# PAPER CHROMATOGRAPHIC AND ELECTROPHORETIC SEPARATION AND IDENTIFICATION OF SOME NATURALLY OCCURRING TETRONIC ACIDS

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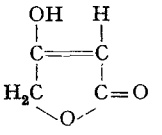
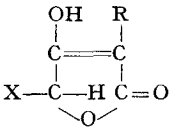
A number of moulds are known to produce substances related to tetronic acid (I) when grown on certain defined liquid media[1-5]. These acids have the general formula shown below (II).



(I)



(II)

In this account the behaviour of five such tetronic acids is recorded. Table I shows the common names of these acids with their corresponding substituent groups.

TABLE I

| *Common name* | *Group X* | *Group R* |
|---|---|---|
| Carolic acid (hydrated form) | $-CH_3$ | $-CO\cdot CH_2\cdot CH_2\cdot CH_2OH$ |
| Carlic acid (hydrated form) | $-CH_2COOH$ | $-CO\cdot CH_2\cdot CH_2\cdot CH_2OH$ |
| Carlosic acid | $-CH_2COOH$ | $-CO\cdot CH_2\cdot CH_2\cdot CH_3$ |
| Terrestric acid (hydrated form) | $-CH_3$ | $-CO\cdot CH_2\cdot CH_2\cdot CHOH\cdot C_2H_5$ |
| Viridicatic acid | $-CH_2COOH$ | $-CO\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_3$ |

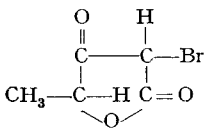
The first three of these acids have been isolated from *Penicillium charlesii* G. Smith[1]; terrestric acid has been isolated from *Penicillium terrestre* Jensen[3]; whilst terrestric and viridicatic acids have been obtained from *Penicillium viridicatum* Westling[5]. HAYNES AND PLIMMER[6] have recently discussed the properties and, in particular, the strongly acidic nature of some tetronic acids.

## DETECTION OF TETRONIC ACIDS

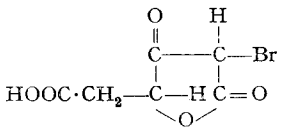
In the course of investigations into the metabolism of *Penicillium charlesii* G. Smith, the need arose for a sensitive and specific method for the separation and detection

of tetronic acids. REIO[7] included the tetronic acids in his chromatographic survey of mould metabolites, and HAYNES, PLIMMER AND STANNERS[8] have described the chromatographic behaviour of carolinic acid (formula II, R = $COCH_2CH_2COOH$; X = $CH_3$). At the time when the present work was started, however, only the latter report had appeared in the literature. REIO[7] detected the acids on paper chromatograms using a bromophenol blue indicator spray. He also made use of the reaction between aqueous $FeCl_3$ and certain tetronic acids[1,3–5]. Present work confirmed his results that spots containing 25 to 50 $\mu$g of a tetronic acid do not show up strongly with the bromophenol blue spray. In order to detect tetronic acids with an aqueous $FeCl_3$ spray, similar amounts are necessary but again the reaction is weak and the yellow spots are often difficult to see.

The detection method used in the present work is based on the reaction between the tetronic acids and bromine as described by CLUTTERBUCK *et al.*[9,10]. In 50 % glacial acetic acid carolic and carolinic acids gave mainly $\alpha$-bromo-$\gamma$-methyl-tetronic acid (III), whilst with carlosic and carlic acids an analogous reaction occurred with production of $\alpha$-bromo-$\gamma$-carboxymethyl-tetronic acid (IV).



(III)



(IV)

The tetronic acids are converted to their corresponding bromo derivatives on paper chromatograms by spraying with bromine in acetic acid solution. The bromo compounds are then revealed by spraying with a solution of starch–KI. Similar spray reagents have been used for the detection of peptides[11] and other nitrogenous compounds[12].

*Spray reagent and procedure*

A solution of 0.1 ml $Br_2$ in 100 ml 50 % glacial acetic acid is sprayed on to the dry chromatograms; a light spray suffices. When the paper is again completely dry, after 10–20 min in air, a second spray of 1 % (w/v) soluble starch solution containing 2 % (w/v) KI is applied. The tetronic acids show up immediately as blue or brown spots on a white background. The spots are marked as soon as the paper is dry as they tend to fade, particularly after using an alkaline chromatography solvent. The reagent easily detects 5 $\mu$g of each of the acids tested, after chromatography using any of the solvents discussed below. Following chromatography using alkaline or neutral solvents the sensitivity of the reagent may be increased by spraying lightly with *N*/10 HCl after the starch–KI spray.

*Specificity of the reagent*

The specificity of the spray reagent was tested against some common organic acids on

Whatman No. 1 paper. The paper was developed with the $Br_2$/starch–KI reagent as described above. The following acids, at levels as high as 200 $\mu$g, gave no colour with the reagent: citric, isocitric, *cis*-aconitic, malic, succinic, fumaric, tartaric, and itaconic. A faint blue colour was given by 100 $\mu$g of oxoglutaric acid. With 100 $\mu$g, pyruvic, oxaloacetic and malonic acids gave strong blue colours. The reagent apparently detects acids which are capable of enolising. (Malonic acid may be regarded as an enol[13].)

