

## THE IDENTIFICATION OF THE THEARUBIGINS AS POLYMERIC PROANTHOCYANIDINS

A. G. BROWN, W. B. EYTON, A. HOLMES and W. D. OLLIS

Department of Chemistry, The University, Sheffield S3 7HF

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**Abstract**—Of the thearubigins associated with black tea, those which are extracted from aqueous black tea liquor have been degraded to flavan-3-ols, flavan-3-ol gallates, anthocyanidins, and gallic acid. These results establish that the thearubigins are polymeric proanthocyanidins.

### INTRODUCTION

THE THEARUBIGINS constitute about 10–20 per cent by weight of black tea,<sup>1</sup> but in spite of several earlier investigations<sup>2–5</sup> the class of natural products to which the thearubigins could be assigned was not identified. In a recent publication,<sup>6</sup> the earlier chemical studies of the thearubigins have been reviewed and it was reported that the thearubigins belong to the proanthocyanidin class of natural products. The experimental evidence in support of this proposal is now presented.

The thearubigins were named by Roberts,<sup>7</sup> who isolated them from an aqueous black tea liquor<sup>8</sup> by successive extractions with ethyl acetate and *n*-butyl alcohol. However, the material isolated was found to contain nitrogen in the form of caffeine, and our isolation procedure includes an initial extraction of the liquor with chloroform to remove the caffeine. The tea liquor was then successively extracted with ethyl acetate and with *n*-butyl alcohol, followed by acidification and further extraction with *n*-butyl alcohol. The resulting three extracts yielded material which by fractional precipitation (see Fig. 1) gave five thearubigin fractions, TR-1, TR-2, TR-3, TR-4, and TR-5. These fractions are not homogeneous, but each consists of thearubigins of similar solubilities and chromatographic behaviour.

### RESULTS

#### *Formation of Anthocyanidins*

The five thearubigin fractions were separately hydrolysed by heating with hydrogen chloride in anhydrous isopropyl alcohol,<sup>9</sup> and this yielded cyanidin (I), delphinidin (II),

<sup>1</sup> E. A. H. ROBERTS, in *The Chemistry of Flavonoid Compounds* (edited by T. A. GEISSMAN), p. 468, Pergamon Press, Oxford (1962).

<sup>2</sup> L. VUATAZ and H. BRANDENBERGER, *J. Chromatogr.* **5**, 17 (1961).

<sup>3</sup> R. L. WICKREMASINGHE, *Tea Quart.* **38**, 205 (1967).

<sup>4</sup> D. J. MILLIN and D. W. RUSTIDGE, *Process Biochem.* **2**, 9 (1967).

<sup>5</sup> D. J. CRISPIN, R. H. PAYNE and D. SWAINE, *J. Chromatogr.* **37**, 118 (1968).

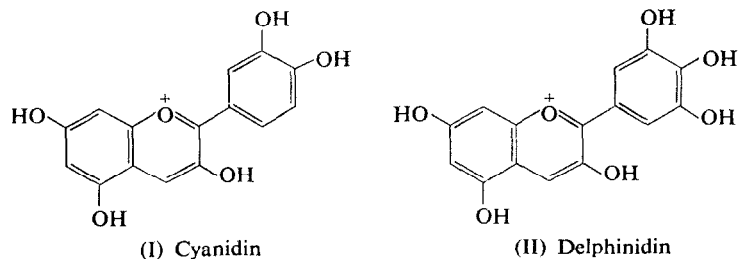
<sup>6</sup> A. G. BROWN, W. B. EYTON, A. HOLMES and W. D. OLLIS, *Nature* **221**, 742 (1969).

<sup>7</sup> E. A. H. ROBERTS, *J. Sci. Food Agr.* **9**, 212 (1958).

<sup>8</sup> E. A. H. ROBERTS, R. A. CARTWRIGHT and M. OLDSCHOOL, *J. Sci. Food Agr.* **8**, 72 (1957).

