CCXX. ABSORPTION SPECTRA OF THE METABOLIC ACIDS OF *PENICILLIUM CHARLESII* AND THEIR RELATIONSHIP TO THE ABSORPTION SPECTRUM OF ASCORBIC ACID.

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It has been shown [Clutterbuck *et al.*, 1934] that *Penicillium Charlesii* G. Smith produces from glucose a polygalactose, a polymannose and a variety of metabolic acids. Evidence concerning the structure of the acids has recently been published by Clutterbuck *et al.* [1935, 1, 2]. Simultaneously with the work there recorded investigations of the absorption spectra of these substances were carried out in this laboratory with the idea that correlation of the data with the results of chemical transformations would be of assistance in the allocation of structural formulae. The present paper deals with the absorption spectra of carlic, carolic, carolinic and carlosic and other acids from the point of view of structural relationships and it will be seen that the observations now recorded give strong support to the constitutional formulae advocated by Clutterbuck *et al.* [1935, 1, 2].

Since α -ethyltetronic acid (I, $R_1 = \text{Et}$; $R_2 = \text{H}$) and α -acetyltetronic acid (II, $R_1 = \text{CH}_3$; $R_2 = \text{H}$) occupy special positions as reference substances in connection with the subject matter of this paper their absorption spectra will be

I

II

III

considered first. Aqueous solutions of α -ethyltetronic acid are characterised by a single intense band with head at $\lambda 258 m\mu$, the corresponding value of the molecular extinction coefficient ($\epsilon_{\text{max.}}$) being 12,000. In acidified aqueous solutions the values are $\lambda 233 m\mu$, and $\epsilon_{\text{max.}}$ 12,000 respectively, but in alkaline solutions the head of the band is at $\lambda 258 m\mu$ and the value of $\epsilon_{\text{max.}}$ is 18,000. This behaviour resembles very closely that of ascorbic acid (III) which has a single band at $\lambda 265 m\mu$ in alkaline solution and at $\lambda 245 m\mu$ in acidified aqueous solution (non-ionised condition) [Herbert *et al.*, 1933; Baird *et al.*, 1934]. Reference to the formulae shows a close structural similarity between the two substances

and ascorbic acid is in fact an example of formula I with $R_1 = \text{OH}$ and $R_2 = -\text{CHOH} \cdot \text{CH}_2\text{OH}$. Both ascorbic acid and ethyltetronic acid possess a lactic CO group forming part of a system of conjugated double bonds to which the observed absorption is attributable. There is however one point of difference sufficiently noteworthy to require further comment. The $-\text{C}(\text{OH})=\text{C}(\text{R})-$ group in α -ethyltetronic acid is much less sensitive to oxidation in alkaline solution than is the corresponding dienol group of ascorbic acid. For this reason the absorption of the ionised form of α -ethyltetronic acid is readily ascertainable but special precautions are necessary when solutions containing ionised ascorbic acid are under examination. For example when the absorption in pure water is being measured it is important to exclude all traces of metallic catalysts [cf. Mawson, 1935; Zilva, 1935]. Owing to the intensity of the band the aqueous solutions generally used for absorption work are excessively dilute (about $M/10,000$) and under these conditions ascorbic acid (for which $\log K = -4.1$) is mainly but not entirely in the ionised state. The absorption spectrum is therefore composite and the full intensity of the band at $\lambda 265 m\mu$ characteristic of the ionised acid is not developed. The absorption at $\lambda 245 m\mu$ is not sufficiently intense to be observed as a band and the main feature is the band at $\lambda 265$ which has $\epsilon_{\text{max.}}$ 10,000. If to such a solution having $\epsilon_{\text{max.}}$ 10,000 acetate buffer containing a trace of potassium cyanide ($M/1000$) is added to bring the p_{H} to 5 the ascorbic acid becomes completely ionised, $\lambda_{\text{max.}}$ remains at $265 m\mu$ and the value of $\epsilon_{\text{max.}}$ rises to 13,000. It is of course possible that in addition to ionisation other changes in the molecule affecting absorption may be promoted by the alteration from acid to alkaline conditions, but it is important to note that oxidation is not responsible for the lower value in water as compared with that in the acetate buffer [cf. Robertson, 1934]. In accordance with expectation concentrated aqueous solutions of ascorbic acid ($N/10$), which have a lower p_{H} value, display the absorption band ($\lambda_{\text{max.}}$ $245 m\mu$) of the non-ionised acid [Herbert, Hirst and Wood, 1933]. Numerous synthetic analogues of ascorbic acid, many of which are entirely devoid of antiscorbutic activity display similar absorption bands and it will be shown below that all the natural products referred to in this paper possess bands in close proximity to that of *l*-ascorbic acid (vitamin C). For these reasons it is obvious that spectrophotometric methods for the assay of vitamin C should be used only with great caution.

