[CONTRIBUTION FROM THE U. S. PUBLIC HEALTH SERVICE, TUBERCULOSIS RESEARCH LABORATORY, CORNELL UNIVERSITY MEDICAL COLLEGE]

Aromatic Biosynthesis. X.[1] Identification of an Early Precursor as 5-Dehydroquinic Acid[2,3]

BY ULRICH WEISS, BERNARD D. DAVIS AND ELIZABETH S. MINGIOLI

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An early intermediate in aromatic biosynthesis in bacteria has been isolated in crystalline form from culture filtrates of a mutant strain of *Escherichia coli*. This substance III, $C_7H_{10}O_6$, is shown to be 5-dehydroquinic acid (5-keto-1,4α,3β-trihydroxycyclohexane-1-β-carboxylic acid), a previously unknown compound. The proof of identity rests essentially upon its conversion to 5-dehydroshikimic acid (II) by mild treatment with acid, to protocatechuic acid by pyrolysis, and to quinic acid (IV) by catalytic hydrogenation.

Earlier work from this Laboratory has yielded evidence on the path by which microörganisms form the benzene ring in five metabolites: tyrosine, phenylalanine, tryptophan, *p*-aminobenzoic acid and *p*-hydroxybenzoic acid.[4] Certain mutants of *Escherichia coli*, blocked in the synthesis of these compounds (Fig. 1), were found to accumulate shikimic acid (I) in their culture filtrates; other mutants, blocked in earlier reactions in the same sequence, can utilize I to replace the above five required metabolites. These observations establish I with great probability as a common precursor of these compounds. In turn, certain mutants, accumulate, and others utilize, a precursor of I, which has been isolated and identified[1] as 5-dehydroshikimic acid (II). Finally, some mutants accumulate, and others utilize, an even earlier intermediate in this biosynthetic sequence, provisionally designated as compound W.[5]

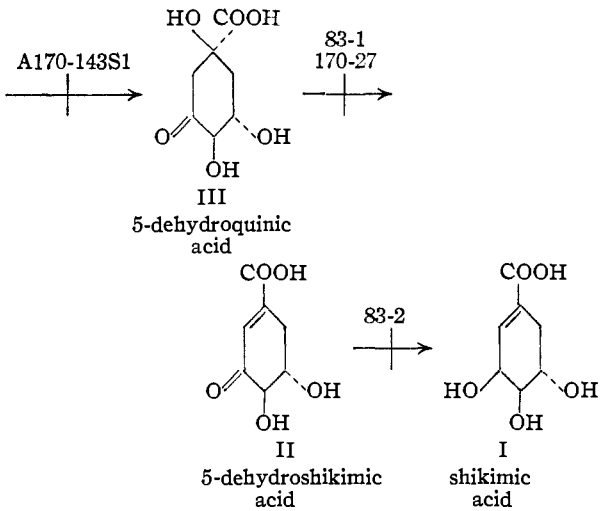


Fig. 1.—Intermediates in aromatic biosynthesis. Numbers refer to mutants blocked in the reactions indicated.

This paper will describe the isolation and identification of compound W. The procedures which revealed its existence, and certain complications in its utilization as a growth factor, have been described elsewhere.[5] This earlier work also showed that compound W is closely related chemically to its presumed biosynthetic product, II, since W in culture filtrates was converted to II on heating.[5] This ready conversion became understandable when W was identified, as will be shown below, as 5-dehydroquinic acid (III).

Appropriate mutants accumulate III in culture filtrates in considerable amounts (several hundred γ/ml.). Orienting experiments with such filtrates revealed the extreme instability of the compound to alkali and its moderate stability in acidic media; aqueous solutions at *p*H 1–5 could be heated at 65° without loss of activity, but at 100° were partly inactivated and partly converted into II.[5] All steps in the isolation of III were therefore carried out at moderate temperatures and without exposure to alkaline or to strongly acidic reaction. These steps were controlled by bioassay, which could be conveniently carried out with a mutant of *Aerobacter aerogenes* (A170-143S1) that responds well to III (as well as II or I) as a growth factor.[5]

Compound III was isolated from culture filtrates of *E. coli* mutant 170-27 by adsorption at *p*H 3–4 on a charcoal column, elution with 2.5% ethanol, and precipitation in alcoholic solution as the brucine salt. The brucine was removed in aqueous medium as the insoluble picrate, excess picric acid was extracted with ether, and the aqueous phase was evaporated. Treatment of the glassy residue with ether gave III as granular crystals. These had almost the same growth-factor activity, weight for weight, as I. Recovery was rather low in the elution from charcoal, presumably due to partial destruction of the very sensitive compound in the adsorbed state; yields were nearly quantitative in the precipitation and decomposition of the brucine salt; they were again low in the final step, the losses being in large part mechanical since III forms crystals strongly adhering to the glass.