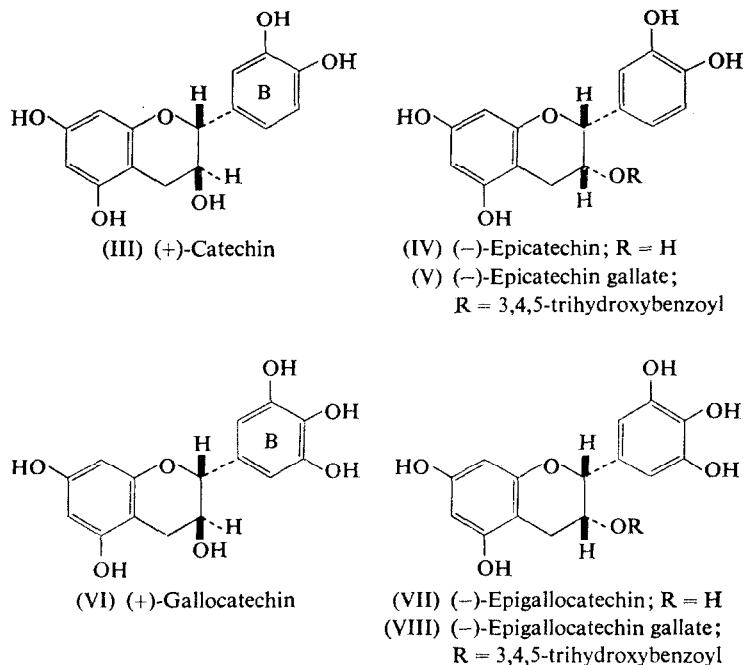
<sup>9</sup> D. G. ROUX and M. C. BILL, *Nature* **183**, 42 (1959).

and their isopropyl ethers. The anthocyanidins were identified by chromatographic comparison in several solvent systems with authentic samples. The yields of the anthocyanidins were determined spectrophotometrically<sup>10, 11</sup> and were found to be of the order of 20 per cent from each fraction. No attempt was made to estimate the yield of each anthocyanidin individually, but it was clear from the intensity of the chromatographic spots that cyanidin (I) was present in relatively greater amount than delphinidin (II).



#### *Reductive Hydrolysis with Aqueous Sulphurous Acid*

Quesnel has drawn attention to the use of aqueous sulphurous acid for the reductive hydrolysis of condensed tannins,<sup>12</sup> and has reported that the hydrolysis of the condensed tannins<sup>13</sup> from *Theobroma cacao* yields epicatechin (IV) and sulphur-containing compounds.<sup>14</sup>



<sup>10</sup> W. PIGMAN, E. ANDERSON, R. FISCHER, M. A. BUCHANAN and B. L. BROWNING, *TAPPI* **36**, 4 (1953).

<sup>11</sup> T. SWAIN and W. E. HILLIS, *J. Sci. Food Agr.* **10**, 63 (1959).

<sup>12</sup> V. C. QUESNEL, *Tetrahedron Letters* 3699 (1964).

<sup>13</sup> V. C. QUESNEL, *Phytochem.* **7**, 1583 (1968).

<sup>14</sup> W. G. C. FORSYTH and J. B. ROBERTS, *Biochem. J.* **74**, 374 (1960).

Treatment of the thearubigin fractions, TR-1, TR-2, TR-3, TR-4, and TR-5 with aqueous sulphurous acid yielded flavan-3-ols, flavan-3-ol gallates, and sulphur-containing compounds. The flavan-3-ols were identified as catechin (III), epicatechin (IV), and gallocatechin (VI) with certainty, and it is believed that epigallocatechin (VII) was also formed from the fraction TR-3. The flavan-3-ol gallates were identified as epicatechin gallate (V) and epigallocatechin gallate (VIII). However, not all the flavan-3-ols and flavan-3-ol gallates mentioned above were identified as degradation products from each thearubigin fraction. The sulphur-containing compounds were separated from the flavan-3-ols and flavan-3-ol gallates by cellulose column chromatography. Treatment of the sulphur-containing compounds with dilute acid<sup>14</sup> yielded cyanidin (I) and delphinidin (II).

#### *The Confirmation of Gallate Ester Residues*

In relation to the formation of the gallates (V) and (VIII) by sulphurous acid hydrolysis of the thearubigins, the equivalent yield of gallic acid produced by direct acid hydrolysis of the thearubigins has been examined and was found to be 2–4 per cent.

