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SHORT COMMUNICATION

THE DETERMINATION OF D-QUINIC AND D-SHIKIMIC ACIDS BY MICROBIOLOGICAL ASSAY IN THE CHARACTERIZATION OF CHLOROGENIC ACIDS AND RELATED COMPOUNDS

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Abstract—D-Quinic and D-shikimic acids can be quantitatively determined in microgram quantities with a mutant of *Aerobacter aerogenes* which is blocked prior to 5-dehydroquinic acid. The bioassay was used for the quantitative and stereospecific determination of quinic acid from the hydrolysis of micro amounts of chlorogenic acid and related depsides either from the previously isolated material or from paper chromatograms.

EXAMINATION of plant extracts by chromatographic techniques has revealed the widespread distribution of many compounds closely related to chlorogenic acid, 3-*O*-caffeoyl-D-quinic acid. These compounds may be position isomers of chlorogenic acid; they may contain more than one aromatic moiety; they may have *p*-coumaroyl, feruloyl, or galloyl substituents in place of the caffeoyl group or quinic acid may be replaced by shikimic acid.[1] Structure determinations of these substances are often hampered by the small amounts of material available and by difficulties involved in the quantitative, chemical determination of quinic and shikimic acids. In this report we describe a microbiological assay for the quantitative, stereospecific determination of microgram quantities of D-quinic and D-shikimic acids and demonstrate the application of the procedure to structural work with chlorogenic acid and related compounds. We used an *Aerobacter aerogenes* mutant, A 170–143 SIO, obtained by Davis and Weiss[2] and shown by them to be suitable for microbiological assays of quinic and shikimic acids.

From Fig. 1 it is seen that the growth response of *A. aerogenes*, A 170–143 SIO to D-quinic and D-shikimic acid is linear between concentration levels of 0·005 and 0·06 μg/ml and that the sensitivity of the assay is drastically increased by the addition of 5 μg/ml of L-tryptophan to the medium. That the utilization of the acid is stereospecific was demonstrated by the use of DL-shikimic, DL-quinic and L-quinic acids. The growth response to the racemates was half that of the D-isomers. There was no stimulation of growth with 0·035 μg/ml of L-quinic acid and the addition of a tenfold excess of L-quinic acid to D-quinic acid had no effect on the utilization of the D-isomer. Since quinic and shikimic acids can be readily separated by one-dimensional paper chromatography[3] combination of this procedure with bioassays can be used for the specific, quantitative determination of mixtures of these two acids. Ninety-five per cent recoveries are obtained when mixtures containing as little as 5 μg of each acid are

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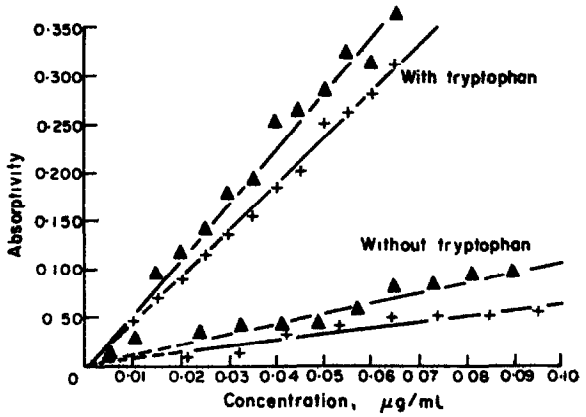

FIG. 1. GROWTH CURVE FOR *A. aerogenes* AFTER 18 HR AT 30° ON MINIMAL MEDIUM A SUPPLEMENTED WITH TYROSINE AND PHENYLALANINE. ▲ response to D-quinic acid; + response to D-shikimic acid.

chromatogramed, the separated spots eluted with water and the eluates assayed with the *A. aerogenes* mutant.

The bioassay can also be applied to structural work with microgram quantities of the naturally occurring depsides, if an alkaline hydrolytic procedure is used (Table 1).

