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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

* From the Biochemistry Department, The Connecticut Agricultral Experiment Station, New Haven, Conn. *Received June 25, 1965.* This investigation was supported, in part, by grant GB-2626 from the National Science Foundation. The findings reported in this paper were presented at a meeting of the Plant Phenolics Society of North America, Boston, Mass., 1964.

TABLE I: R_{cg} Values of Corresponding Cinnamoyl and Hydroxycinnamoyl Conjugates.

Carbohydrate Moiety	Position of Substitution	Aromatic Moiety			Ratio of R_{cg} Values	
		Cinnamoyl R_{cg}	*p*-Coumaroyl R_{cg}	Caffeoyl R_{cg}	Caf/Cin	Caf/*p*-Cou
Quinic acid	1	0.69[a]		1.60[b]	2.32	
Quinic acid	3	0.42[a]	0.70[c]	1.00	2.38	1.43
Quinic acid	4	0.51[a]	0.89[d]	1.22[e]	2.39	1.37
Quinic acid	5	0.63[a]	1.14[d]	1.56[f]	2.48	1.37
Quinide	1	0.22[a]		0.53[g]	2.41	
Quinide	5	0.30[h]		0.71[i]	2.37	
Shikimic acid	3		0.56[j]	0.77[k]		1.38
β-D-Glucose	1	0.80[l]	1.28[l]	1.81[l]	2.26	1.41
				Mean:	2.37	1.39

[a] Synthetic compounds (Hanson, 1965). [b] Synthetic compound (Rúveda *et al.*, 1964b). Although the R_{cg} value for this compound is slightly greater than that for neochlorogenic acid (the 5-isomer), the two compounds are not resolved. They are, however, resolvable by countercurrent distribution (Rúveda *et al.*, 1964b). [c] The *O*-*p*-coumaroylquinic acid, with this R_{cg} value, from the potato may be enzymatically oxidized to 3-*O*-caffeoylquinic acid (Hanson and Zucker, 1963). An *O*-*p*-coumaroylquinic acid from apples (Williams, 1958) has been shown to be the 3-isomer by comparison with the synthetic compound (Haslam *et al.*, 1961). The apple and potato conjugates behaved in a similar manner on the analytical column (Hanson and Zucker, 1963). The apple and potato conjugates have now been compared by the more sensitive isotope technique described in the text. The radioactive *p*-coumaroyl conjugate was obtained from potato tuber slices maintained on L-phenylalanine, quinate, and tracer amounts of [α-^{14}C]cinnamate and cochromatographed with the conjugate from apples. [d] Obtained by the action of potassium phosphate buffer, pH 7, on the 3-isomer for 30 min at 90° (see preceding paper, Table III, Hanson, 1965). [e] Band-510 substance (see text). [f] Neochlorogenic acid (see text). [g] Synthetic compound (Johnson). [h] Tentative value based on heating and acid treatment experiments (Hanson, 1965). The compound has not been isolated. [i] Hauschild's substance (Hanson and Zucker, 1963), identified as the lactone of neochlorogenic acid by Rúveda *et al.* (1964a). [j] It was shown previously by enzymatic oxidation that the *O*-*p*-coumaroyl- and *O*-caffeoylshikimic acids accumulating in the potato tuber are substituted in the same position (Hanson and Zucker, 1963). [k] A sample of 3-*O*-caffeoylshikimic acid provided by Dr. Maier had the same R_{cg} value as the compound accumulating in the potato tuber. This value agreed with that obtained by Maier *et al.* (1964). The 4- and 5-isomers had R_{cg} values of 1.00 and 0.89, respectively. [l] The cinnamoyl conjugate was not observed in earlier experiments in which tuber slices were maintained on phenylalanine and quinate (Hanson and Zucker, 1963) but accumulated when cinnamate was supplied in place of phenylalanine (experiments to be described elsewhere). The presence of glucose in the conjugate was detected with the aid of the Glucostat reagent (glucose oxidase, peroxidase, *o*-dianisidine, Worthington Biochemical Corp.). The conjugate, previously described by Harborne and Corner (1961), was hydrolyzed by dilute acid or by the β-D-glucoside glucohydrolase (emulsin) from almonds (Sigma Chemical Co.). The *p*-coumaroyl and caffeoyl conjugates have been described by both Harborne and Corner (1961), and by Birkofer *et al.* (1961). Insufficient material was available to study the caffeoyl conjugate in detail and the assignment is therefore conjectural. The conjugates have not been compared with samples of authentic material.