### DISCUSSION

The degradative reactions which have been carried out on the thearubigin fractions TR-1, TR-2, TR-3, TR-4, and TR-5 (see Fig. 1) isolated by solvent extraction of black tea liquor, may be summarized as follows: (a) Treatment with anhydrous isopropyl alcohol-hydrogen chloride gives cyanidin (I) and delphinidin (II), which are partially transformed into their corresponding isopropyl ethers. (b) Reductive hydrolysis of the thearubigins with aqueous sulphurous acid gives mixtures including the flavan-3-ols (III, IV, VI, and VII) and flavan-3-ol gallates (V and VIII). In addition sulphur-containing compounds are also isolated from this reaction, but although the constitution of these substances is not yet established in detail, it is probable that they are 4-substituted flavan-3-ols and flavan-3-ol gallates because they yield cyanidin (I) and delphinidin (II) on treatment with acid. (c) Acidic hydrolysis of the thearubigins gives gallic acid.

These results demonstrate that the thearubigins are mixtures of polymeric proanthocyanidins containing flavanoid residues of the types (IX), (X), (XI), and (XII). Consideration may now be given to the types of interflavanoid linkage which are compatible with the degradative evidence described above (see reactions *a*, *b*, and *c*) and with opinions regarding the formation of the thearubigins.

During the manufacture of black tea from green tea leaf, an enzymatic oxidative process occurs in which the flavanoid compounds (III, IV, V, VI, VII, and VIII) present in the green leaf are almost completely transformed into the theaflavins and the thearubigin pigments characteristic of black tea.

It is well established<sup>15, 16</sup> that the creation of C—C bonds in oxidative coupling processes involves positions which are located *ortho*- and *para*- with respect to phenolic hydroxyl groups in the phenolic precursors. Thus, the formation of the thearubigins by an oxidative process could involve<sup>17–19</sup> the positions 6, 8, 2', 5', 6', 2'', and 6'' as indicated in the diagrams

<sup>15</sup> E. HASLAM, in *The Chemistry of Vegetable Tannins*, p. 81, Academic Press, London (1966) and references therein.

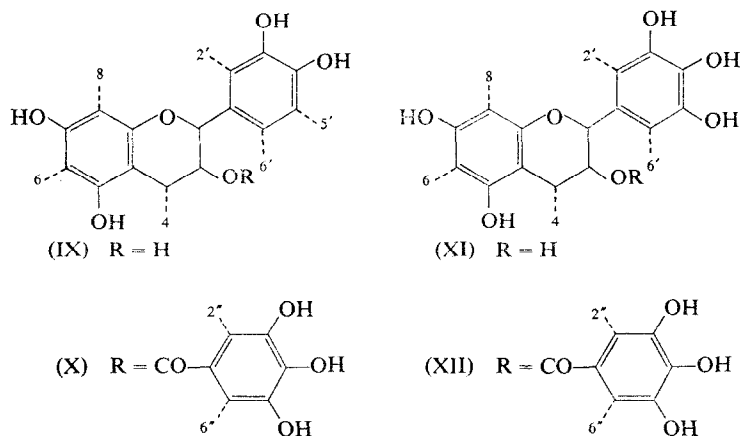
<sup>16</sup> *Oxidative Coupling of Phenols* (edited by A. R. BATTERSBY and W. I. TAYLOR), Arnold, London (1967).

<sup>17</sup> J. L. GOLDSTEIN and T. SWAIN, *Phytochem.* **2**, 371 (1963).

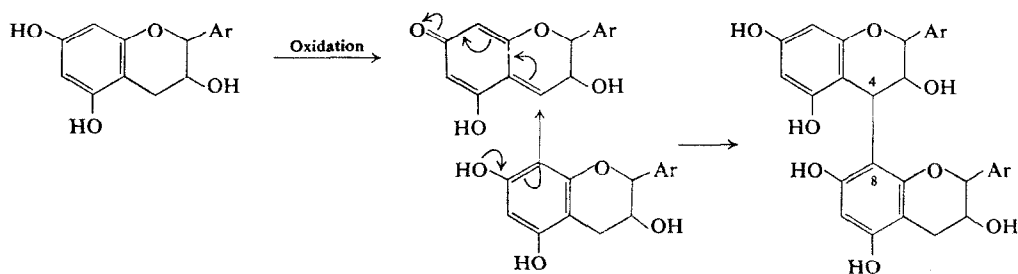
<sup>18</sup> K. WEINGES and K. FREUDENBERG, *Chem. Commun.* 220 (1965) and references therein.

<sup>19</sup> S. E. DREWES, D. G. ROUX, S. H. EGGERS and J. FEENEY, *J. Chem. Soc. (C)*, 1217 (1967); S. E. DREWES and D. G. ROUX, *Chem. Commun.* 1 (1968).