The transition from α -ethyltetronic acid to α -acetyltetronic acid (II, $R_1 = \text{CH}_3$; $R_2 = \text{H}$) involves the addition of another unsaturated grouping to the molecule and a complex system of conjugated double linkages results. The absorptive power is thereby profoundly modified and is now characterised by two intense bands¹ situated respectively at $\lambda 265 m\mu$ and at $\lambda 230 m\mu$ the value of $\epsilon_{\text{max.}}$ being 15,000 for both. A further difference is that there is now no appreciable change in the wave-length at the heads of the bands when aqueous acid or aqueous alkaline solutions are used in place of solutions in pure water.

The following four metabolic acids of *P. Charlesii*, carlic, carolic, carolinic and carlosic acids, display in aqueous solution absorption spectra characterised by two strong bands, the positions and intensities of which offer the closest possible parallel with the corresponding data for α -acetyltetronic acid. Furthermore on reduction these acids give, by replacement of one oxygen by two hydrogen atoms, tetrahydro-derivatives which show single banded absorption spectra, the position and intensity of this band being closely similar to those of α -ethyltetronic acid. The absorption data therefore suggest strongly that in aqueous

¹ It is not possible, from the data available, to apportion the contributions of the various groupings to these two bands.

solution these four acids possess markedly similar structures and that they are related to their tetrahydro-derivatives in the same manner as acetyltetronic acid is related to α -ethyltetronic acid. In conjunction with the chemical evidence put forward by Clutterbuck *et al.* these results provide strong evidence in favour of the formulation of the four substances as the following derivatives of acetyltetronic acid (formula II):

Substance	R_1	R_2
Carolinic acid	$-(CH_2)_3 \cdot COOH$	$-CH_3$
Carlosic acid	$-(CH_2)_3 \cdot CH_3$	$-CH_2 \cdot COOH$
Carolic acid*	$-(CH_2)_3 \cdot CH_2OH$	$-CH_3$
Carlic acid*	$-(CH_2)_3 \cdot CH_2OH$	$-CH_2 \cdot COOH$

* Hydrated form.

The tetrahydro-derivatives are derived from formula I in the following way.

Substance	R_1	R_2
Tetrahydrocarolinic acid	$-(CH_2)_3 \cdot COOH$	$-CH_3$
Tetrahydrocarlosic acid	$-(CH_2)_3 \cdot CH_3$	$-CH_2 \cdot COOH$
Tetrahydrocarolic acid	$-(CH_2)_3 \cdot CH_2OH$	$-CH_3$
Tetrahydrocarlic acid	$-(CH_2)_3 \cdot CH_2OH$	$-CH_2 \cdot COOH$

These four acids are separated into two distinct groups by virtue of important differences in behaviour between carolinic and carlosic acids on the one hand and carolic and carlic acids on the other hand. Whereas carolinic and carlosic acids display normal properties and have similar absorption spectra in aqueous and non-aqueous solvents, carlic and carolic acids crystallise with one molecule of water less than is represented by the above formulae and the anhydro-acids contain no appreciable active hydrogen (hydroxyl groups) when tested in anisole by the Zerewitinow method. The crystalline acids dissolve in water giving the hydrated forms indicated above, but the anhydrous forms of carolic acid and carlic acid have polycyclic structures. For suggestions concerning the nature of these ring systems the paper by Clutterbuck *et al.* [1935, 2] should be consulted. It suffices to say here that examination of the absorption spectra has revealed that in a non-aqueous medium (dry alcohol) both carolic and carlic acids display a single absorption band ($\lambda 270-272m\mu$) in place of the two well marked bands found in aqueous solutions. Strong indications are thus afforded of a difference in structure between the hydrated and non-hydrated forms and the close resemblance in the behaviours of carolic and carlic acids points to the operation of similar causes in both cases. In this respect the correlation of the chemical evidence with the absorption data presents difficulties in view of the structural differences between the formulae for carlic and carolic acids suggested by Clutterbuck *et al.*

