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Chlorogenic Acid Biosynthesis. Chemical Synthesis and Properties of the Mono-*O*-cinnamoylquinic Acids*

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ABSTRACT: Rational syntheses of the 1- and 3-*O*-cinnamoylquinic acids are reported. Negligible wandering of the cinnamoyl group occurred under the mildly acidic conditions used to remove the protecting groups from the substituted quinic acid molecule. The 4- and 5-*O*-cinnamoylquinic acids were isolated from the mixture formed when barium hydroxide acted on 1-*O*-cinnamoylquinide. Once the lactone ring was opened, the cinnamoyl group migrated from hydroxyl to hydroxyl group.

The results of experiments with [U-^{14}C]quinic acid establish that the base-catalyzed migration of cinnamoyl groups about the quinic acid molecule is an intramolecular process. Three lines of evidence establish that migrations occur in the sequence 1 $\rightleftharpoons$ 5 $\rightleftharpoons$ 4 $\rightleftharpoons$ 3: (a) studies of migration when the carboxyl is methylated; (b) studies of migration when the 4,5-hydroxyl groups are blocked by an isopropylidene group; and (c) studies of the rates of migration in buffered solutions (pH 6–10). It is improbable that a 1 $\rightarrow$ 3 migration, by way of a cinnamic–quinic mixed anhydride, is of importance.

Chlorogenic acid (3-*O*-caffeoylquinic acid), and a variety of closely related hydroxycinnamoyl conjugates, are widely distributed in the roots, stems, leaves, and flowers of plants (Herrmann, 1956; Sondheimer, 1964). Despite the ubiquitous occurrence of these compounds their functions in the life of the plant and the main facts concerning their biosynthesis have yet to be established. The compounds, or their derivatives, may play an important role in morphogenesis; thus they may act as mediators of photoperiodic effects (Zucker *et al.*, 1965; Bottomley *et al.*, 1965) and be associated with the action of plant growth hormones (Zenk and Müller, 1963). They may also act as cofactors in oxidation–reduction systems (Marrè *et al.*, 1962) or in photophosphorylation (Haberman, 1963), and they may influence the activity of specific enzymes or types of enzyme (Schwimmer, 1958).

Levy and Zucker (1960) postulated that 3-*O*-cinnamoyl- and 3-*O*-*p*-coumaroylquinic acids are intermediates in the biosynthesis of chlorogenic acid. In order to investigate this hypothesis further it was necessary to prepare 3-*O*-cinnamoylquinic acid by rational chemical synthesis. At an early point in the investigation it became apparent that the cinnamoyl group is liable to migrate from hydroxyl to hydroxyl group about the quinic acid molecule. To prove conclusively that the 3-isomer had been synthesized all four mono-*O*-cinnamoylquinic acids had to be obtained and characterized. The present paper describes (1) the synthesis of these isomers, and (2) a study of the base-catalyzed process by which cinnamoyl group migration occurs.

Related problems in the synthesis of the *p*-coumaroyl, caffeoyl, and galloyl esters of quinic acid have been concurrently explored in two laboratories. Investigators at the University of Rome have reported the synthesis of 1,4-di- (Panizzi *et al.*, 1954); 1-, and 4,5-di- (Scarpati *et al.*, 1958); 3- (Panizzi *et al.*, 1956); and 4-, 5-, and 1,3-di-*O*-caffeoylquinic acids (Scarpati *et al.* 1964). In view of later work by this group (Scarpati and Esposito, 1964) and results reported in the present paper, mixtures of isomers were probably obtained in the synthesis of the 1,4-di- and 3-*O*-caffeoylquinic acids. The Sheffield University group has synthesized 3-*O*-*p*-coumaroylquinic acid (Haslam *et al.*, 1961); 1-, 4-, and 5-*O*-*p*-coumaroylquinic acids; and 5-*O*-caffeoylquinic

* From the Biochemistry Department, The Connecticut Agricultural Experiment Station, New Haven, Conn. *Received June 25, 1965.* This work was supported, in part, by grant GB-2626 from the National Science Foundation. A preliminary account of the synthetic studies has appeared (Hanson, 1963). The evidence for assigning an absolute configuration to quinic acid has been reviewed (Hanson, 1962). The various carbon atoms in the cyclohexane ring of quinic acid are indicated by the system of stereospecific numbering generally employed for this compound.

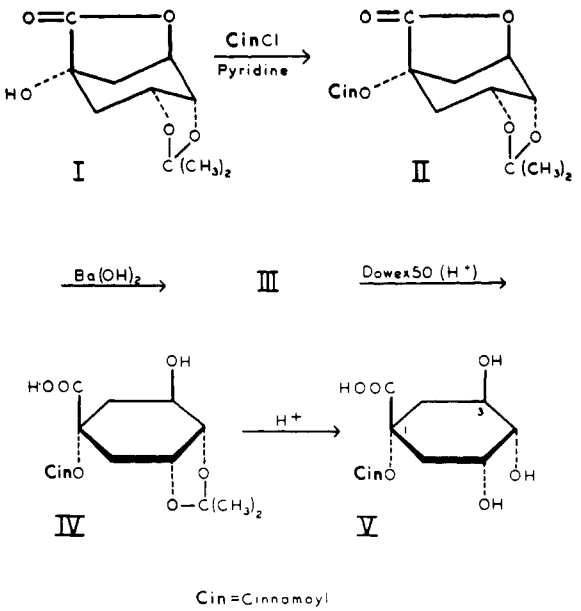

FIGURE 1: Synthesis of 1-*O*-cinnamoylquinic acid. Quinic acid → I (55% yield), I → II (80%), II → III, hydrated barium salt of IV (66%, the gross yield from the reaction is closer to 80%), migration to the 3-position does not take place; III → V without isolation of IV (95%); over-all yield from quinic acid 28%.

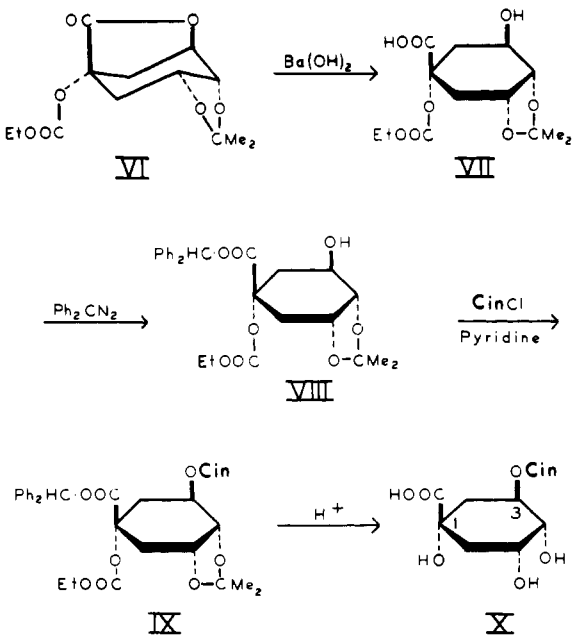

FIGURE 2: Synthesis of 3-*O*-cinnamoylquinic acid. I → VI (85% yield), VI → VII (30%), VII → VIII (85%), VIII → IX (85%), IX → X (46%); over-all yield from quinic acid 8%. For the control experiments to stage IX → X see Table II.

acid (Haslam *et al.*, 1964); also 1-, 3-, 4-, 5-, 4,5-di-, 3,4,5-tri-, and 1,3,4,5-tetra-*O*-galloylquinic acids (Haslam *et al.*, 1963).

Syntheses

Both the 1- and 3-isomers were obtained by rational synthesis. Suitable control experiments established that negligible wandering of the cinnamoyl group occurred under the mildly acidic conditions used to remove the protecting groups. The 1-isomer was obtained in high yield without recourse to column chromatography and the synthesis of this compound is, therefore, described first. The 4- and the 5-isomers were obtained as a result of cinnamoyl group migration in the presence of alkali.

1-O-Cinnamoylquinic acid (*V*) was synthesized as shown in Figure 1. The isopropylidene group was removed from the acid IV either by leaving a solution of the acid at room temperature overnight or by heating a solution for 3 hr at 100°. The yield in this step is essentially quantitative. In control experiments the *O*-cinnamoylquinic acids believed to be the 3- and 5-isomers and the product of the above synthesis were each heated in aqueous solution at 100° for 3 hr. When these were examined with aid of the analytical silica gel column described previously (Hanson and Zucker, 1963), it was established that no detectable (<0.1%) isomerization, hydrolysis, or lactone formation took place. If it is accepted, at this point, that migration from the 1- to the 3-position did not take place in the presence of barium hydroxide, *i.e.*, II gives III (the barium salt of IV) and not its isomer, then the sequence of reactions I to V constitutes a rational synthesis of the 1-isomer.

The presence of a substituent in the 1-position was confirmed by oxidizing the compound with ceric sulfate in a Warburg respirometer (Table I). No carbon dioxide was evolved, whereas α-hydroxy acids give on oxidation one molar proportion of carbon dioxide. On periodate oxidation the expected two molar proportions of periodate were consumed (Table I).