Zucker, 1963; for apparatus modifications see preceding paper (Hanson, 1965)]. The system employs a rectilinear solvent gradient of 10% cyclohexane–chloroform to 30% *t*-butyl alcohol–chloroform. Over a significantly broad range of conditions the ratio of the peak effluent volumes for any two compounds resolved is independent of the applied gradient and of the length of the column. [The "peak effluent volume" is defined as "that volume of effluent collected while a given compound moves from the top of the column to the bottom and is measured at the point at which the greatest concentration of the compound is eluted" (Marvel and Rands, 1950).] The conjugates studied were therefore characterized in terms of their R_{cg} values: the ratio of the peak effluent volume of the compound relative to the peak effluent volume for chlorogenic acid. As the exact chemical structure of many of these compounds was unknown, it was not possible at that time to examine the relationship between R_{cg} values and chemical structure. Once the mono-*O*-cinnamoylquinic acids had been synthesized and their structures established (Hanson, 1963; Hanson, 1965) a significant pattern began to appear.

In Table I the R_{cg} values for all known or assumed pairs of caffeoyl and cinnamoyl, or caffeoyl and *p*-coumaroyl conjugates are listed. For a given position of esterification with a carbohydrate molecule the mean R_{cg} ratio caffeoyl to *trans*-cinnamoyl conjugate is 2.4, and the ratio caffeoyl to *p*-coumaroyl conjugate (leading major peak) is 1.4. Not all the structural assign-

ments in Table I are firmly established, but it is shown in the Theoretical Section that the occurrence of R_{cg} structural factors of the above type is consistent with the known properties of the analytical gel column and with the theoretical relationship between partition coefficients and chemical structure proposed by Martin (1950). These factors may therefore be used to justify structural assignments pending additional or definitive evidence.

The first application of the factorial relationship was to assign structures to band-510 substance and neochlorogenic acid. It was argued previously (Hanson and Zucker, 1963) that these compounds are the only known naturally occurring isomers of chlorogenic acid, *i.e.*, that one of the four possible isomers remained to be discovered. The so-called isochlorogenic acid was found to be a mixture of at least three related compounds. [It has now been shown that the isochlorogenic acid fraction is a mixture of di-*O*-caffeoylquinic acids (Corse *et al.*, 1965; Scarpati and Guiso, 1964), together with traces of related conjugates. Corse *et al.* (1965) have identified by nuclear magnetic resonance spectroscopy the fractions termed by us a, b, and c as the 3,4-, 3,5-, and 4,5-isomers.] As ceric sulfate oxidation served to distinguish 1-*O*-cinnamoylquinic acid from its isomers, similar experiments were performed on caffeic acid, on the above mono-*O*-caffeoylquinic acids, and on a sample of 1-*O*-caffeoylquinic acid. Caffeic acid and the synthetic 1-isomer gave carbon dioxide at approximately the same slow rate, whereas the naturally occurring compounds evolved carbon dioxide at an initially rapid rate which then fell to a rate attributable to the caffeoyl moiety (Figure 1). About one molar proportion of carbon dioxide, estimated by extrapolation, was derived from the quinic acid moiety. Since the *O*-cinnamoylquinic acids were eluted from the analytical column in the sequence 3-, 4-, 5-, and 1-, and since chlorogenic acid (the 3-isomer) in the set of caffeoyl conjugates is followed by band-510 substance and neochlorogenic acid, it was inferred that these last two compounds are the 4- and 5-isomers, respectively.

The same assignment has been made on purely chemical grounds by Scarpati and Esposito (1963), and, with the aid of nuclear magnetic resonance spectroscopy, by Waiss *et al.* (1964). The former group treated the three naturally occurring compounds and the synthetic 1-isomer with diazomethane and then titrated the products with periodate. Application of this technique to the caffeoyl conjugates has been reported to give equivocal results in other hands (Waiss *et al.*, 1964). The assignments recorded here were made before the publication of these alternative proofs. The three arguments taken together leave little doubt as to the validity of the conclusions reached.

Although the above relationships may be used to assign structures to a series of isomers when all of the isomers are available, the method must be applied with caution when the behavior of only a few of the relevant isomers is known. From the R_{cg} value for 1-*O*-cinnamoylquinide it seemed probable that peak-substance 6 (Hanson and Zucker, 1963) is 1-*O*-caffeoylquinide. An