Crude III was purified by dissolving at room temperature in acetone and adding chloroform; the cloudy mixture yielded fine granular crystals on standing in the refrigerator; recoveries were rather low.[6] Alternatively, crude III could be

(1) Paper IX, I. I. Salamon and B. D. Davis, THIS JOURNAL, **75**, 5567 (1953).

(2) Presented at the 122nd National meeting of the American Chemical Society, Atlantic City, N. J., September, 1952.

(3) Aided by a grant from the Squibb Institute for Medical Research.

(4) (a) B. D. Davis, *Experientia*, **6**, 41 (1950); (b) *J. Biol. Chem.*, **191**, 315 (1951); (c) *J. Bact.*, **64**, 729 (1952); (d) for a general discussion of mutant methods see G. W. Beadle, *Physiol. Revs.*, **25**, 643 (1945).

(5) B. D. Davis and U. Weiss, *Arch. exp. Path. Pharmakol.*, **219**, 549 (1953).

(6) The bioassay of III, though adequate for controlling the steps in its isolation, is not very precise on account of the flatness and variability of the growth curves compared with those given by I.[5] It is presumably for this reason that some crude crystallizates appeared to lose growth-factor activity on further purification. No such discrepancies were found when the concentration of III was measured by an im-

recrystallized from boiling ethyl acetate, but yields were equally unsatisfactory and the product obtained was less pure.

III is very soluble in water or various polar organic solvents, and not appreciably soluble in nonpolar organic solvents. The product after recrystallization from acetone–chloroform melts at 140–142°[8] after some sintering; on further heating the melt crystallizes again, to remelt at 198–200°. Mixed melting point and reaction with $FeCl_3$ identified this pyrolysis product as protocatechuic acid (*cf.* the analogous formation of this compound on pyrolysis of II[1]). This substance is also formed from III, as from II,[1] by brief warming with concentrated HCl.

Elementary analysis gave values in excellent agreement with the formula $C_7H_{10}O_6$. III therefore differs from II, $C_7H_8O_5$, by the elements of one molecule of water.

The acidity of the compound (*p*H of a 0.5% aqueous solution, 2.42) and the peak at 5.8 μ in the infrared spectrum[9] (see Fig. 3) show the presence of a carboxyl group. Attempts to prepare carboxyl-substituted derivatives failed to give crystalline products.

A carbonyl group could be demonstrated in III by the presence in aqueous solution of a typical ultraviolet band of low intensity (λ_{max} 269 mμ, ϵ 44). Furthermore, reaction of III with thiosemicarbazide in water[10] gave a thiosemicarbazone (not isolated) which showed the characteristic bands[11] of such a derivative, although the intensities, especially of the maximum at the shorter wave length, were low: λ_{max} 268–270 mμ, ϵ 17,600, and 230 mμ, ϵ 9,640.[26] The presence of a ketol grouping is suggested by the fact that III, like II,[1] reduces Fehling and Tollens reagents rapidly at room temperature, and it develops color with orcinol. Attempts to obtain well-characterized derivatives with carbonyl reagents were not encouraging.[12]

The conversion of III to II, noted above to have been observed with filtrates,[5] was studied in more detail with pure III. The reaction was found to take place at 100° with 0.1 *N* HCl, or with water in the presence of a strongly acidic ion exchange resin. However, the conversion is accompanied by other reactions, so that II could not be isolated in pure form from the resulting mixture. Its presence was demonstrated by bioassay with a mutant that responds to II but not to III; by paper chromatography; by adsorption on a charcoal column and characteristic elution with 60–75% ethanol[1]; and by the ultraviolet absorption spectrum of its thiosemicarbazone (see Fig. 2).