1-*O*-Cinnamoylquinic acid was previously obtained by Josephson (1928) who treated II with hydrochloric acid in aqueous acetone and isolated 1-*O*-cinnamoylquinide (XI) together with a small quantity of V. Lactone opening under acidic conditions has been employed as the final step in the synthesis of the corresponding caffeoyl, *p*-coumaroyl, and galloyl compounds, but the conditions of hydrolysis employed were more drastic than those used here: 1-*O*-*p*-coumaroylquinic acid was obtained by refluxing 1-*O*-(*O*-acetyl-*p*-coumaroyl)-4,5-*O*-isopropylidenequinide in acetone and 3 N hydrochloric acid (1:1 by volume) for 2.5 hr (Haslam *et al.*, 1964); 1-*O*-caffeoylquinic acid, by heating 1-*O*-caffeoylquinide in 0.1 N hydrochloric acid at 100° for 15 min (Rúveda *et al.*, 1964b); 1-*O*-galloylquinic acid, by heating aqueous 1-*O*-galloylquinide at 100° for 40 hr (Haslam *et al.*, 1963). In each instance countercurrent distribution was required to isolate the products. The acyl substituents were as-

TABLE I: Properties of the Mono-*O*-cinnamoylquinic Acids.

	O-Cinnamoylquinic Acids				Quinic Acid
	1-	3-	4-	5-	
R_{cq} values	0.69	0.42	0.51	0.63	
Mp (°C)	194	166 (146)	157	198	
$[\phi]^{24}D$ (deg) (*c* 2.00, 95% ethanol)	+18.0	−168	−265	−21.8	
ORD (300-mμ region)[a]	−	+	−	+	−
$Ce(SO_4)_2$ oxidation (moles of CO_2/mole)[b]	0.04	0.94	1.05	0.90	1.00
HIO_4 oxidation (moles consumed/mole)[c]					
10 min	1.79	1.01	0.10	0.70	2.00
30 min	2.00	1.03	0.15	0.88	2.09
60 min	2.06	1.10	0.25	0.94	2.09
100 min	2.06	1.10	0.37	1.00	2.09

[a] See Experimental Section. In view of the complexity of the optical rotatory dispersion curves the lack of a simple correlation between molecular rotation and the position of substitution when the galloyl, caffeoyl, *p*-coumaroyl and cinnamoyl series are compared is not surprising. [b] The oxidations were performed in a Warburg respirometer at 30° as described by Meister (1952). The substance to be oxidized (8–10 μmoles) with HCl (10 μmoles) was added to the side arm and $Ce(SO_4)_2$ (200 μmoles) and H_2SO_4 (1000 μmoles) to the flask; final volume 2 ml. [c] The reaction mixture consisted of the cinnamoyl conjugate (12 mg, 37.2 μmoles) and periodic acid (90 μmoles) in 50% ethanol (500 μl). Samples (100 μl) were taken at the times indicated and the periodate consumed was estimated by the arsenite–iodine method of Fleury and Lange (1933).

TABLE II: Effect of Hot Aqueous Acetic Acid on the Mono-*O*-cinnamoylquinic Acids. Control Experiments for the Synthesis of 3-*O*-Cinnamoylquinic Acid.

Treated Isomer[a]	Isomers Produced (%)				Major Lactone Peak (%)	Minor Lactone Peaks (%)	Cinnamic Acid (%)
	1-	3-	4-	5-			
1-	78	1	2	−	16	2	1
3-	−	97	2	0.2	−	0.3, 0.5	−
4-	−	−	82	8	7	2, 1	−
5-	−	2	5	80	10	2, 1	−

[a] The substances indicated (4 mg) were heated in 50% acetic acid for 2 hr at 100°, and the products were examined with the analytical column (yields as percentages of the total ultraviolet light absorbing material). Where sufficient material was available the compounds associated with the various peaks were examined by electrophoresis at pH 7. The major lactone peaks (zero charge), and the minor peaks, fell in the region R_{cq} 0.2–0.3. The single major lactone peak was assumed to correspond to the treated isomer. The reproducibility of the R_{cq} values in this region is not exact and the order of elution of the lactones is thus uncertain. A dash indicates that the compound was undetectable or, in the case of the 1- and 5-isomers, that the presence of a trace of either isomer cannot be detected in the presence of an excess of the other. When either the 1- or the 5-isomer was heated in 80% acetic acid at 100° for 3 hr, four mono-*O*-cinnamoylquinic acid peaks and three major peaks in the lactone region were obtained. Similar mixtures of isomers and lactones are produced when any of the isomers are heated at 200° for 3 min, but the peaks are frequently double, suggesting that *cis–trans* isomerization takes place on heating.

sumed not to migrate. The effect of alkali on such 1-substituted quinides is discussed below.

3-O-Cinnamoylquinic Acid (*X*) was synthesized as shown in Figure 2. The route was developed by Haslam *et al.* (1961) for the preparation of 3-*O*-*p*-coumaroylquinic acid and subsequently employed to synthesize the corresponding galloyl compound (1963). Scarpati *et al.* (1956) in their earlier study of the synthesis of chlorogenic acid employed a methyl group, rather than the acid-labile diphenylmethyl group, to block the carboxyl function.

The conditions required to remove the protecting groups from IX were investigated with the aid of the analytical silica gel column. When IX was heated in 50%

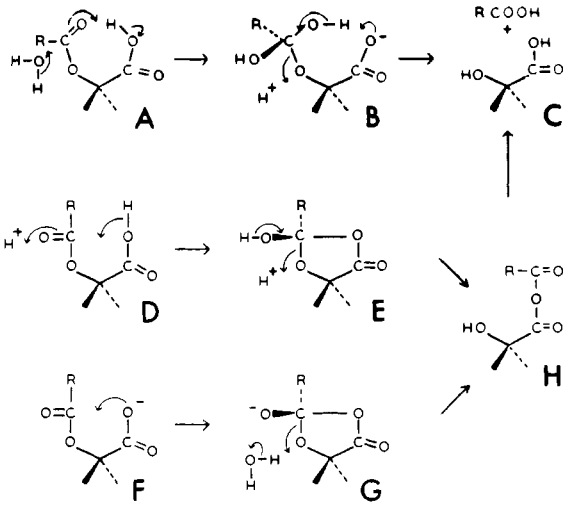

FIGURE 3: Possible mechanisms for the catalysis of ester hydrolysis by an adjacent carboxyl group or carboxylate ion: A, B, C by proton donation; D, E, H or F, G, H by nucleophilic attack to form a mixed anhydride (H) which is subsequently hydrolyzed.

acetic acid at 100° for 2 hr, the major product was 3-*O*-cinnamoylquinic acid (57% of the total ultraviolet light absorbing material, R_{cg} 0.42). No peaks were observed in the region associated with the other cinnamoylquinic acids (R_{cg} 0.51–0.69). The only other important component (32% yield, R_{cg} 0.28) was shown on electrophoresis at pH 7 to be negatively charged and is assumed to be 3-*O*-cinnamoyl-1-*O*-ethoxycarbonylquinic acid. Further treatment of this compound with 50% acetic acid gave more 3-*O*-cinnamoylquinic acid. Compound IX migrated with the elution front. The material eluted in the R_{cg} region 0–0.10, amounting to 11% of the total, was uncharged at pH 7, and presumably consisted of IX and diphenylmethyl 3-*O*-cinnamoyl-1-*O*-ethoxycarbonylquinate (IX less isopropylidene). For preparative purposes a longer period of hydrolysis in 80% acetic acid was employed.

It was necessary to show that the protecting groups were removed from IX without the cinnamoyl group migrating from the 3-position. The product of the above synthesis, 1-*O*-cinnamoylquinic acid, and the compounds believed to be the 4- and 5-*O*-cinnamoylquinic acids were heated in 50% acetic acid at 100° for 2 hr (Table II). These conditions are more drastic than those employed for the synthesis of the 1-isomer: both migration of the cinnamoyl groups and lactone formation were found to occur. The presumed 3-isomer was recovered in 97% yield. If the major lactone peak corresponds to the isomer treated, then the extent of migration in each case was less than 11%. The sequence of reactions in Figure 2, therefore, constitutes a rational synthesis of the 3-isomer. Its properties listed in Table I are consistent with the assigned structure.