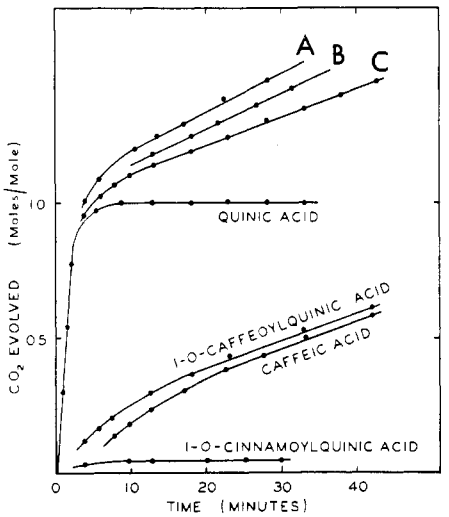

FIGURE 1: Ceric sulfate oxidations. Curve A, chlorogenic acid; B, neochlorogenic acid; C, band-510 substance (3-, 4-, and 5-*O*-caffeoylquinic acids; see text). The curves for the evolution of carbon dioxide from the 3-, 4-, and 5-*O*-cinnamoylquinic acids closely resembled the curve for quinic acid (Hanson, 1965). 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) were added to the flask; final volume 2 ml.

authentic sample of 1-*O*-caffeoylquinide showed the expected R_{cg} value. (Peak-substance 6, a conjugate of caffeic and quinic acids with zero charge at pH 7, is present in the potato tuber in very low concentrations.) This assignment was shown to be incorrect as follows. Slices of potato tuber were maintained on L-phenylalanine, quinate, and tracer amounts of [α-^{14}C]cinnamate, and the accumulated conjugates were chromatographed on the analytical column (the experiment will be described in detail elsewhere). The effluent record showed that the ultraviolet absorption peak 6 exactly corresponded to a peak in radioactivity. The peak fractions were combined and the compound again was chromatographed with the same result. Finally 1-*O*-caffeoylquinide was added to the recovered material and the mixture was chromatographed. The radioactivity peak preceded the absorption peak by 0.03 R_{cg} unit. It follows that the natural product is not identical with the synthetic compound, though it may be identical with one of its isomers. The method of comparison is probably sensitive to 0.01 R_{cg} unit, or less.

The limited resolving power of paper chromatography has been previously noted (Hanson and Zucker, 1963), and attempts to develop reproducible thin layer systems have been unsuccessful. It seems probable, therefore, that the above method of comparison will be of great value when it is desired to establish the identity

of two similar conjugates, and the unknown conjugate has not yet been isolated in crystalline form.

Theoretical Section

The Relationship between R_{cg} Values and Chemical Structure. A theoretical account of the relationship between partition coefficients and chemical structure has been given by Martin (1950). His proposal may be stated as follows. If a solute A is composed of a group M and a group X, then the free energy $\Delta^{A}F$ involved in transferring 1 mole of solute A from the stationary phase at unit activity to the mobile phase at unit activity may be treated as the sum of the free energies involved in transferring the group M and the group X, *i.e.*, $\Delta^{A}F = \Delta^{M}F + \Delta^{X}F$. If a second substance B contains a group Y in place of the group X, then $\Delta^{(Y-X)}F = \Delta^{B}F - \Delta^{A}F$. This hypothesis leads to eq 1 where ${}^{A}\alpha$ is the concentration of solute A at equilibrium in the stationary phase divided by the concentration in the mobile phase. [Dilute solutions are assumed in this derivation. Martin (1950) defined ${}^{A}\alpha$ in terms of mole fractions, but it may be shown that the same equation applies if ${}^{A}\alpha$ is defined in terms of concentrations.] It is implied that the replacement of a group Y by a group X in a molecule changes α by a factor which depends on X and Y and on the phases employed, but not on the rest of the molecule.

$$\Delta^{(Y-X)}F = RT \ln ({}^{B}\alpha/{}^{A}\alpha) \quad (1)$$

A simple relationship exists between the R_F, or the elution volume, of a compound and its partition coefficients when it is chromatographed in a system of constant solvent composition. Many series of compounds have been studied by gas and paper chromatography, and the validity of eq 1 has been established. The paper chromatography of phenolic compounds has been extensively investigated from this point of view (Bate-Smith and Westall, 1950; Marcinkiewicz *et al.*, 1963). For various systems and series of compounds $\Delta^{(OH-H)}F$ is constant. The several parts of a molecule are not, however, entirely independent, and isomers may differ in their partition coefficients. In the study reported here the R_{cg} values for the compounds separated on the analytical column were determined under conditions of changing solvent composition. The Martin equation is thus not sufficient to account for the relationships established in Table I.