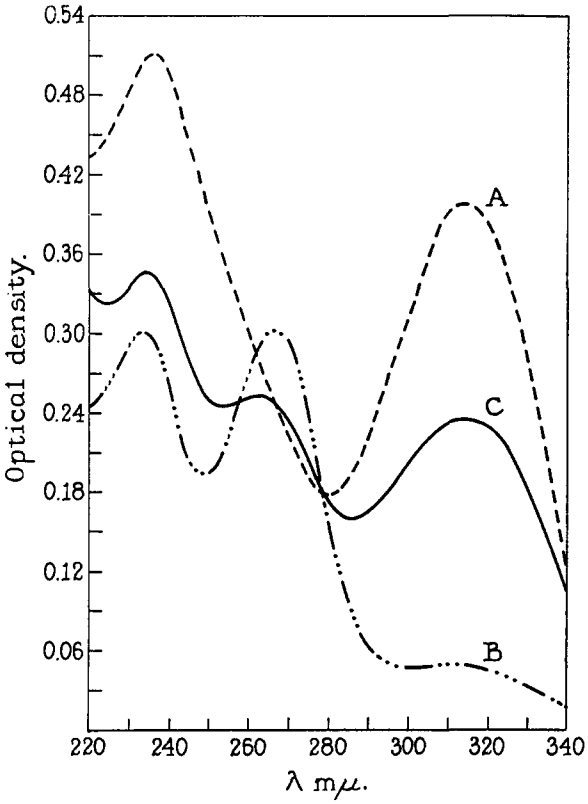
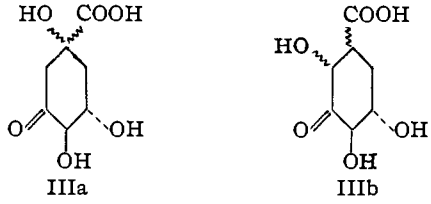


Fig. 2.—Conversion of III to II, all curves at equal concentrations: spectra after 48 hours of incubation with thiosemicarbazide: A, authentic II; B, authentic III; C, acid-treated III. For the low extinctions in curve B, see ref. 26. The instability of the 269 mμ band of III thiosemicarbazone may explain the absence of an isosbestic point around 260 mμ.

This conversion of III to II, together with the empirical formulas of the two substances and the known structure of II,[1] proves the constitution of compound III to be either IIIa or IIIb. This question was decided in favor of IIIa by the yellow color-reaction of the compound with aqueous $FeCl_3$, characteristic of α-hydroxy acids,[15] and by its reduction to quinic acid (see below).



Formulation IIIa is in harmony with the properties noted above, and also with the fact that III

proved method developed since the completion of this work.[7] This method involves enzymatic conversion of III into II and estimation of the latter compound by its ultraviolet absorption. Recrystallized III underwent almost quantitative conversion to II in this procedure.

(7) S. Mitsuhashi and B. D. Davis, unpublished results.

(8) Melting points were taken on a Fisher–Johns hot stage; they are not corrected for stem exposure.

(9) Kindly taken by Dr. Herbert Jaffe, Rockefeller Institute for Medical Research.

(10) *Cf.* R. Donovick, G. Rake and J. Fried, *J. Biol. Chem.*, **164**, 173 (1946); proof of the presence of a carbonyl group in streptomycin.

(11) L. K. Evans and A. E. Gillam, *J. Chem. Soc.*, 568 (1943).

(12) A peculiar reaction of II and III was found when bacterial filtrates containing III were investigated by paper chromatography for the possible presence of α-keto acids that might be revealed by the ultraviolet absorption of their semicarbazones.[13] An area of strong light-blue fluorescence was observed at the position of III. Fluorescence was also produced under these conditions by crystalline II[14] and III, but not by a variety of related compounds, including I, IV, the semicarbazone of II,[14] and that of III. The fluorescence therefore appears to be due to some reaction of II or III, or their semicarbazones, with semicarbazide HCl during the treatment at 100° prescribed in the test used.

(13) B. Magasanik and H. E. Umbarger, THIS JOURNAL, **72**, 2308 (1950).

(14) Kindly furnished by Dr. I. I. Salamon.

(15) A. Berg, *Bull. Soc. Chim.*, [3] **11**, 882 (1894).