The finding that the ethoxycarbonyl group was removed under the conditions employed was surprising (Haslam *et al.*, 1961). When methyl 3-*O*-cinnamoyl-1-*O*-ethoxycarbonyl-4,5-*O*-isopropylidenequinate (IXa: IX with CH_3 in place of Ph_2HC–) was heated at 100° in 50%

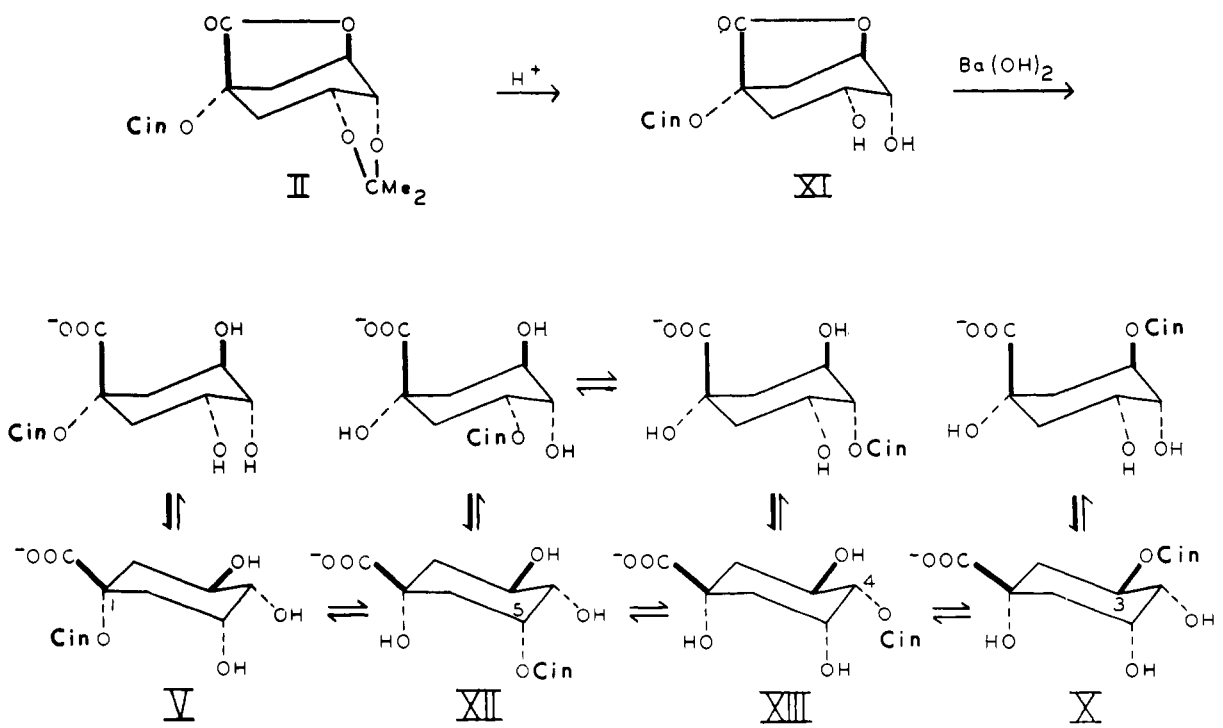

FIGURE 4: The action of barium hydroxide on 1-*O*-cinnamoylquinide (XI). The carboxyl-axial conformations for the mono-*O*-cinnamoylquinic acids are probably favored. For equilibria and rates of migration seeTable III.

acetic acid for 2, 3, or 5 hr, only the isopropylidene group was removed (87, 85, and 94% preparative scale yields). Clearly the carboxyl group plays an important role in eliminating the 1-substituent. Two interpretations of this role are possible. Either the carboxyl group acts as a conveniently placed proton donor in catalyzing the transfer of the ethoxycarbonyl group to water (Figure 3, partial formulas A, B, and C), or the ethoxycarbonyl group migrates to the carboxyl group to form a mixed anhydride which is subsequently hydrolyzed (D, E, and H). A decision between these alternatives is not at present possible. It is shown below, however, that such mixed anhydrides do not play a significant role in the migration of the cinnamoyl group from the 1-position under neutral and alkaline conditions (route F, G, and H).

The 4- and 5-O-Cinnamoylquinic Acids. A mixture of the four possible mono-*O*-cinnamoylquinic acids was obtained by treating 1-*O*-cinnamoylquinide (XI) in dioxane with 1 equiv of aqueous barium hydroxide (Figure 4). When the reaction mixture was examined with the analytical column, the presence of six major compounds with absorption maxima in the ultraviolet was revealed. These were, in order of elution, cinnamic acid (R_{cg} 0.04, 30% yield); 1-*O*-cinnamoylquinide (XI, R_{cg} 0.22, 10%); and 3-, 4-, 5-, and 1-*O*-cinnamoylquinic acids (X, XIII, XII, and V; R_{cg} 0.42, 0.51, 0.63, and 0.69; yields 23, 20, 9, and 7%). The separation of a mixture of the isomeric acids is illustrated in Figure 5.

The method for isolating the components of the reaction mixture and establishing their identities is given in the Experimental Section. Of the two new isomers, the substance with R_{cg} 0.51 did not react with periodate (Table I) and was therefore held to be blocked in the 4-position. The substance with R_{cg} 0.63 consumed one molar proportion of periodate. As the substance of R_{cg} 0.42 is the 3-isomer, this compound is the 5-isomer. When 1-*O*-cinnamoylquinic acid was treated with 1.5 equiv of barium hydroxide, a mixture of the four cinnamoylquinic acids, in which the 1- and 3-isomers predominated, was obtained.

The behavior of XI toward alkali appears to differ from that of 1-*O*-caffeoylquinide. Dilute potassium hydroxide on this compound gave 1-*O*-caffeoylquinic acid (Scarpati *et al.*, 1958). Scarpati and Esposito (1964) report that a caffeoyl group does not migrate from the 1-position to an appreciable extent under the alkaline conditions which cause *p*-coumaroyl and galloyl groups to migrate. Rapid migration between the 3-, 4-, and 5-positions does, however, take place. Deulofeu and his associates also report that the rate of alkali-catalyzed migration from the 1-position is relatively slow. The rate is of the same order as the rate of hydrolysis (Rúveda *et al.*, 1964a).

The Base-Catalyzed Isomerization Process

The above syntheses and proofs of structure do not rest on a knowledge of the route of cinnamoyl group wandering. The matter of base- and acid-catalyzed migrations, however, is of considerable importance in the chemistry of the acyl derivatives of quinic acid. It

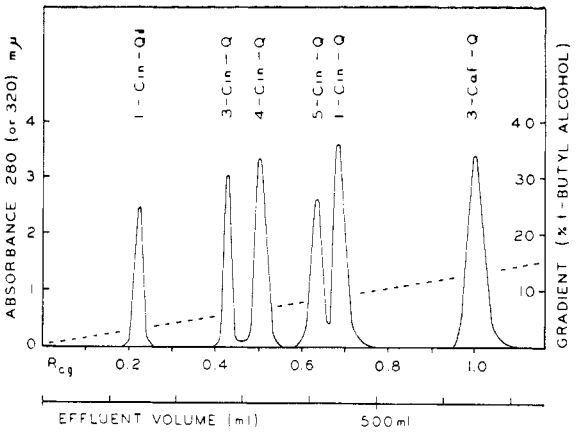

FIGURE 5: Separation of the mono-*O*-cinnamoylquinic acids on the analytical silica gel column (Hanson and Zucker, 1963). The standard gradient is indicated by a dashed line. The fraction volumes were 7 ml. Compounds in order of elution: 1-*O*-cinnamoylquinide (XI), 3-, 4-, 5-, and 1-*O*-cinnamoylquinic acids (X, XIII, XII, and V), 3-*O*-caffeoylquinic acid (chlorogenic acid).

is also possible that certain of the naturally occurring conjugates of quinic acid are formed from other isomers by enzymatic or nonenzymatic acyl migration. The following studies were therefore undertaken.

Inter- or Intramolecular Migrations? 1-*O*-Cinnamoylquinide (25 mg, 82 μmoles) was treated with barium hydroxide (83 μequiv) in the presence of [U-^{14}C]quinic acid (13,000 cpm, 1.3 μmoles) [the labeled compound was a gift of Dr. Weinstein (Weinstein *et al.*, 1962)]. The products of the reaction were separated on the analytical column. No detectable radioactivity appeared in the several ester fractions, and all of the recovered activity was associated with the quinic acid fraction. Free quinic acid is thus not an intermediate and, since there is no reason why trace quantities of di-*O*-cinnamoylquinic acids should act as intermediates, the base-catalyzed process is intramolecular.

1 → 5 or 1 → 3 Migrations? Two alternative routes for the migration of the cinnamoyl group from the 1-position may be considered. The simplest hypothesis is to suppose that the lactone gives rise to the 1-isomer, and that migration of the cinnamoyl group takes place in the sequence 1 ⇌ 5 ⇌ 4 ⇌ 3, as shown in Figure 4. If the several isomers exist in aqueous solution primarily as a mixture of two possible chair conformations in dynamic equilibrium (Hanson, 1962), then migration of the cinnamoyl group between the 1- and 5-positions, and also between the 3- and 4-positions, can take place only when the carboxyl group is equatorial. The nmr spectra of quinic acid and its four mono-*O*-caffeoyl esters show that the COOH-equatorial conformation is strongly favored in deuteriopyridine at 90° (Waiss *et al.*, 1964). In all probability, this is also the more favored conformation in aqueous solution.