The series of observations was extended by the investigation of various structurally related acids, of which *l*- γ -methyltetronic acid (I , $R_1 = H$; $R_2 = Me$) is a metabolic product of *P. Charlesii*. Its absorption falls exactly into line with others of this general type. Another example is γ -carboxymethyltetronic acid (I , $R_1 = H$; $R_2 = CH_2 \cdot COOH$) which has a similar absorption spectrum. In addition two other acids of this series were encountered by Clutterbuck *et al.* [1935, 3], namely, ramigenic acid and verticillic acid which have been shown by these authors to be products derived by the condensation of *l*- γ -methyltetronic acid with acetone. The absorption spectra are characterised by a single band with high values of ϵ_{max} , corresponding with the high molecular weight and the number of absorbing centres of the tetronic acid type present in the molecule.

Details of absorption spectra.

The measurements were made with solutions containing about 2 mg. of the substance under examination in 100 ml. of solution. The figures given in Table I are molecular extinction coefficients, calculated from the expression $\log I_0/I = \epsilon lc$ where c is the concentration in terms of g. mols. per litre. Figs. 1 and 2 show

Table I. *In water.*

Substance	$\epsilon_{\max.}$	λ	$\epsilon_{\min.}$	λ	$\epsilon_{\max.}$	λ	$\epsilon_{\min.}$	λ
α -Acetyltetronic acid	15,000	265	9000	243	15,000	230	1000*	200*
Carolic acid	13,500	265	7500	245	13,000	230	1000*	200*
Carlic acid	16,400	266	7500	245	14,200	230	1000*	200*
Carolinic acid	13,800	265	8600	242	12,600	230	1000*	200*
Carlosic acid	14,000	265	7500	245	13,200	230	1000*	200*
α -Ethyltetronic acid	12,000	258						
<i>l</i> - γ -Methyltetronic acid	17,000	248						
γ -Carboxymethyltetronic acid	11,500	248						
Tetrahydrocarolinic acid	11,500	252						
Tetrahydrocarlosic acid	9,000	252						
Tetrahydrocarlic acid	12,000	258						
Tetrahydrocarolic acid	12,500	257						
Ramigenic acid	17,000	252						
Verticillic acid	53,000	250						

* Approximate value.

Table II. *In acid solution (N/40 H₂SO₄).*

Substance	$\epsilon_{\max.}$	λ	$\epsilon_{\min.}$	λ	$\epsilon_{\max.}$	λ	$\epsilon_{\min.}$	λ
α -Acetyltetronic acid	15,000	265	9000	243	15,000	230	—	—
Carolic acid	14,000	268	6000	245	8,500	230	1000*	200*
Carlic acid	14,200	266	6500	245	8,200	230	1000*	200*
Carolinic acid	13,800	265	8600	242	12,600	230	1000*	200*
Carlosic acid	14,000	265	7500	245	13,200	230	1000*	200*
α -Ethyltetronic acid	12,000	233						
<i>l</i> - γ -Methyltetronic acid	14,000	226-7						
γ -Carboxymethyltetronic acid	9,000	228						
Tetrahydrocarolinic acid	11,500	232						
Tetrahydrocarlosic acid	9,500	235						
Tetrahydrocarlic acid	12,000	234						
Tetrahydrocarolic acid	11,000	233						
Ramigenic acid†	16,000	235						
Verticillic acid†	38,000	228						

* Approximate value.