(IX)–(XII). These positions could in principle be involved in either C—O<sup>20</sup> bond formation or C—C<sup>21</sup> bond formation. For ease of presentation, the mechanism of formation and the general constitutional aspects of the formulation of the thearubigins are discussed in terms of C—C bond formation, *but equivalent processes involving C—O bond formation are not excluded*. The creation of interflavanoid bonds involving positions 6, 8, 2', 5', 6', 2'', and 6'' (see IX—XII) can be interpreted either in terms of phenoxyl radical coupling processes or their ionic equivalent; these processes are now so well known<sup>15, 16</sup> that further comment is not required.



Interflavanoid bonds involving position 4 are also possible and are likely to have their origin<sup>22</sup> in nucleophilic attack by a phenolate anion upon an intermediate quinonemethide as shown in Scheme 1.



SCHEME 1. FORMAL MECHANISTIC PROPOSAL FOR THE CREATION OF INTERFLAVANOID BOND INVOLVING POSITION 4 VIA A QUINONEMETHIDE INTERMEDIATE.

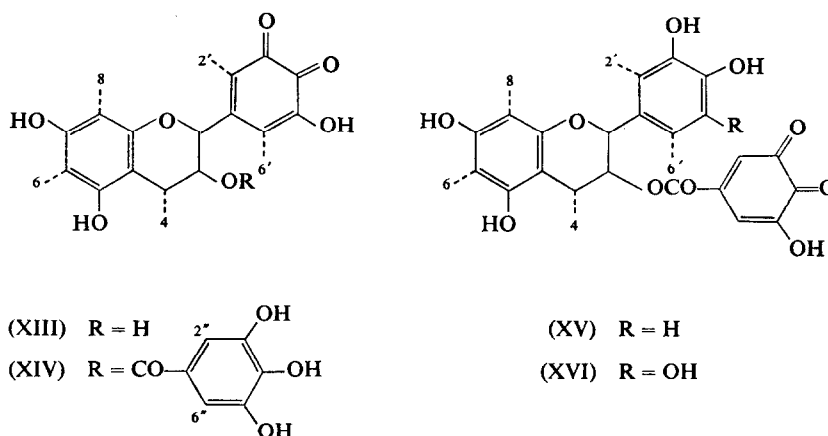
The suggestion<sup>1</sup> that the thearubigins characteristic of black tea are formed from the flavanoid precursors present in the green leaf is clearly well based, but there is a further point requiring comment. If it is assumed that thearubigin formation is directly related to the flavanoid precursors (III, IV, V, VI, VII, and VIII), then it might have been expected that

<sup>20</sup> M. J. BETTS, B. R. BROWN, P. E. BROWN and W. T. PIKE, *Chem. Commun.* 1110 (1967).

<sup>21</sup> K. D. SEARS and R. L. CASEBIER, *Chem. Commun.* 1437 (1968).

<sup>22</sup> A. ROBERTSON, W. B. WHALLEY and J. YATES, *J. Chem. Soc.* 2013 (1951).

the flavanoid residues in the thearubigin polymer would have been related to the relative proportions present in the green leaf tea. This is not the case. Thus, the approximate proportions of the flavanoid precursors in green leaf are, 3',4'-dioxygenated derivatives (III, IV, and V) approximately one part, and 3',4',5'-trioxygenated derivatives (VI, VII, and VIII) approximately three parts, whereas by the degradation of the thearubigins with isopropyl alcohol-hydrogen chloride, the 3',4'-dioxygenated cyanidin (I) is formed in greater amount relative to the 3',4',5'-trioxygenated delphinidin (II). This apparent inconsistency may be accounted for by one or both of the following postulates. During thearubigin formation, it is possible that a number of the 3',4',5'-trihydroxyflavanoid residues (XI and XII) are oxidized further to hydroxy-*o*-quinones (XIII and XIV). This hypothesis is additionally attractive in that such hydroxy-*o*-quinone residues could be associated with the acidity of thearubigins; this is an alternative to the suggestion made by Roberts<sup>1</sup> that the acidity of the thearubigins is due to the presence of carboxyl groups. The second possibility to account for the relatively lower yield of delphinidin with respect