The migration of acyl groups between proximate

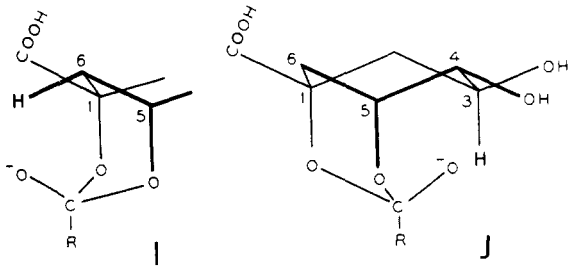

FIGURE 6: Structures for ortho acid diester ions formed as intermediates in the 1 → 5 migration of acyl groups. Structure J is sterically impossible.

hydroxyls is believed to involve the intermediate formation of an ortho acid diester or its ion (Fischer, 1920; Bender, 1951). The rate of this process is a function, among other things, of the steric arrangement of the hydroxyls and of the bulk of the groups involved. [A rate of base-catalyzed migration 40,000 times the rate of hydrolysis has recently been reported for an acyl migration between *cis*-hydroxyl groups on a five-membered ring (McLaughlin and Ingram, 1964).] It is therefore pertinent to examine the structures of the cyclic systems that may be involved in the migration of the cinnamoyl group about the quinic acid molecule.

Inspection of Dreiding Stereomodels reveals that an ortho ester bridge can be formed between the 1- and 5-positions, but only with distortion of bond angles. In the strainless ring (chair form) shown in partial formula J (Figure 6, R = styryl) the ionized oxygen of the ortho ester and the C-3 hydrogen are almost in the same position, which is impossible. In partial formula I, the strainless ring is in the boat conformation, and the ionized oxygen is 1.6 A from the C-6 hydrogen, *i.e.*, the van der Waals' radii overlap by about 1.0 A. The extent to which the radii overlap may be reduced by flattening and twisting the cyclohexane ring. The formation of ortho ester five-membered rings between the 3,4- and 4,5-positions of quinic acid also tends to deform the cyclohexane ring but to a smaller extent (less strain is involved in bridging *cis*-hydroxyl groups on a five-membered ring). Stereochemically, therefore, 1 ⇌ 5 and 3 ⇌ 4 ⇌ 5 migrations are similar, but it is impossible to predict relative migration rates. The search to find an example of acyl-group wandering analogous to the 1 ⇌ 5 migration under discussion has not been successful.

An alternative route for the migration of acyl groups from the 1-position has been proposed by Haslam *et al.* (1964). By analogy with the hydrolysis of acetylsalicylic acid in the pH range 2–8, it is assumed that, at pH 2 and above, the *O*-acyl group at C-1 is attacked by the nucleophilic carboxylate ion to form a mixed anhydride (Figure 3; F, G, and H) (Edwards, 1950; Bender *et al.*, 1958; Bender, 1960). The acyl group may then be hydrolyzed, returned to the C-1 hydroxyl, or, when the ring is in the less favored carboxyl-axial conformation, transferred to the C-3 hydroxyl.

The analogy with acetylsalicylic acid hydrolysis cannot be sustained in a satisfactory manner. The proposed mechanism for 1 → 3 migration requires that both the rate of hydrolysis of the anhydride, and the rate of direct hydrolysis of the acyl group, are slow compared to the rates of acyl migration between the carboxyl group and the C-1 and C-3 hydroxyl groups. The available information on acetylsalicylic acid hydrolysis suggests that the rate of hydrolysis of a quinic–cinnamic mixed anhydride would be fast compared to the other processes, *i.e.*, the observations that the rate of hydrolysis of acetylsalicylic acid is constant over the pH range 5–8, and that this process is not subject to general acid–base catalysis, are held to show that the rate of hydrolysis of the anhydride intermediate is very much faster than the steps leading to its formation (Bender *et al.*, 1958, and references therein). Also by analogy, the rate of direct hydrolysis of the cinnamoyl group is likely to be faster than the rate of anhydride formation at alkaline pH (*e.g.*, above pH 8).

In order to distinguish experimentally between the two hypotheses, three studies were carried out. In the first two, blocking groups were employed. In the third, the results of maintaining the *O*-cinnamoylquinic acids at 90° in buffers with the pH range 6–10 were examined.

BLOCKING CARBOXYL GROUP PARTICIPATION. Direct evidence that 1 → 5 migration may take place was obtained by treating methyl 1-*O*-cinnamoylquinate (Va, R_{cg} 0.39) with phosphate buffer, pH 7, 0.2 M, at 90° for 90 min (see below and Table III, line 12, for the effect of these conditions on the free acid). Only a small amount of hydrolysis of the ester linkages took place. The main products of the reaction (85% yield) were a mixture of methyl esters (R_{cg} 0.24–0.39, zero charge on electrophoresis). The only other detectable products were cinnamic acid (2%), and the 4 , 5-, and 1-*O*-cinnamoylquinic acids (4, 4, and 5%). On briefly treating milligram quantities of the 3-, 4-, and 5-*O*-cinnamoylquinic acids with diazomethane, compounds with R_{cg} values in the region 0.24–0.39 were obtained. The amounts of the methyl esters of the (3- and 4-), 5-, and 1-isomers produced in the isomerization reaction were approximately as 20:25:39% (order of elution). The rate of migration from the 1-position appeared to be somewhat faster for the methyl ester than for the free acid.

BLOCKING THE 4- and 5-HYDROXYL GROUPS. Evidence that 1 → 3 migration does not take place was obtained by studying the action of barium hydroxide on 1-*O*-cinnamoyl-4,5-*O*-isopropylidenequinide (II) in greater detail (Figure 1). If a 1 → 3 migration can occur, then barium 3-*O*-cinnamoyl-4,5-*O*-isopropylidenequinate should be present in the residues from the preparation of the corresponding 1-isomer (III). The residues from a typical preparation were therefore treated with Dowex 50-H^+, and the acidic solution obtained was heated to remove the attached isopropylidene groups. As this process was essentially quantitative for the preparation of 1-*O*-cinnamoylquinic acid, it should also be quantitative for the preparation of 3-*O*-cinnamoylquinic acid. The products from this treatment were examined with the analytical column. The major peak in the elution

TABLE III: Base-Catalyzed Isomerization of the Mono-*O*-cinnamoylquinic Acids.

Line	Buffer (0.2 M)[a]	Time at 90° (min)	Treated Isomer[b]	Isomers Produced[c] (%) 1-	3-	4-	5-	Unidentified Peaks[d] (%)	Cinnamic Acid (%)
1	A, pH 7.0	3	3-	—	72	16	8	3	1
2	A, pH 7.0	9	3-	—	42	25	22	9	2
3[e]	A, pH 7.0	30	3-	—	28	25	39	—	8
4[e]	A, pH 7.0	90	3-	—	28	25	36	—	11
5[e]	A, pH 7.0	90	4-	—	23	34	37	—	6
6	A, pH 7.0	3	5-	—	8	35	56	0.8	0.2
7[e]	A, pH 7.0	90	5-	—	30	29	37	—	4
8	A, pH 6.1	9	3-	—	82	12	4	1.1	0.9
9	B, pH 7.0	90	3-	—	93.8	6.1	0.1	—	—
10	A, pH 6.1	90	1-	97.7	0.5	0.7	1.0	—	0.6
11	A, pH 6.7	90	1-	86	2	3	5	—	4
12[e]	A, pH 7.0	90	1-	72	7	7	10	—	4
13[e]	A, pH 7.8	90	1-	42	14	12	18	8	6
14	B, pH 7.0	90	1-	99.4	—	0.1	0.2	—	0.3
15	C, pH 8.5	90	1-	80	5	5.1	8	—	1.6
16[e]	C, pH 9.0	90	1-	70	7	6	10	—	7
17[e]	C, pH 9.75	90	1-	39	11	10	10	—	30

[a] A = phosphate (K^+), B = Tris (Cl^-), C = glycine (K^+). The pH at 90° was not measured, but for phosphate buffer 0.025 M with a pH of 6.88 at 20°, the pH is 6.89 at 95° (Bates, 1964). Much larger changes in the pH of Tris and glycine buffers with temperature are likely (Bates and Pinching, 1949). [b] The compound indicated (10 mg or less) was brought to the desired pH in the appropriate buffer at 21°. The pH was unchanged after the solution had been heated to 90° and cooled. [c] A dash indicates that the compound was undetectable or, in the case of the 1- and 5-isomers, that the presence of a trace of the 1-isomer cannot be detected in the presence of an excess of the 5 isomer. Some uncertainty exists as to the relative amounts of the 1- and 5-isomers in lines 10 to 17. [d] The nature of two small peaks observed in the region R_{cg} 0.2–0.3 has not been investigated. [e] The equilibrium mixture 3-:4-:5-*O*-cinnamoylquinic acids contain these isomers in ratios close to 1:0.9:1.3. For the *p*-coumaroyl and caffeoyl series, ratios close to 1:1.15:1.5 were obtained (90 min at 90°, pH 7, 5% hydrolysis).

record corresponded to 1-*O*-cinnamoylquinic acid, indicating that the initial yield of III was about 80%. The compound was crystallized and its identity was confirmed. In addition to cinnamic acid (yield 14%) and 1-*O*-cinnamoylquinide (1%) some unknown material, R_{cg} 0.10 (3%), was detected. A peak was not observed in the 3-*O*-cinnamoylquinic acid region (yield less than 0.02%).