## PAPER CHROMATOGRAPHY

A series of buffered solvent systems were used because the pH could be adjusted in order to effect particular separations. These are prepared as follows: *n*-butanol is saturated by shaking up with *M*/10 phosphate buffer of definite pH. Whatman No. 1 papers are thoroughly soaked by pulling the papers through a trough of the same buffer. The papers are then hung to dry so that the excess buffer drips off the paper in the same direction as the eventual chromatography solvent flow. The papers are allowed to dry in air for 6 h and are then irrigated with the buffer-saturated butanol. Buffer systems of pH 3, 5 and 7 (designated A, B and C respectively) gave the best separations of the available acids. Chromatograms were also irrigated with the top layer of a *n*-butanol–water–pyridine (5:4:1, by vol.) mixture (D). All chromatograms were run for 16 h on Whatman No. 1 paper using the descending flow method. Chromatograms were irrigated in the complete absence of any "aqueous phase" since the best separations were achieved under these conditions with all the solvents which were used. Some typical $R_F$ values are listed in Table II. These values were not found to be strictly reproducible but the relative mobilities of the acids were constant for a particular solvent system.

TABLE II

$R_F$ VALUES OF TETRONIC ACIDS

| *Acid* | $R_F \times 100$ | | | |
|---|---|---|---|---|
| | *A* | *B* | *C* | *D* |
| Carolic | 26 | 27 | 25 | 38 |
| Carlic | 8 | 6 | 0 | — |
| Carlosic | 26 | 22 | 2 | 9 |
| Terrestric | 73 | 58 | 52 | 60 |
| Viridicatic | 47 | 43 | 5 | 24 |

## HIGH VOLTAGE PAPER ELECTROPHORESIS

The apparatus used was a modification of that described by GROSS[14]. Tap water circulating through the precision-ground aluminium plates provides the cooling system. Wicks of folded Whatman 3 MM strips complete the connection between the electrodes and the electrophoretic strip. The wicks are wrapped in cellophane (British Cellophane Ltd., grade 300 P.T.) in order to minimise the inflow of buffer from the wicks to the strip which otherwise may cause movement and distortion of spots. The electropho-

retic strips (4 1/2 × 22 1/4 in. Whatman 3 MM papers) are dipped through a trough of buffer and then passed through a domestic clothes wringer to remove excess buffer. The mixture to be separated is spotted on to the wet strip, or preferably, applied as a thin streak using a device similar to that described by OSBORNE AND BAWDEN[15]. Of the buffers tested, a pyridine–acetic acid–water mixture (10:0.4:190, by vol.) of

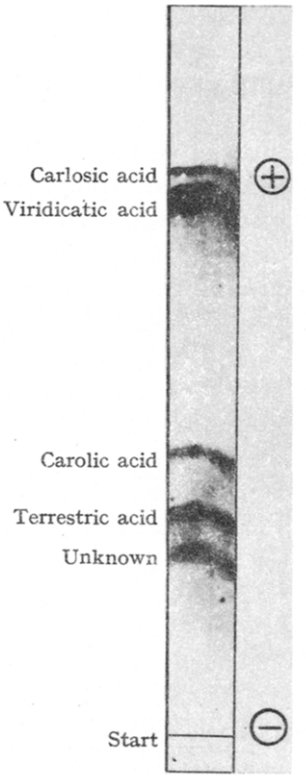



Fig. 1. Electrophoresis of a mixture containing approximately 20 μg each of terrestric, carolic, viridicatic and carlosic acids, and an unknown tetronic acid, applied as a streak 6 in. from cathode end. Conditions: 190 V/cm; 1 mA/cm; 35 min; Whatman No. 3 MM 4 1/2 × 22 1/4 in. strip; pyridine–acetic acid–water buffer (10:0.4:190, by vol.), pH 6.5. Spraying reagents: 0.1 ml $Br_2$ in 100 ml 50% acetic acid; 1% starch solution containing 2% KI.

pH 6.5 gave the best separation of the available acids. Fig. 1 shows a typical separation.

In Table III are listed some electrophoretic mobilities. These values are only

TABLE III

ELECTROPHORETIC MOBILITIES OF TETRONIC ACIDS

| *Acid* | *Mobility* $cm^2/V \cdot sec \times 10^5$ |
|---|---|
| Terrestric | 4.0 |
| Carolic | 4.8 |
| Viridicatic | 7.4 |
| Carlosic | 8.0 |

approximate since the actual voltage across the paper strips is less than the applied voltage and no correction was made for endosmotic flow. Nevertheless under constant conditions the mobilities are strictly reproducible.