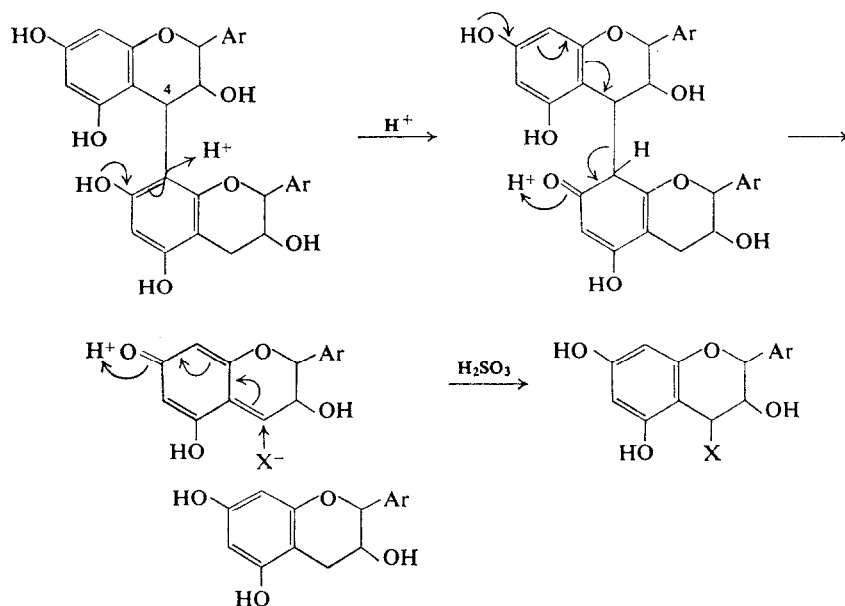
to cyanidin is, of course, associated with the possibility that the interflavanoid linkages can involve positions 2' and/or 6'<sup>17</sup> of the trioxygenated flavan-3-ols and flavan-3-ol gallates (see XI and XII).

Acidic hydrolysis of the thearubigins with methanol-hydrochloric acid gave a mixture of gallic acid and methyl gallate, and the equivalent yield was 2-4 per cent. This is much lower than that expected for the incorporation of epicatechin gallate (V) and epigallocatechin gallate (VIII) in proportions related to the relative amounts of these gallate esters (65 per cent) in the flavanoids present in green tea. The low yield of gallic acid from the hydrolysis of the thearubigins may be explained by suggesting either that the galloyl residues are oxidized to the corresponding hydroxy-*o*-quinones (XV and XVI) or that there are inter-aryl bonds involving positions 2'' and/or 6'' of the gallate residues (see X and XII).

In addition to the possibility that C—C and C—O bonding could involve positions 6, 8, 2', 5', 6', 2'', or 6'', the possibility that position 4 is also involved has been recognized (see Scheme 1). This possibility is supported by the isolation of sulphur-containing compounds<sup>12-14</sup> from the treatment of the thearubigins with aqueous sulphurous acid in that reasonable reaction mechanisms can be postulated for the cleavage of bonds involving position 4 (see Scheme 2). In Scheme 2 the group X is the sulphur-containing residue. It

could be a sulphonic acid group, but detailed proposals are not possible until these sulphur-containing compounds are fully characterized. Studies directed towards this objective are in progress.

The studies now reported show that the thearubigins are polymeric proanthocyanidins and the general nature of the flavanoid residues is clear. Some comment can be made upon the types of interflavanoid linkages which are present, but a complete definition of the general constitutional features of the thearubigins must await the isolation of "homogeneous" material. In many respects, the problems presented by the thearubigins are very similar in form to that associated with the structural investigation of all condensed tannins of the proanthocyanidin type.<sup>17-21</sup> Our present knowledge of the thearubigins indicates that there are clear differences in the physical characteristics of various thearubigin fractions



SCHEME 2. A MECHANISTIC PROPOSAL FOR THE CLEAVAGE OF THE 4-8 INTERFLAVANOID BOND BY AQUEOUS SULPHUROUS ACID.

and these differences may well be related to variations in the degree of polymerization, the oxidation level of flavanoid residues, and the molecular shape.<sup>13, 23</sup>

## EXPERIMENTAL

### Fractionation Procedure

Black tea (75 g) was added to boiling distilled  $H_2O$  (1500 ml) and the mixture was boiled gently (10 min). The liquor was filtered through a cotton wool plug and cooled immediately to room temperature. The combined liquors from three such extractions of black tea ( $3 \times 75$  g) were treated as shown in Fig. 1.