The partition coefficient ${}^{A}\alpha_v$ for a compound A moving in a gradient system is a function of the volume of solvent v which has passed the point of maximum solute concentration at some stage in the development of the column (Hanson and Zucker, 1963). The observation that the ratio of the peak effluent volumes for any two compounds separated on the analytical column was independent of the length of the column led to the approximate relationship shown in eq 2. Both ${}^{A}h$ and n are constants dependent upon the gradient, but n is independent of the solute.

$$1/{}^{A}\alpha_v = {}^{A}hv^n \quad (2)$$

Equation 1 may be applied to two compounds A and B separated by the column in the situations where the same volume of solvent has passed the two maxima, *i.e.*, the solvents are of the same composition. From eq 2, and the similar equation for B

$$\Delta^{(Y-X)}F = RT \ln ({}^{A}h/{}^{B}h) \quad (3)$$

It was shown previously, however, that if eq 2 applies, then ${}^{A}h/{}^{B}h = ({}^{B}V/{}^{A}V)^{n+1}$ where ${}^{B}V$ and ${}^{A}V$ are the elution volumes for compounds B and A. Since the distinction between peak effluent volumes and elution volumes may be ignored, ${}^{B}V/{}^{A}V = {}^{B}R_{cg}/{}^{A}R_{cg}$. Equation 4 follows. If n is zero (${}^{A}\alpha$ and ${}^{B}\alpha$ are constants), eq 4 reduces to the equation for the no-gradient situation.

$$\Delta^{(Y-X)}F = (n+1)RT \ln ({}^{B}R_{cg}/{}^{A}R_{cg}) \quad (4)$$

Equation 4 implies that the R_{cg} ratio will be constant for any pair of compounds A and B that differ only in that A contains the group X and B the group Y. Such constancy is in fact observed (Table I).

Values for $\Delta^{(OH-H)}F$ (the free energy required to transfer a hydroxyl group at 1 M concentration from the aqueous stationary phase to the organic mobile phase, less the free energy required to transfer a hydrogen at 1 M concentration) may be calculated as follows. From the previous study (Hanson and Zucker, 1963) n is about 3.5 under standard conditions, also $2.303(n+1)RT$ is about 6070 kcal/mole. From Table I, $\Delta^{(OH-H)}F$ for the first OH group is about 1.37 kcal/mole and for the second OH group 0.91 kcal/mole. Interaction between the two phenolic groups accounts for the fact that the second OH group is not equivalent to the first. Similarly interactions between the OH groups and the COOH group of the quinic acid and shikimic acid moieties account for the fact that isomeric conjugates of these acids are separated on the column.

Acknowledgments

Appreciation is expressed to Dr. M. Zucker for his valuable suggestions and comments, to Dr. H. B. Vickery for his aid in the preparation of this manuscript, to Katherine A. Clark for her skillful technical assistance, to Drs. R. Johnson and V. Deulofeu for details of their experiments prior to publication, and to the following persons for gifts of materials: Dr. E. Sondheimer for neochlorogenic acid and band-510 substance (Sondheimer, 1958), Dr. V. P. Maier for 3-*O*-caffeoylshikimic acid (Maier *et al.*, 1964), Dr. A. H. Williams for 3-*O*-*p*-coumaroylquinic acid (Williams, 1958), Dr. R. Johnson of the Brown and Williamson Tobacco Corp., Louisville, Ky., for 1-*O*-caffeoylquinide, and Dr. V. Deulofeu for 1-*O*-caffeoylquinic acid (Rúveda *et al.*, 1964a).

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The Molecular Biology of *Euglena gracilis*. III. General Carbon Metabolism*

E. S. Kempner and J. H. Miller

ABSTRACT: Glutamic acid, supplied as a sole carbon source, enters the metabolic pathways of *Euglena gracilis* principally *via* transamination by glutamic–oxalacetic transaminase.

Synthesis of purines and pyrimidines and the interconversions of amino acids suggest that general carbon metabolism is similar to that of most microorganisms.

The development of a simple growth medium and standard culture conditions for *Euglena gracilis* have been reported (Kempner and Miller, 1965a). The gross chemical composition of the cells and the kinetics of carbon assimilation among several cellular fractions were also determined (Kempner and Miller, 1965a,b). The continuing studies of the molecular biology of *Euglena gracilis* have now been extended to a very general study of carbon metabolism.

The biochemical pathways in *Euglena* have not been fully elucidated, although several reports have appeared in recent years concerning selected aspects of carbon metabolism. Two reports (Danforth, 1953; Hurlbert and Rittenberg, 1962) have been particularly significant with respect to intermediary metabolism. These studies have shown the existence of the Krebs cycle and Embden–Meyerhof and hexose monophosphate pathways. Under our growth conditions, glutamic acid is supplied as a sole carbon source; the question of which routes of entry into the various major cell pathways were utilized by the glutamic acid and some information about the synthesis of nucleic acids and amino acids have been considered. Most of the experimental techniques were those developed in the studies of the bacterium *E. coli* at the Carnegie Institution of Washington. On the basis of the published data and the results given here, a general picture of the carbon flow in *Euglena gracilis* can be drawn.

Materials and Methods

Composition of the growth medium and details of culture conditions for *Euglena gracilis* strain z have been reported previously (Kempner and Miller, 1965a). Radioactive compounds were obtained from the Nuclear

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