## IDENTIFICATION OF TETRONIC ACIDS

Acids appearing in the culture media of *Penicillium charlesii* G. Smith have been tentatively identified in the usual way by running "markers" on chromatograms or electrophoretic strips. In addition the identity of some tetronic acids has been confirmed by measuring the U.V. spectra of material eluted from chromatograms or electrophoretic strips. The spectra of "possible tetronic acids", *i.e.* substances which give a positive reaction with the $Br_2$/starch–KI spray, may be similarly examined.

From the data recorded by HERBERT AND HIRST[16] it is clear that the tetronic acids can be divided into two groups showing characteristic U.V. spectra. The first group, typified by α-ethyltetronic acid (formula II; R = $C_2H_5$; X = H) show absorption maxima at 265 mμ in alkali and at 230 mμ in acid media. The second group, which consists of acids similar in structure to α-acetyltetronic acid (formula II; R = $CO \cdot CH_3$; X = H) have absorption maxima at 265 and 230 mμ in both acid and alkali. The acids described in this account belong to the second group.

A careful re-examination of the U.V. spectra of the available acids revealed important differences between the acids which have not been reported previously. Thus, it was found that in acid media the absorption maximum at 230 mμ can be completely suppressed, and the strength of acid necessary to completely suppress this maximum at 230 mμ is characteristic for a particular tetronic acid. Also the absorption maximum at 265 mμ is shifted towards the longer wavelengths in acid media so that when the maximum at 230 mμ is completely suppressed a single maximum at 274 mμ remains. These characteristic shifts in U.V. spectra are useful in the identification of tetronic acids. Table IV shows the strength of HCl required to

TABLE IV

CONCENTRATION OF HCl REQUIRED TO SUPPRESS ABSORPTION MAXIMUM AT 230 mμ

| *Acid* | *HCl concn. N* |
|---|---|
| Carolic | 0.1 |
| Carlic | 0.05 |
| Carlosic | 2.0 |
| Terrestric | 0.005 |
| Viridicatic | 2.0 |

suppress the various maxima at 230 mμ. (The maximum at 230 mμ is considered to be suppressed completely when the spectrum shows no obvious maximum at 230 mμ and when the optical density reading at 230 mμ is less than that at 224 mμ. With the instrument available (Unicam S.P. 500 spectrophotometer) it is not possible to decide

whether the maximum at 230 mμ is removed or merely shifted to a wavelength below 220 mμ.)

### A PROCEDURE FOR THE PARTIAL CHARACTERISATION OF NEW TETRONIC ACIDS

All the tetronic acids so far isolated from fungal sources have either a methyl or a carboxymethyl group attached to the γ-carbon atom of the ring (dehydrocarolic acid[4] is an exception, having a methylene group instead). After bromination in acetic acid solution these tetronic acids yield either α-bromo-γ-methyl-tetronic acid (III) or α-bromo-γ-carboxymethyl-tetronic acid (IV). These α-bromo derivatives are easily separable by paper chromatography so that it is possible to characterise a new tetronic acid according to the type of α-bromo derivative which it yields on bromination.

The following experiment illustrates the type of procedure that has been applied to an unknown compound. The compound ($C_9H_{10}O_5$), which gave the two-banded U.V. spectrum characteristic of some tetronic acids, was isolated from culture filtrates of *Penicillium charlesii* G. Smith. This compound gave an orange colour with aqueous $FeCl_3$ solution which is a typical reaction of tetronic acids[1,3–5] and also gave a positive reaction with the $Br_2$/starch–KI reagent on paper. Spots containing 50 μg of carolic, carlosic, terrestric and viridicatic acids and the unknown substance were applied along the starting line of a chromatogram (Whatman No. 1). The area containing these spots was lightly sprayed with the $Br_2$ in 50% glacial acetic acid reagent in order to convert the tetronic acids to their corresponding α-bromo derivatives. After drying in the fume cupboard for 2–6 h the chromatogram was irrigated for 16 h by the descending flow method using the *n*-butanol–water–pyridine system described earlier. The separated bromo derivatives were revealed by spraying the dry chromatogram with the starch–KI reagent, followed immediately by a light spray with *N*/10 HCl which increases the sensitivity of the reagent. From the results set out in Table V

TABLE V

$R_F$ VALUES OF TETRONIC ACID BROMO DERIVATIVES

| *Acid* | $R_F \times 100$ *solvent D* |
|---|---|
| Carolic | 49 |
| Carlosic | 8 |
| Terrestric | 49 |
| Viridicatic | 8 |
| Unknown ($C_9H_{10}O_5$) | 49 |

it seems probable that the "unknown tetronic acid" contains a γ-methyl-tetronic acid ring structure like that of carolic and terrestric acids.

SUMMARY

1. A sensitive spray reagent is described for the detection of some naturally occurring tetronic acids on paper chromatograms and electrophoretic strips.

2. New chromatography solvents are described and $R_F$ values are reported.

3. The high voltage electrophoresis of tetronic acids is reported and their electrophoretic mobilities are recorded.

4. The identification of tetronic acids by means of their U.V. spectra is discussed; some relevant new data are recorded.

5. A paper chromatographic procedure is described for the partial characterisation of tetronic acids.

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