EFFECTS OF pH. The above results fail to establish conclusively that the anhydride route is not followed for the *O*-cinnamoylquinic acids themselves. The introduction of an isopropylidene or a methyl group may affect the equilibrium between the two chair conformations of quinic acid and therefore affect migration rates. A study of the migration of the cinnamoyl group under various conditions of pH (6–10) and in various buffers gives results which cannot easily be explained in terms of the anhydride route. The slowness of the analytical method precluded a detailed kinetic investigation, but the following conclusions may be drawn from Table III.

(i) The rate of migration to the 1-position is very slow (undetectable) compared to the rates of 3 $\rightleftharpoons$ 4 $\rightleftharpoons$ 5 migration (lines 1–9). These rates, however, are fast compared to the rates of hydrolysis (last column, Table III). Similarly the rate of migration from the 1-position is slow compared to the rates of the 3 $\rightleftharpoons$ 4 $\rightleftharpoons$ 5 migrations (lines 10–17). A difference between the 1 $\rightleftharpoons$ 5 and 3 $\rightleftharpoons$ 4 $\rightleftharpoons$ 5 rates is entirely reasonable on steric grounds. The difference in the 1 $\rightarrow$ 5 and 5 $\rightarrow$ 1 rates (lines 12 and 6) may be interpreted in terms of a difference in the standard free energies of the two isomers. The equilibrium, if measurable, would strongly favor the 5-isomer. In contrast the equilibria for the 5 $\rightleftharpoons$ 4 and 4 $\rightleftharpoons$ 3 reactions are of the order of unity (Table III, footnote *e*). Appreciable migration to the 1-position takes place if the 1-isomer is removed by lactonization as it is formed; *i.e.*, when the 3-, 4-, or 5-isomers are heated, or are treated with acid, 1-*O*-cinnamoylquinide is present in the mixture of products (Table II).

(ii) Under conditions where the 3-, 4-, and 5-isomers are still far from equilibrium, the amounts of these isomers formed from the 1-isomer are 5- > 4- > 3- (lines 10, 11, and 14). This result would be expected if the migration sequence is 1 $\rightarrow$ 5 $\rightarrow$ 4 $\rightarrow$ 3 and the rates of 5 $\rightleftharpoons$ 4 $\rightleftharpoons$ 3 migration are approximately equal (lines 6, 1, and 2).

(iii) The rate of migration from the 1-position (lines

10 to 13, 15 to 17), like the rates of 3 ⇌ 4 ⇌ 5 migration (lines 8 and 2), is pH dependent. If attack by the carboxylate ion on the C-1 substituent were the rate-determining step, the rate of migration from the 1-position would be pH independent (see Bender *et al.*, 1958).

(iv) The rate of migration from the 1-position, like the rates of 3 ⇌ 4 ⇌ 5 migration, appears to be a function of the buffer employed. A reservation is necessary on this point since the pH at 90° for the Tris and glycine buffers may differ appreciably from the pH at 22° (Bates and Pinching, 1949). For the 3 ⇌ 4 migration, phosphate buffer is about 250-fold more effective than Tris buffer (lines 1 and 9). For the migration from the 1-position, phosphate buffer is about 100-fold more effective than Tris buffer (lines 12 and 14) and is also more efficient than glycine buffer (lines 10 to 13 compared with 15 to 17). Again, if carboxylate ion attack were the rate-determining step, the reaction would not be subject to general-base catalysis (see Bender *et al.*, 1958).

It remains to discuss the observations of Haslem *et al.* (1964) cited to support the proposal that 1 → 3 migrations occur. The predominance of the 3-isomer in the products from heating 1-*O*-*p*-coumaroylquinic acid with sodium hydrogen carbonate solution could arise if the 4- and 5-isomers are hydrolyzed more rapidly than the 3-isomer. The formation of methyl *p*-coumarate, when the above compound is treated with aqueous methanol and barium hydroxide, may indicate that alcoholysis takes place more rapidly than hydrolysis (Bender and Glasson, 1959). In the absence of any other evidence, the possibility that 1 → 3 migration occurs to a significant extent under neutral or basic conditions may be disregarded.

Acid-Catalyzed Migrations. Although a detailed study of acid-catalyzed migrations has not been made, it is probable that the rate of migration first decreases as shown in Table III and then increases with decreasing pH. A minimal rate of migration in the region of pH 4–5 is reasonable (see the control experiments for the synthesis of the 1- and the 3-isomers). Samples of cinnamoyl and caffeoyl conjugates obtained from the analytical column in acid-equilibrated *t*-butyl alcohol–chloroform solution have been stored in this mixture for 6 months at −10° without any sign that isomerization or hydrolysis had taken place.

Acyl Group Wandering in Plant Tissues. The above studies raise the possibility that the mixtures of isomeric hydroxycinnamoyl conjugates of quinic acid found in certain tissues may arise by the enzymatic or nonenzymatic migration of their acyl groups (Sondheimer, 1958; Zucker *et al.*, 1965). As acidic conditions are normally maintained during extraction and analysis, these mixtures are not artifacts of the isolation procedure. For each set of acyl derivatives of quinic acid studied (cinnamoyl, *p*-coumaroyl, and caffeoyl) the ratio at equilibrium of the 3-, 4-, and 5-isomers is close to 1:1:1.3 (Table III, footnote *e*), and therefore the preponderance of a particular hydroxycinnamoyl isomer must be explained in terms of enzyme specificity, either of synthesis or of further metabolism. In the potato tuber, acyl migration, whether enzymatic or nonenzymatic, is apparently not important; only the 3-isomers are observed.

3-O-Cinnamoylquinic Acid as a Biosynthetic Intermediate. Experiments, to be reported elsewhere, in which potato tuber slices were maintained on [α-^{14}C]-cinnamic acid and synthetic 3-*O*-cinnamoylquinic acid (pH adjusted to 6), showed that very little radioactivity was incorporated into the added conjugate although extensive incorporation into 3-*O*-*p*-coumaroyl- and 3-*O*-caffeoylquinic acids took place. If this conjugate is an intermediate, then either the active pool of the compound is very small and is contained within a cell compartment whose membrane is relatively permeable to phenylalanine and cinnamic acid but not to the conjugate itself, or the compound is an enzyme-bound intermediate.

Experimental Section

Carbon, hydrogen, and molecular weight determinations were performed by Schwarzkopf Microanalytical Laboratories. Infrared absorption spectra of samples as Nujol mulls were determined with a Perkin-Elmer Model 21 spectrophotometer. Ultraviolet absorption spectra were recorded with a Cary Model 11 spectrophotometer, but measurements at single wavelengths were made with a Beckman D.U. instrument. Melting points were determined with a Fisher-Johns apparatus. Radioactivity was measured with a Nuclear-Chicago 720 liquid scintillation counter. Solutions were concentrated in a rotary evaporator with the bath temperature at 30 or 40°. The petroleum ether fraction employed for recrystallizations etc. had bp 30–60°.

Partition Chromatography on Silica Gel. The analytical silica gel column (17 × 0.8 cm) was prepared from 7 g of dried gel and 3.5 ml of 0.5 N sulfuric acid as described previously (Hanson and Zucker, 1963). The column was developed under the standard conditions (rectilinear gradient: mixing vessel, 800 ml of acid-equilibrated cyclohexane–chloroform (10:90 by volume); reservoir, 800 ml of acid-equilibrated *t*-butyl alcohol–chloroform (30:70 by volume). The apparatus previously described was modified as follows. In place of polyethylene catheter tubing, Teflon tubing (Pennsylvania Fluorocarbon, Philadelphia, Pa.; A.W.G. 15, 0.059 in. id) was used between the column and the monitor and between the monitor and the fraction collector. The junction to the column was made by threading the tubing into a hole bored in a piece of Teflon rod and joining the rod to the column by a Teflon Tube Fitting (Beckman, size 408). The junction to the quartz cell of the recorder was made by threading the tubing into a similarly bored Teflon rod the same size as the cell entrance and exit tubes. The rod was joined to the cell by Teflon tubing (A.W.G. 9; 0.118 in. id). In this way contamination by ultraviolet light absorbing materials from the polyethylene was eliminated. Teflon tubing and tube fittings were also used to

eliminate greased ground-glass joints from the apparatus.

For preparative purposes a silica gel column (44 × 4 cm, in a 60-cm glass tube), consisting of 350 g of dried gel to which 150 ml of 0.5 N sulfuric acid had been added, was prepared with chloroform as the suspending medium. A flow rate of approximately 240 ml/hr was maintained with a Micropump constructed of Teflon and glass (Buchler Instruments). For the pump valves to operate, it was necessary to place the large gradient device below the pump and the column above the pump. The pump, the column, and the mixing vessel were connected by Teflon tubing (A.W.G. 9) and tube fittings (size 500). The column was developed with 1.5 l. of acid-equilibrated chloroform, followed by 12 further l. of acid-equilibrated chloroform, followed by 12 further l. of solvent with a rectilinearly increasing concentration of *t*-butyl alcohol (2% increase in concentration/l.).