† In N/25 H₂SO₄.Table III. *In alkaline solution (aqueous NaOH).*

Substance	$\epsilon_{\max.}$	λ	$\epsilon_{\min.}$	λ	$\epsilon_{\max.}$	λ	$\epsilon_{\min.}$	λ
(d) α -Acetyltetronic acid	15,000	263-5	8,000	243	15,000	230	6,500	213
(a) Carolic acid	13,500	265	7,500	245	13,000	230	6,500	214
(b) Carlic acid	16,400	266	7,500	245	14,200	230	5,500	214
(c) Carolinic acid	13,800	265	11,000	244	14,000	230	9,400	216
(d) Carlosic acid	15,000	265	8,000	245	13,500	230	12,500	217
(d) α -Ethyltetronic acid	18,000	258	3,000	225				
(a) <i>l</i> - γ -Methyltetronic acid	20,400	250	3,400	220				
(d) Carboxymethyltetronic acid	17,500	250	1,800	218				
(a) Tetrahydrocarolinic acid	16,000	255-60	1,600	220				
(a) Tetrahydrocarlosic acid	19,000	260	3,500	225				
(d) Tetrahydrocarlic acid	19,000	258	4,000	225				
(d) Tetrahydrocarolic acid	17,000	258	4,000	226				
(e) Ramigenic acid	21,000	254	4,500	212				
(f) Verticillic acid	64,000	250	9,000	218				

(a) N/100 NaOH, (b) N/200 NaOH, (c) N/20 NaOH, (d) N/50 NaOH, (e) N/500 NaOH, (f) N/150 NaOH.

Table IV. *In alcohol.*

Substance	$\epsilon_{\max.}$	λ	$\epsilon_{\min.}$	λ
Carolic acid	15,000	272	4000	238*
Carlic acid	16,500	270	5000	236*
Tetrahydrocarlic acid	12,000	230	—	—

* Below this wave-length the value of ϵ increases regularly and reaches 7500 at λ 210 $m\mu$ (see Fig. 1).

typical curves given under different conditions by substances of formulae I and II respectively. Since the shapes of the corresponding curves vary little from substance to substance within each group it is not necessary to give detailed graphs for every substance and it is sufficient to tabulate the essential points of

Fig. 1.

Fig. 2.

Fig. 1. I, *l*- γ -methyltetronic acid in *N*/100 NaOH (1 mg. per 100 ml.; *l*, 1 cm.); II, *l*- γ -methyltetronic acid in water (1 mg. per 100 ml.; *l*, 1 cm.); III, *l*- γ -methyltetronic acid in *N*/40 H_2SO_4 (1 mg. per 100 ml.; *l*, 1 cm.); IV, carolic acid in alcohol (2.2 mg. per 100 ml.; *l*, 1 cm.).

Fig. 2. I, carolic acid in water (2.7 mg. per 100 ml.; *l*, 1 cm.); II, carolic acid in *N*/100 NaOH (2.7 mg. per 100 ml.; *l*, 1 cm.); III, carolic acid in *N*/40 H_2SO_4 (2.7 mg. per 100 ml.; *l*, 1 cm.).

interest, namely, the values of λ and ϵ at the maximum and minimum points on the curves. These are given in Tables I–IV. It should be noted that the tendency, shown by curve II on Fig. 1, to broaden out towards the base is apparent even more markedly with aqueous solutions of other substances of formula I. This is

almost certainly due to the presence in the aqueous solution of a small proportion of the substance in the form which gives the band shown in curve III (see remarks above on the absorption spectra of ascorbic acid). The figures for carlic and carolic acids in Tables I-IV are based on the formulae $C_{10}H_{10}O_6$ and $C_9H_{10}O_4$ respectively (anhydrous forms).

SUMMARY.

1. The absorption spectra of carolinic, carlosic, carolic and carlic acids (produced by the action of *Penicillium Charlesii* G. Smith on glucose) have been investigated under a variety of conditions.

2. It is shown that the absorption spectra of the four acids show the closest possible similarity with that of α -acetyltetronic acid and that the absorption spectra of the reduced acids resemble those of α -ethyltetronic acid, *l*- γ -methyltetronic acid and *l*- γ -carboxymethyltetronic acid.

3. In dry alcohol carolic and carlic acids show an absorption spectrum different from that given by the hydrated acids in water.

4. It is shown that the absorption data can be correlated with the chemical properties of the substances and the observations are discussed from the point of view of structural relationships.

5. All the above substances are closely related to ascorbic acid and the bearing of the results on the use of the spectrographic method for the estimation of vitamin C is discussed.

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