<sup>23</sup> K. WEINGES, K. GÖRITZ and F. HADER, *Annalen* **715**, 164 (1968).

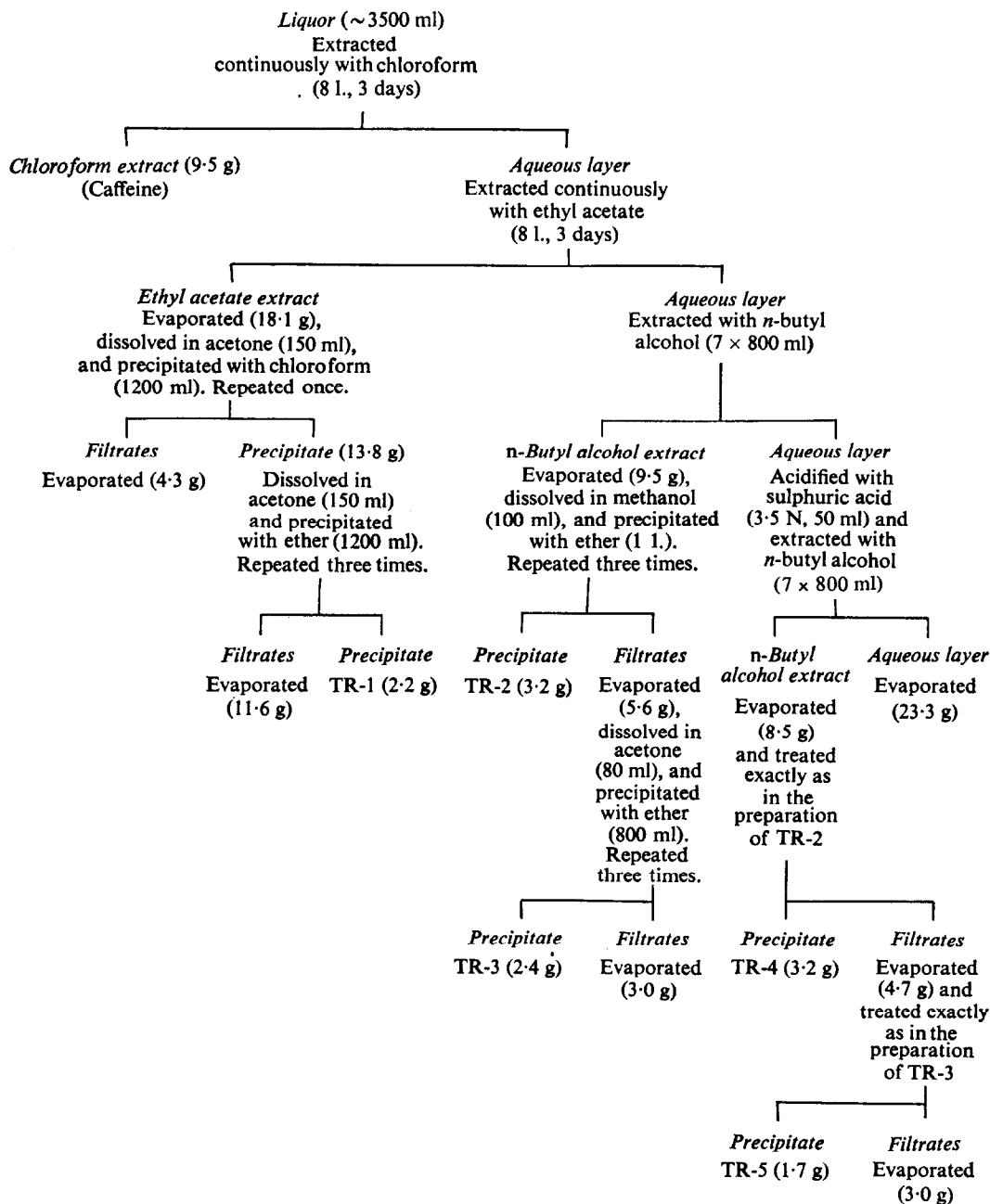


FIG. 1.

*Formation on Anthocyanidins*

Each thearubigin fraction (25 mg) was heated (100°, 15 min, sealed tube) in a solution of HCl in anhydrous isoPrOH (0.035 N, 1.0 ml). The product was then examined by two-dimensional paper chromatography, eluting first with HCO<sub>2</sub>H:3 N-HCl (1:1) followed by H<sub>2</sub>O:HOAc:conc. HCl (10:30:3). Four red spots were observed which were identified from their *R<sub>f</sub>* values<sup>24</sup> as cyanidin (I), delphinidin (II), and their corresponding isopropyl ethers. The identification of cyanidin (I) and delphinidin (II) was confirmed by co-chromatography.