Paper Chromatography. For purposes of comparison R_F values for various related conjugates encountered in plant tissues are given: ascending on Whatman No. 1 (Hanson and Zucker, 1963), descending on Whatman 3 mm paper (Levy and Zucker, 1960). The order of citation of R_F values is cinnamic, *p*-coumaric, and caffeic acids; Cin conj (cinnamoyl conjugates): 1-*O*-cinnamoylquinide, 1-, 3-, 4-, and 5-*O*-cinnamoylquinic acids; 1-*O*-cinnamoyl-β-D-glucose; *p*-Cou conj (*p*-coumaroyl conjugates): 3-*O*-*p*-coumaroylquinic acid; 1-*O*-*p*-coumaroyl-β-D-glucose; 3-Caf-Q (3-*O*-caffeoylquinic acid).

System A (*n*-butyl alcohol–acetic acid–water, 4:1:5 by volume, upper phase): ascending: 0.93, 0.90, 0.82; Cin conj: 0.91, 0.79, 0.81, 0.81, 0.81, 0.79; *p*-Cou conj: 0.78, 0.72; 3-Caf-Q: 0.62; descending: similar but less resolution.

System B (acetic acid, 5%): ascending: 0.58, 0.40, 0.29; Cin conj: 0.67, 0.84, 0.71, 0.73, 0.80, 0.78; *p*-Cou conj: 0.65, 0.74; 3-Caf-Q: 0.58; descending: similar but less resolution.

System D [*n*-butyl alcohol–ammonium carbonate solution (0.8 M)–ammonium hydroxide solution (1.5 N), 2:1:1, upper phase]: ascending: 0.45, 0.18, —; Cin conj: 0.83, 0.25, 0.33, 0.33, 0.33, 0.70; *p*-Cou conj: 0.14, 0.40; descending: 0.48, 0.24, —; Cin conj: 0.88, 0.26, 0.36, 0.36, 0.36, 0.74; *p*-Cou conj: 0.15, 0.45. A sample of 3-*O*-cinnamoylquinic acid was chromatographed in this system overnight and then rechromatographed on the analytical silica gel column; migration of the cinnamoyl group was found to have occurred (3-:4-:5-isomers as 1:1:0.8). Similar migrations may be assumed to occur in the *O*-*p*-coumaroylquinic acid series.

Ultraviolet Absorption Spectra. The several cinnamoyl esters prepared had essentially the same spectra. A slight difference however was noted between the 1-*O*-cinnamoyl derivatives of quinide and the other compounds. Compounds II and XI: λ_{max} (95% ethanol) 218, 224, 282 mμ (ε 14,800, 13,300, 24,200); λ_{min} 211, 222, 234 mμ (ε 11,000, 12,900, 1800). Compounds IV, V, Va, X, IXa, IXb, XII, and XIII: λ_{max} (95% ethanol) 217, 223, 280 mμ (ε 15,400, 13,400, 22,800); λ_{min} 210, 221, 232 mμ (ε 12,100, 12,900, 2000).

Optical Rotatory Dispersion Curves.[1] The curve for quinic acid in methanol (*c* 0.292) showed a negative Cotton effect with a trough at 250 mμ attributable to the carbonyl of the carboxyl group: [φ] (at 500 mμ) −70, (400) −110, (286) −220, (250) −255, (222) −150, (213) 0°. The curves for the mono-*O* cinnamoylquinic acids (*c* 0.273, methanol) are not simple. Accurate measurements below 300 mμ were impossible owing to the intensity of the 280-mμ absorption band. For D line rotations in ethanol see Table I; 1-*O*-cinnamoylquinic acid: $[\phi]_D$ +21.8° (*c* 2.00), [φ] (at 580 mμ) *ca.* 0, (500) −175, (400) −345, (300) −2660°; 3-*O*-cinnamoylquinic acid: [φ] (at 500 mμ) −250, (400) −335 (trough), (321) 0, (310) +235°; 4-*O*-cinnamoylquinic acid: [φ] (at 500 mμ) − 210, (400) − 335, (303) − 1130°; 5-*O*-cinnamoylquinic acid: [φ] (at 520 mμ) *ca.* 0, (500) +10, (400) +140, (323) +945°.

The relationship between the ORD curves of carboxylic acids and their chemical structures has not been extensively investigated (Jennings and Klyne, 1963). An interpretation of the above curves is not possible at the present time.

Synthesis of 1-O-Cinnamoylquinic Acid

4,5-O-Isopropylidenequinide (I). To a suspension of quinic acid (100 g, 0.52 mole) in pure dry acetone (3 l.), concentrated sulfuric acid (10 ml) was added and the reaction mixture was stirred at room temperature for 2 days. The solution was filtered and the filtrate was neutralized with pyridine. The oily layer which separated was removed, and the acetone solution was concentrated. More oily material separated and was removed before the solution reached the crystallization point. The solid was washed with petroleum ether containing a little acetone and recrystallized from acetone to give long needles of I: mp 138–141° [lit. (Fischer, 1921) mp 140–141°]; 61.4 g, 55% yield; ν_{max} 3450 sh, 3360 (OH), 1770 (lactone C=O) cm^{-1}.

1-O-Cinnamoyl-4,5-O-isopropylidenequinide (II). Cinnamoyl chloride (25 g, 0.15 mole) and I (21.4 g, 0.10 mole) were heated under reduced pressure at oil-bath temperature (110°) until the heat given out by the reaction was sufficient to maintain the evolution of hydrogen chloride. After the reaction mixture solidified (10 min), the temperature was maintained at 110° for a further 20 min. The solid was triturated with ether and filtered (31 g, mp 180–185°). II is readily soluble in acetone, ethyl acetate, chloroform, and benzene; moderately soluble in ethanol; and has little solubility in water, ether, or petroleum ether. Recrystallization from acetone gave long needles of II: 27.6 g, 80% yield; mp 189° (not further raised by sublimation *in vacuo* at 180°); [lit. (Josephson, 1928) mp 189°]; $[\phi]_D^{24}$ + 24° (*c* 2.00, chloroform); ν_{max} 1800 (lactone C=O), 1710

[1] The author is indebted to Professor W. Klyne, University of London, for these determinations.

(cinnamoyl C=O), 1640 (conj C=C), 1570 vw (aromatic C=C) cm^{-1}.

Anal. Calcd for $C_{19}H_{20}O_8$ (344.4): C, 66.27; H, 5.85. Found: C, 66.2; H, 5.62.

1-O-Cinnamoyl-4,5-O-isopropylidenequinic Acid Monohydrate (IV). To a solution at 30° of II (3.44 g, 0.01 mole) in dioxane (40 ml), barium hydroxide solution (0.466 N, 0.01 equiv) was added portionwise with stirring. The reaction mixture was allowed to clarify before each further addition. When all the alkali had been added, the solution was concentrated to near dryness and the resulting crystalline solid was collected and washed with acetone, chloroform, and ether (2.9 g, mp 220°). Recrystallization from ethanol–water gave small needles of barium 1-*O*-cinnamoyl-4,5-*O*-isopropylidinequinate monohydrate (III) (2.90 g, 66% yield), mp 225°. III dissolved slowly in hot 95% ethanol but failed to reprecipitate when the ethanol solution was concentrated. On adding water, crystals formed at once. The tendency of III to dissolve in organic solvents suggests that it is a chelation compound.

Anal. Calcd for $C_{38}H_{42}O_{14}Ba \cdot H_2O$ (878.1): C, 51.97; H, 5.05; Ba, 15.64. Found: C, 52.3; H, 4.99; Ba, 15.6.

No loss in weight occurred on drying at 130° *in vacuo* over P_2O_5 for 5 hr.

A solution of III (2 g, 0.00456 equiv) in 80% aqueous ethanol at 4° was passed through a short column of Dowex 50-H^+ (0.012 equiv). The eluate on being concentrated gave needles of IV, mp 90–95° (1.29 g). Since the isopropylidene group is readily removed from IV, considerable losses occurred when recrystallization from hot solvents was attempted. Recrystallization by adding water to ethanol solutions, or petroleum ether to ethyl acetate or acetone solutions at room temperature, gave needles of IV: mp 104°; $[\phi]_D^{24}$ −74.3° (*c* 2.00, 95% ethanol); ν_{max} 3320, 3360 (OH), 1720 (ester C=O), 1695 (carboxyl C=O), 1632 (conj C=C) cm^{-1}.

Anal. Calcd for $C_{19}H_{22}O_7 \cdot H_2O$ (380.4): C, 59.99; H, 6.36. Found: C, 59.8; H, 6.39.

1-O-Cinnamoylquinic Acid (V). An aqueous solution of IV, obtained from III (2 g, 0.00227 mole) as described above, was heated at 100° for 3 hr. On being concentrated, crude V separated (1.37 g, mp 190–192°, yield 95%). V is readily soluble in chloroform or hot ethanol; slightly soluble in acetone or water; and insoluble in ethyl acetate, ether, or petroleum ether. Recrystallization from aqueous ethanol and ethyl acetate–ethanol gave short prisms of V: mp 195° (gas evolution takes place at temperatures slightly above the melting point) [lit. (Josephson, 1928) mp 188°; the structure for quinic acid assumed by Josephson is now known to be incorrect]; $[\phi]_D^{24}$ +18.0° (*c* 2.00, 95% ethanol) [lit. (Josephson, 1928) $[\phi]_D^{20}$ +19.0° (*c* 2.00, 95% ethanol)]; ν_{max} 3380, 3240 (OH), 1715 (carboxyl C=O), 1695 (cinnamoyl C=O), 1635 (conj C=C), 1590 w, 1568 w (aromatic C=C) cm^{-1}; see also Table I.