TABLE 1. THE CONCENTRATION OF D-QUINIC ACID IN DEPSIDES BY MICROBIOASSAY*

Depside	Concentration (μg/ml)	Concentration of D-quinic acid (μg/ml)	
		Found	Calculated
Chlorogenic acid	0·059	0·031	0·032
	0·061	0·031	0·033
Neochlorogenic acid †	0·067	0·034	0·038
	0·074	0·036	0·040
3-Feruloylquinic acid	0·068	0·037	0·035
	0·072	0·037	0·036

* Determined on hydrolyzed depsides by incubation for 18 hr at 30°.
† $\epsilon_{323mu} = 17{,}000$ in H_2O.

Equally good results were obtained when a crystalline preparation of chlorogenic acid was used directly or when it was eluted from chromatograms. In either case alkaline hydrolysis must precede the quinic acid determination since the growth response to unhydrolyzed chlorogenic acid is very weak. Even when caffeic, *p*-coumaric or ferulic acids were present at ten times the concentration of the D-quinic acid, the growth response was unchanged. Gallic acid at concentrations above 0·1 μg/ml causes some interference due to formation of colored oxidation products. However, this can be corrected by measuring the absorptivity at 560 mμ of the clarified broth against a sterile medium blank in the spectrophotometer. Since the *A. aerogenes* mutant can utilize only the D-isomer of quinic acid, the data in Table 1 provide proof that the quinic acid moiety of neochlorogenic acid, 5-*O*-caffeoylquinic acid, and 3-*O*-feruloylquinic acid has the D-configuration.

EXPERIMENTAL

Microbioassay Procedure

The basic medium consisted of minimal medium A as described by Davis and Mingioli[4] supplemented with 20 μg/ml L-tyrosine, 40 μg/ml DL-phenylalanine and 5 μg/ml L-tryptophan. Solutions of D-quinic or D-shikimic acid were prepared in 2·5 ml glass-distilled water and autoclaved for 20 min at 15 lb/in^2 in cotton-plugged 25 ml Erlenmeyer flasks. The growth medium was added by cold sterilization through "Millipore" filters (pore diameter = 0·45 mμ) to a final volume of 5 ml. Solutions were inoculated with 0·01 ml of fresh culture. Inoculated cultures were shaken at 30° for 18 hr and the absorptivity measured at 560 mμ against a culture containing no quinic or shikimic acid. Each assay consisted of four unknowns and fourteen known concentrations of D-quinic of D-shikimic acid ranging from 0 to 0·07 μg/ml. A standard curve was prepared and the concentration of the unknown calculated by the method of least squares. It was found advisable to prepare a standard curve for each determination. Calculation of the linear regression showed some variation in the slope and the line of best fit did not always intersect exactly at the origin.

Saponification of the Quinic Acid Depsides

Solutions containing 0·06 N sodium hydroxide were heated for 5 min at 100°, cooled to room temperature and neutralized with dilute hydrochloric acid. Appropriate aliquots, 5 μg quinic acid derivative in 25 ml water, were assayed with the mutant.

Chromatography

Chlorogenic acid was chromatogramed on Whatman No. 1 filter paper with *n*-butanol: acetic acid: water (4:1:5 v/v).[5] The compound was detected by its fluorescence when exposed to u.v. light. D-quinic acid, R_f 0·22, and D-shikimic acid, R_f 0·46, were separated by descending chromatography on Whatman No. 1 filter paper with *n*-butanol: formic acid: water (4:1:5 v/v).[3] Spots were located by chromatography of knowns adjacent to the experimental material and spraying of these with 4% bromophenol blue.

Preparation of DL-*Quinic Acid and* L-*Quinic Acid*

DL-Quinic acid was prepared from D-triacetylquinide by heating at 240° for 4 hr, and hydrolyzing with potassium hydroxide.[6] The L-isomer was obtained through the brucine salt and recrystallization with methanol.[7] The brucine salt, 2 g, was passed over 35 g Dowex 50W–X 12, previously washed with water, 1 N NaOH, water, 1 N HCl and water. The acidic fractions were collected, the solvent evaporated under reduced pressure, and the residue crystallized from ethanol; Yield 366 mg; m.p. 166·5–167°, $[\alpha]_D^{25}$ +42·6° (*c* 4·8 H_2O).

DL-Shikimic acid was a gift from Dr. E. E. Smissman. He and his coworkers[8] reported its synthesis from the Diels Alder reaction of *trans*, *trans*-1,4-diacetoxy-1,3-butadiene and methyl-acrylate.

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