<sup>24</sup> J. B. HARBORNE, in *Comparative Biochemistry of the Flavonoids*, p. 7, Academic Press, London (1967).

The yields of anthocyanidins were determined by repetition of the reaction on each thearubigin fraction followed by dilution of the product with EtOH. Spectrophotometric measurement of the resulting solution was made against a blank<sup>10,11</sup> consisting of an unheated, freshly prepared mixture of the same quantities of the thearubigin fraction and the reagents. The total yield of anthocyanidin was determined by comparison of the corrected optical densities at 550 nm with a standard solution of cyanidin in EtOH:conc. HCl (0.01%). The yields of anthocyanidins in these reactions are variable and the yields quoted are typical and not average.

Thearubigin fraction	TR-1	TR-2	TR-3	TR-4	TR-5
Yield of anthocyanidins (per cent)	24	20	26	16	18

#### *Reductive Hydrolysis with Aqueous Sulphurous Acid*

SO<sub>2</sub> was passed through a heated (75–80°) solution of each thearubigin fraction (30 mg) in EtOH (1 ml) and H<sub>2</sub>O (2 ml) for 2.5 hr. The product was examined by two-dimensional paper chromatography eluting with aqueous HOAc (6%) followed by a mixture of 2-butanol:HOAc:H<sub>2</sub>O (14:1:5). The chromatograms were developed using a mixture (1:1) of FeCl<sub>3</sub> (0.3%) and K<sub>3</sub>Fe(CN)<sub>6</sub> (0.3%) which shows flavan-3-ols and flavan-3-ol gallates as dark-blue spots; their identities were confirmed by co-chromatography. As the *R<sub>f</sub>* values of catechin and gallic acid are almost identical in the solvent systems used, it was necessary to distinguish between these two compounds by spraying with KCN (5%); gallic acid gives a deep red-brown spot, whereas catechin gives a yellow spot which develops slowly.

The FeCl<sub>3</sub>–K<sub>3</sub>Fe(CN)<sub>6</sub> solution also revealed on the chromatogram a number of spots of high *R<sub>f</sub>* in HOAc (6%) and low *R<sub>f</sub>* in the 2-butanol:HOAc:H<sub>2</sub>O (14:1:5) solvent. These compounds of high *R<sub>f</sub>* gave anthocyanidins as red spots when sprayed with aqueous *p*-toluenesulphonic acid. In order to examine the compounds of high *R<sub>f</sub>* a large-scale SO<sub>2</sub> hydrolysis was performed using 150 mg of the thearubigin fraction, and the reaction mixture was applied directly to a cellulose column (55 × 2 cm) and eluted with H<sub>2</sub>O. Two-dimensional paper chromatography revealed that the fraction first eluted consisted of the compounds with high *R<sub>f</sub>*, which were shown by a positive Lassaigne test to contain covalently bound sulphur.

#### *Confirmation of Gallate Ester Residues*

The thearubigin fraction (20 mg) was dissolved in MeOH (1 ml) containing conc. HCl (0.3 ml) and N<sub>2</sub> was passed through the heated mixture (65°, 24 hr). The solution was fractionated on two-dimensional paper chromatograms (Whatman 3MM) using the same solvents as above, and the spots due to gallic acid and methyl gallate (u.v. illumination, deep purple) were cut out, combined, and extracted into hot MeOH (3 × 10 ml). The residue from the extract was dissolved in MeOH (40 ml) containing one drop of dilute HCl and the combined yield of gallic acid–methyl gallate was determined spectrophotometrically using the following spectral parameters for methyl gallate<sup>25</sup> ( $\lambda_{\max}$  275 nm,  $\epsilon_{\max}$  10,000).

The validity of the above analytical method was established using methyl gallate as a model compound, which gave recoveries of 94–98 per cent. The yield of gallic acid–methyl gallate from the five thearubigin fractions was 2–4 per cent.

*Acknowledgement*—We thank the Tea Research Association (India) for their support of this investigation.

<sup>25</sup> A. HILLMER, *Ber.* 66, 1600 (1933).