Anal. Calcd for $C_{16}H_{18}O_7$ (322.3): C, 59.62; H, 5.63. Found: C, 59.4; H, 5.61; mol wt (ethanol vapor pressure osmometer), 290; equiv wt (CO_2 from $NaHCO_3$ in Warburg respirometer), 320.

When V (450 mg) in ethanol was treated with excess diazomethane and the product was repeatedly recrystallized from aqueous ethanol, a small amount (50 mg) of the methyl ester Va was obtained, mp 130°, $[\phi]_D^{23}$ +65.5° (*c* 1.36, 95% ethanol). Diazomethane is known to attack the double bond of cinnamoyl esters and form heterocyclic compounds (Pechmann and Burkard, 1900). The low yield is attributed to the effect of this side reaction. Esterification may be performed on a microscale in a quantitative manner (see above section on "1 → 3 or 1 → 5 migrations?") if the exposure to diazomethane is brief.

Anal. Calcd for $C_{17}H_{20}O_7$: C, 60.71; H, 5.99. Found: C, 60.8; H, 5.79.

Effect of Heat on V and Its Isomers. When V (100 mg) was heated at 200° for 5 min under reduced pressure, evolution of gas (water vapor) took place. A small amount of 1-*O*-cinnamoylquinide (XI), mp 160–164° (p unchanged on admixture with authentic material), was obtained on recrystallization from ethanol. In a similar experiment with 3 mg of V the reaction products were examined with the analytical silica gel column. In addition to XI and cinnamic acid a small amount of a second lactone was formed on heating (zero charge on electrophoresis at pH 7, R_{cg} 0.31), possibly 5-*O*-cinnamoylquinide. When 5-*O*-cinnamoylquinic acid (XII) was heated in the same way, the same two compounds were formed apparently in roughly equal amounts, in addition to trace amounts of the 3-, 4- and 5-*O*-cinnamoylquinic acids. 3-*O*-Cinnamoylquinic acid (X) when heated likewise gave a mixture of lactones and acids, but the starting material predominated.

Synthesis of 3-O-Cinnamoylquinic Acid

1-O-Ethoxycarbonyl-4,5-O-isopropylidenequinic Acid (VII). Attempts to repeat the preparation of VII described by Panizzi *et al.* (1956) were unsuccessful, and an alternative procedure was therefore developed. Haslam *et al.* (1961) have also reported an improved preparation of this compound.

1-*O*-Ethoxycarbonyl-4,5-*O*-isopropylidenequinide (VI) was synthesized from I by the method of Josephson (1928) in a yield of 85%. To a solution of VI (14.3 g, 0.05 mole) in methanol (140 ml) containing phenolphthalein as an indicator at 50°, barium hydroxide solution (0.5 N, 0.05 equiv) was added with agitation. As soon as the indicator color disappeared (3.5 min) the reaction mixture was brought to 0°. The solution was concentrated and the barium carbonate removed by centrifugation. The aqueous solution was extracted with three 100-ml portions of chloroform, the chloroform solution was washed with water (50 ml), and the aqueous solution and wash fluid were combined (it was convenient in some experiments to store the aqueous solution at this point at −10°). The solution was then passed at 0° through a Dowex 50-H^+ column (25 ml) and at once extracted with ethyl acetate previously cooled to −10° (200, 100, and 100 ml). The extracts were dried for 1 hr over sodium sulfate, combined, and concentrated to give crystals of VII (6.61 g). Material once recrystallized from ethyl acetate was suitable for the next stage of the synthesis; 6.4 g, mp

150–153° [lit. (Panizzi *et al.*, 1956) mp 150–152°]; 30% yield.

The temperature of 50° for the initial reactions was established as optimal after the reaction had been performed on 0.005-equiv quantities at temperatures from 0 to 100°. Temperatures of 70 and 35° gave only slightly less material than at 50°. No attempt was made to determine the optimum amount of barium hydroxide required for the reaction.

Diphenylmethyl 3-O-Cinnamoyl-1-O-ethoxycarbonyl-4,5-O-isopropylidenequinate (IX). Diphenylmethyl 1-*O*-ethoxycarbonyl-4,5-*O*-isopropylidenequinate (VIII) was prepared from VII and diphenyldiazomethane, as described by Haslam *et al.* (1961), in 85% yield. To VIII (2 g, 0.00425 mole) in pyridine (0.65 ml, 0.008 mole), cinnamoyl chloride (0.8 g, 0.0048 mole) in chloroform (6 ml) was added and the reaction mixture left at 25° for 2 days. The solution was then heated under reflux for 1 hr, methanol (3 ml) was added, and heating was continued for 1 hr. It was then taken to dryness and the solid was triturated with petroleum ether. Recrystallization from methyl alcohol–acetone and benzene–petroleum ether gave a mat of fine needles of IX (2.13 g, 84% yield): mp 151°; $[\phi]_D^{24}$ −124° (*c* 0.40, 95% ethanol); λ_{max} (95% ethanol) 280 mμ (ε 23,300); λ_{min} 235 mμ (ε 3250); ν_{max} 1741 (covalent carbonate and ester C=O), 1707 (cinnamoyl C=O), 1638 (conj C=C) cm^{-1}.

Anal. Calcd for $C_{35}H_{36}O_9$ (600.7): C, 69.99; H, 6.04. Found: C, 69.9; H, 5.87.

3-O-Cinnamoylquinic Acid (X). To IX (3.6 g, 0.006 mole) in hot glacial acetic acid (10 ml), water (2.5 ml) was added and the reaction mixture was heated under reflux for 8 hr (these are the conditions employed by Haslam *et al.*, 1961). The solution was then concentrated. Silica gel (7 g) was added to the resultant gum and the mixture was ground to give a free-flowing powder. The powder was added in chloroform suspension to the preparative column, and the column was developed as described above. The fractions corresponding to the front, back, and middle of the major peak were separately combined and each set was concentrated to dryness. In each case recrystallization from aqueous ethanol gave short needles of X (total yield 0.89 g, 46%, mp 146°). Recrystallization from ethyl acetate–petroleum ether gave prisms, mp 166°, on occasion. Recrystallization from aqueous ethanol of the material mp 166° always gave the form of mp 146°: ν_{max} 3460 and 3400 sharp, 3220 broad (OH); form with mp 165°: 3430 and 3200 broad (OH); both forms: 1710 (carboxyl and cinnamoyl C=O), 1630 (conj C=C), 1568 w (aromatic C=C) cm^{-1}; see also Table I.

Anal. Calcd for $C_{16}H_{18}O_7$ (322.3): C, 59.62, H, 5.63. Found: sample mp 146°, C, 59.6, H, 5.78; mol wt (Rast), 309; equiv wt (CO_2 from $NaHCO_3$ in Warburg respirometer), 319.

The above conditions of hydrolysis may not be optimal for preparative purposes (see Results and Table II). A pilot experiment of the above type (40-mg scale), in which the reaction mixture was applied to the analytical silica gel column, gave an elution record with seven peaks of which three minor peaks were in the region associated with the mono-*O*-cinnamoylquinic acids. Presumably these compounds and their lactones are produced from 3-*O*-cinnamoylquinic acid during the prolonged acid treatment.

Methyl 3-O-Cinnamoyl-1-O-ethoxycarbonyl-4,5-O-isopropylidenequinate (IXa). Diazomethane in ether was added to solid VII (6.08 g, 0.020 mole) until no further evolution of nitrogen took place. The solution was filtered to remove undissolved material and concentrated, and the resultant gum was dissolved in dry chloroform (25 ml). Cinnamoyl chloride (4 g, 0.025 mole) and pyridine (4 ml, 0.049 mole) were added and the reaction mixture was left in the dark for 4 days at room temperature. Ethanol (10 ml) was then added and after 30 min the bulk of the solvent was removed. On adding ethanol to the gum, colorless needles of crude IXa separated (8.1 g, mp 105–110°). This was recrystallized from ethanol (7.0 g, mp 110–113°, 78% yield). The substance is readily soluble in acetone, ethyl acetate, ether, chloroform, and benzene; moderately soluble in ethanol; and insoluble in water and petroleum ether. Further recrystallization gave IXa: mp 113°; $[\phi]_D^{21}$ −106° (*c* 2.00, chloroform); $[\phi]_D^{23}$ −93° (*c* 2.00, 95% ethanol); ν_{max} 1750 (covalent carbonate), 1735 (ester C=O), 1712 (cinnamoyl C=O), 1640 (conj C=C), 1575 w (aromatic C=C) cm^{-1}.

Anal. Calcd for $C_{23}H_{28}O_9$ (448.5): C, 61.60; H, 6.29. Found: C, 61.6; H, 6.53.

