acid, was added and the reduction started in the usual way; 2 mols. of hydrogen per molecule of carlic acid were absorbed in just over 1 hour. The catalyst was then filtered off and washed with a little NaOH and then with water, the filtrate and washings acidified and extracted several times with an equal volume of ether. After removal of the ether, a crystalline residue remained, which after recrystallisation from ether (containing a trace of alcohol)-light petroleum melted at 157·5°. (Found: C, 51·88, 51·92; H, 6·15, 6·09 %. $C_{10}H_{14}O_6$ requires C, 52·16; H, 6·13 %.)

0·1050 g. required 9·05 ml. $N/10$ NaOH for neutralisation to phenolphthalein corresponding to an equivalent of 116 ($C_{10}H_{14}O_6$ titrating as a dibasic acid requires 115).

It appears therefore that in the reduction of carlic acid $C_{10}H_{10}O_6$ (as with carolic acid), 2 mols. of hydrogen are absorbed and the reduced acid $C_{10}H_{14}O_6$ contains $2H_2$ more than carlic acid. This result is explained on the ketonic structure of carlic acid by assuming that on dissolving in water, 1 mol. of H_2O is first added in order to open the ring systems, and that the CO group of the side-chain is then reduced to CH_2, giving structure XIV.

Carlosic acid (1 g. in 90 ml. water) was reduced under exactly the same conditions as above. It absorbed 2 mols. of hydrogen in just over 1 hour. The reduced acid was almost insoluble in water and was precipitated during the reaction. The catalyst and acid were filtered off and extracted with NaOH, and the alkaline filtrate on acidification gave a crystalline precipitate which after recrystallisation from hot water melted at 217°. (Found: C, 56·01, 55·94; H, 6·61, 6·54 %. $C_{10}H_{14}O_5$ requires C, 56·06; H, 6·59 %.)

80·7 mg. required 7·50 ml. $N/10$ NaOH for neutralisation to phenolphthalein corresponding to an equivalent of 107·6 ($C_{10}H_{14}O_5$ titrating as a dibasic acid requires 107).

It appears therefore that in the reduction of carlosic acid $C_{10}H_{12}O_6$, 2 mols. of hydrogen are absorbed and that the product has the empirical formula $C_{10}H_{14}O_5$. This is readily explained by assuming that the CO group immediately outside the tetronic acid ring is reduced to CH_2, as in structure XV. The behaviour of carlosic acid on reduction is therefore completely analogous to that of carolinic acid.

The reduction of carlic and carlosic acids proceeds more slowly under our conditions than does the reduction of true unsaturated compounds, taking place at a rate almost identical with that of α-acetyltetronic acid, in which the CO group of the side-chain is reduced to CH_2 with production of α-ethyltetronic acid. The reductions of carlic and carlosic acids therefore support the ketonic structures assigned to these substances. This view, as will be shown in a subsequent publication, is strongly supported by the change of absorption spectrum on reduction, the double-banded spectra of carlic and carlosic acids giving way to single-banded spectra of the reduced acids. The behaviour of carlic and carlosic acids on reduction is therefore completely analogous to that of carolic and carolinic acids, respectively. This view is further supported by the fact that whereas carlic and carlosic acids give orange precipitates with $FeCl_3$, the corresponding reduced acids both give red colours with $FeCl_3$ indistinguishable from those given by γ-methyltetronic and α-ethyltetronic acids.

E. *Reactions of carlic and carlosic acids with 2:4-dinitrophenylhydrazine.*

Carlic acid, after standing with Brady's reagent either overnight or for a fortnight, gave no precipitate. The solution was extracted five times with ether and the ethereal solution extracted with aqueous $NaHCO_3$ when unchanged 2:4-dinitrophenylhydrazine remained in solution in the ether. The $NaHCO_3$ solution was acidified and re-extracted with ether. This ethereal solution on evaporation gave a red oil which could not be obtained crystalline. It appears therefore that carlic acid does react with the reagent, but the product is very soluble in water and has not been obtained in the crystalline condition. When a solution of carlic acid in Brady's reagent was allowed to stand for 8 weeks, a small amount of precipitate separated towards the end of this period. The precipitate proved however to be the bis-dinitrophenylhydrazone of diacetyl, M.P. 318° (unchanged on admixture with an authentic specimen), which must therefore have resulted from slow but complete hydrolytic degradation of carlic acid by the 2*N* HCl of the reagent.