Methyl 3-O-Cinnamoyl-1-O-ethoxycarbonylquinate (IXb). Compound IXa (4.5 g, 0.01 mole) in aqueous acetic acid (8 ml, 50% by volume) was heated for 3 hr at 100°. The reaction mixture was then diluted with water (4 ml) and the solution allowed to cool. Crude IXb separated (3.6 g, mp 149°, 88% yield). The substance is readily soluble in acetone, ethyl acetate, dioxane, and ether; moderately soluble in ethanol and benzene; and insoluble in water and petroleum ether. Recrystallization from ethanol–petroleum ether or ethyl acetate–petroleum ether gave thick prisms of IXb: mp 150°; $[\phi]_D^{21}$ +132° (*c* 2.00, chloroform); $[\phi]_D^{23}$ +64° (*c* 2.00, 95% ethanol); ν_{max} 3500, 3480 (OH), 1750 (covalent carbonate C=O), 1735 (ester C=O), 1698 (cinnamoyl C=O), 1635 (conj C=C) (aromatic 1575 band absent) cm^{-1}.

Anal. Calcd for $C_{20}H_{24}O_9$ (408.4): C, 58.82; H, 5.92. Found: C, 59.0; H, 5.85; equiv wt (from alkoxyl determination), 201.

Synthesis of the 4- and 5-O-Cinnamoylquinic Acids

1-O-Cinnamoylquinide (XI). To a solution of II (10 g, 0.029 mole) in glacial acetic acid (40 ml) at 100°, water (6 ml) was added, and the reaction mixture was heated at 100° for 3 hr. Water (160 ml) was then added to the hot reaction mixture and the resulting cloudy solution was chilled at 4° overnight. Large flaky crystals of crude XI were collected and washed with sodium hydrogen carbonate solution (5%) and with water (8.24 g, mp 159–166°). The product was recrystallized from ethanol (5.4 g, mp 165–167°, 61% yield). It is readily soluble in ethanol, acetone, and ethyl acetate; mod-

erately soluble in chloroform; and insoluble in water, benzene, and petroleum ether. Further recrystallization gave hexagonal plates of XI: mp 167° [lit. (Josephson, 1928) mp 165°]; $[\phi]_D^{21}$ −75° (*c* 0.50, chloroform); $[\phi]_D^{23}$ −59° (*c* 2.00, 95% ethanol) [lit. (Josephson, 1928) $[\phi]_D^{20}$ −56° (*c* 2.00, acetone)]; ν_{max} 3390, 3350 (OH), 1780 (lactone C=O), 1712 (cinnamoyl C=O), 1631 (conj C=C), 1575 w (aromatic C=C) cm^{-1}.

Anal. Calcd for $C_{16}H_{16}O_6$ (304.3): C, 63.15; H, 5.30. Found: C, 63.3; H, 5.45.

4- and 5-O-Cinnamoylquinic Acids (XIII and XII) from 1-O-Cinnamoylquinide (XI). Initial experiments were carried out on 100-mg samples of XI and the products of the reaction were examined with the aid of the analytical silica gel column as described in the Results section. For preparative purposes the scale was increased 50-fold. To a solution of XI (5 g, 0.0164 mole) in dioxane (40 ml) at 40°, barium hydroxide solution (4.584 N, 0.0164 equiv) was added, and the reaction mixture was maintained at 40° for 15 min. The reaction mixture was then concentrated to one-third of its volume, sufficient 5 N sulfuric acid was added to precipitate all of the barium, and the suspension was further concentrated to give a sticky gum mixed with solid. Dried silica gel (20 g) was added to the gum, the mixture was ground to give a free-flowing powder which was added in chloroform suspension to the preparative column, and the column was developed as described above. The ultraviolet absorption record at 280 mμ of the effluent fractions showed six major peaks and two very minor peaks as in the small-scale experiments. The resolution was less satisfactory, but the relative amounts of material in the various peaks were unchanged. The recovery of material, assuming an average extinction coefficient of 23,000, was 93%. The peak substances were, in order of elution, cinnamic acid, starting material (XI), and 3-, 4-, 5-, and 1-*O*-cinnamoylquinic acids (X, XIII, XII, and V). The two very small peaks falling between the XI and X peaks (R_{cg} 0.30 and 0.35) were not investigated. Fractions for concentration were selected with a view to obtaining the least amount of contamination from the adjacent peak substances. Although solid was readily obtained corresponding to each of the cinnamoylquinic acid peaks, recrystallization of the material was much more difficult than in the case of the more highly substituted compounds. The presence of isomeric material was no barrier to crystallization, but well-formed crystals were only obtained with the pure compounds. The identities of the major peak substances were established as follows. All components except the second migrated toward the anode on electrophoresis at pH 7. The ratio of cinnamic acid to quinic acid in each component except the first was close to 1:1 (for method see Hanson and Zucker, 1963). The R_{cg} values for the first three components and the last corresponded to the R_{cg} values of known compounds. The identities of these components were confirmed by means of melting points, mixture melting points, and infrared spectroscopy. The remaining components were considered to be the 4- and 5-*O*-cinnamoylquinic acids on the basis of their various properties, including their reactivity toward periodate (Table I). The amounts of the *O*-cinnamoylquinic acids obtained were: 3-isomer, 800 mg, mp 135–145°, raised to 146° on recrystallization from aqueous ethanol; 4-, 290 mg, mp 154–155°; 5-, 184 mg, mp 175–185°; and 1-, 335 mg, mp 189–192°, raised to 194° on recrystallization from ethanol–ethyl acetate.

Recrystallization of the crude 4-isomer (XIII) from acetone–ethyl acetate gave small plates: mp 157°; ν_{max} 3430 and 3340 sharp, 3180 broader (OH), 1770 w (this unexplained band does not appear to arise from contaminating lactone), 1736 (carboxyl C=O), 1674 (cinnamoyl C=O), 1628 w (conj C=C), 1568 w (aromatic C=C) cm^{-1}; see also Table I.

Anal. Calcd for $C_{16}H_{18}O_7$ (322.3): C, 59.62; H, 5.63. Found: C, 60.2; H, 5.66; mol wt (Rast), 314; equiv wt (CO_2 from $NaHCO_3$ in Warburg respirometer), 338.

Recrystallization of the crude 5-isomer (XII) from ethanol–ethyl acetate and acetone–ethyl acetate gave a mat of fine needles with occasional feathery needles: mp 204°; mmp (with V) 180–183°; ν_{max} 3450 and 3330 (OH), 1705 (carboxyl C=O), 1680 (cinnamoyl C=O), 1626 w (conj C=C), 1600 w and 1575 w (aromatic C=C) cm^{-1}; see also Table I.

Anal. Calcd for $C_{16}H_{18}O_7$ (322.3): C, 59.62, H, 5.63. Found: C, 59.7, H, 5.61; mol wt (Rast), 325; equiv wt (CO_2 from $NaHCO_3$ in Warburg respirometer), 306.

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Chlorogenic Acid Biosynthesis. Relationship between the Chemical Structures of Cinnamoyl and Hydroxycinnamoyl Conjugates and R_{cg} Values from Gradient Chromatography*

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ABSTRACT: When analogous cinnamoyl, *p*-coumaroyl, and caffeoyl conjugates of quinic acid, quinide, shikimic acid, and glucose are separated on the analytical silica gel column (Hanson, K. R., and Zucker, M. (1963), *J. Biol. Chem. 238*, 1105) and their R_{cg} values compared, approximately constant R_{cg} ratios are observed.

For a given position of esterification with a carbohydrate molecule the caffeoyl:*trans*-cinnamoyl ratio is 2.4, and the caffeoyl:*p*-coumaroyl (leading peak) ratio is 1.4. The occurrence of such R_{cg} structural factors is consistent with the known properties of the analytical column, which employs a solvent gradient, and the theoretical relationship between partition coefficients and chemical structure proposed by A. J. P. Martin [(1950), *Biochem. Soc. Symp. 3*, 4]. Band-510 substance and neochlorogenic acid, naturally encountered isomers of chlorogenic acid, are, on the evidence of their chemical properties and R_{cg} values, the 4- and 5-*O*-caffeoylquinic acids, respectively.

Chlorogenic acid (3-*O*-caffeoylquinic acid) is widely distributed in the roots, stems, leaves, and flowers of plants (Herrmann, 1956; Sondheimer, 1964). A number of similar conjugates of the hydroxycinnamic acids also occur. These include such conjugates of caffeic and quinic acid as band-510 substance (Sondheimer, 1958) and neochlorogenic acid (Corse, 1953), various *O*-*p*-coumaroylquinic acids (Williams, 1958), *O*-caffeoyl- and *O*-*p*-coumaroylshikimic acids (Maier *et al.*, 1964; Hanson and Zucker, 1963), and conjugates with various sugars (Harborne and Corner, 1961; Birkofer *et al.*, 1961). In the course of investigations on the biosynthesis of chlorogenic acid a system for partition chromatography on silica gel was developed which would routinely separate mixtures of such conjugates [Hanson and

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