An aqueous solution of carlosic acid gives with Brady's reagent an immediate orange-yellow precipitate. With strong aqueous solutions of carlosic acid, this precipitate consists of a mixture of the monodinitrophenylhydrazone of carlosic acid together with carlosic acid, the latter being only slightly soluble in the 2*N* HCl of Brady's reagent. After several recrystallisations from absolute ethyl alcohol, the monodinitrophenylhydrazone was obtained pure in orange-yellow needles, M.P. 182°. (Found (Schoeller) on a sample dried to constant weight at 128° *in vacuo*: C, 46·74; H, 3·97; N, 13·75 %. $C_{16}H_{16}O_9N_4$ requires C, 47·04; H, 3·95; N, 13·72 %.)

F. *Reactions of carlic and carlosic acids with diazomethane.*

The acids (1 g. in each case) were suspended in commercial (incompletely) dry ether and a solution of diazomethane in ether was added. With carlic acid, as with carolic acid, the reaction proceeded slowly for several hours. With carlosic acid as with carolinic, γ-methyltetronic and α-acetyltetronic acids, the reaction was vigorous and quickly complete. After standing overnight with excess of reagent, the ethereal solutions were evaporated and dried over H_2SO_4 *in vacuo* for several days. In both cases yellow oils resulted. With carlic acid the oil contained 24·1 % OMe ($C_{10}H_8O_4(OMe)_2 + H_2O$ requires 22·8 %), and with carlosic acid 27·2 % OMe ($C_{10}H_{10}O_4(OMe)_2$ requires 24·2 %).

The methylation products were then allowed to stand for 24 hours with 20 ml. *N* NaOH. The material in both cases passed completely into solution. After acidification and ether extraction, removal of solvent and drying, yellow oils were again obtained. The methoxyl content of the dimethyl derivative of carlic acid had fallen to 9·6 % and that of the dimethyl derivative of carlosic acid to 5·7 %.

The behaviour of carlosic acid and of the hydrated form of carlic acid on methylation with diazomethane, and of their dimethyl derivatives on treatment with cold dilute NaOH, is therefore strictly analogous to that of carolinic acid. The product of methylation in each case is a methyl ether methyl ester. In each case the carboxyl group is esterified and is relatively slowly hydrolysed, whereas the enolised carbonyl group in the β-position in the tetronic acid ring methylates to give a methyl ether which is readily hydrolysed, because there are two potentially acidic CO groups in β-positions to it.

The products resulting from the methylation with diazomethane of carlic and carlosic as well as carolic and carolinic acids all have methoxyl contents some-

what higher than the theoretical values, suggesting that the α-CO group of the side-chain is enolised to a slight extent and that the enolised portion is methylated.

SUMMARY.

Penicillium Charlesii G. Smith produces from glucose carlic acid ($C_{10}H_{10}O_6$) and carlosic acid ($C_{10}H_{12}O_6$). The molecular constitutions of these two acids have been investigated and it has been shown that they are closely related derivatives of *l*-γ-carboxymethyltetronic acid.

Carlic acid hydrate has been shown to be *l*-α-[γ-hydroxybutyryl]-γ-carboxymethyltetronic acid, and carlosic acid to be *l*-α-butyryl-γ-carboxymethyltetronic acid. Both acids are therefore closely related structurally to carolic and carolinic acids, which are also metabolic products of *P. Charlesii*, and to ascorbic acid (vitamin C).

A number of derivatives and breakdown products are described.

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