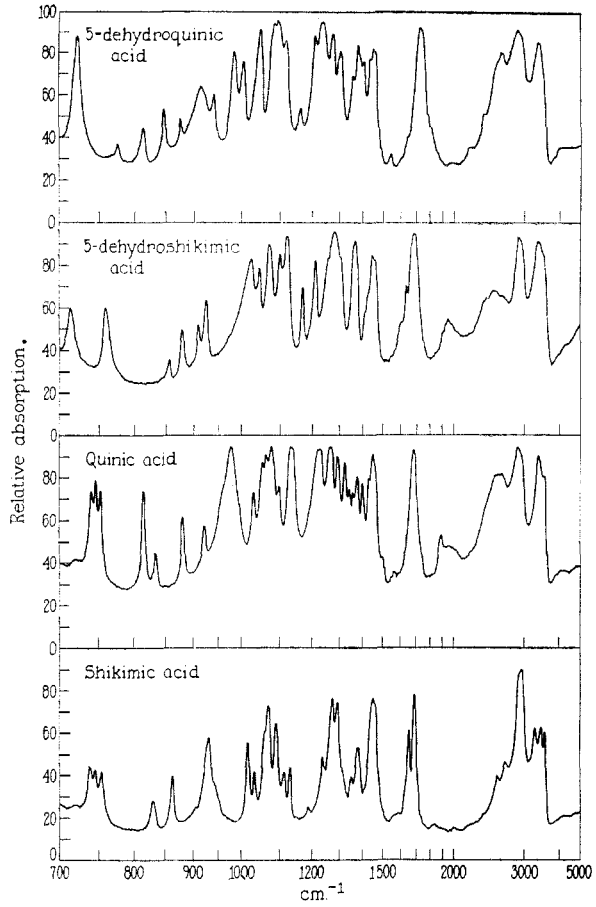


Fig. 3.—Infrared spectra of compounds I to IV.

lacks the characteristic high absorption peak of II at 234 mμ and has a low chromatographic mobility compared to II and I. The ready dehydration of III to II, occurring to a small extent even on evaporation of its solutions under reduced pressure, can presumably be explained by the formation of an extended conjugated double-bond system.

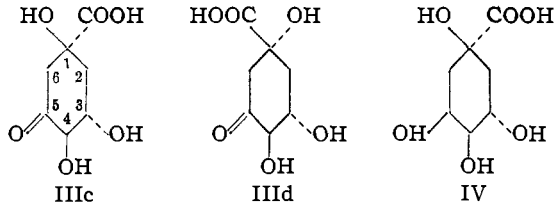
The conversion of III to II also establishes the configuration of the hydroxyls at C_3 and C_4.[16] Their *trans* relationship is further supported by the failure of III, like II,[1] to form an acetonide; on shaking in acetone with an acidic ion exchange resin as catalyst, I and IV (see below) lost most of their biological activity (owing to acetonide formation at the *cis*-hydroxyl groups at C_4 and C_5), but the activity of III persisted undiminished.

The only question still unsettled was the configuration at C_1. Of the two possible stereoisomers, IIIc would be derived from quinic acid (IV), and IIId from the unknown C_1-epimer of IV.[17]

This question was decided in favor of IIIc by catalytic reduction of III in ethanol with Pd–charcoal as catalyst, which yielded IV. This result also verifies the previously established configurations of C_3 and C_4. IV was identified by mixed m.p. with authentic material, by comparison of the R_f values of the free compound and of its

(16) For proof of constitution and configuration of II and its relation to I, see (1); for configuration of I see H. O. L. Fischer and G. Dangschat, *Helv. Chim. Acta*, **18**, 1206 (1935); **20**, 705 (1937).

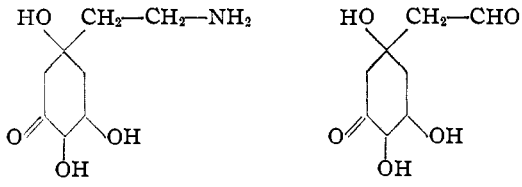
(17) For proof of configuration of IV see H. O. L. Fischer and G. Dangschat, *Ber.*, **65**, 1009 (1932).



IIIc IIId IV

acetonide with those of known samples, by bioassay,[18] and by demonstrating that the biological activity (like that of authentic IV, but unlike that of III) was stable to alkali.

It is of interest that the structure III, established for this earliest known biosynthetic precursor of the aromatic ring, is closely related to structures (*e.g.*, V, VI) postulated much earlier by Robinson[19] for intermediates in biosynthesis of the benzene ring in many alkaloids.



V VI

Experimental

Accumulation.—Culture filtrates containing 5-dehydroquinic acid were obtained in the following way. Medium A[20] without glucose was supplemented with 10 mg. of L-tyrosine, 20 mg. of DL-phenylalanine, 5 mg. of L-tryptophan, 10 γ of *p*-aminobenzoic acid and 10 γ of *p*-hydroxybenzoic acid per liter, and was distributed in 500-ml. amounts in cotton-plugged 1-liter erlenmeyer flasks. These flasks were then sterilized by autoclaving for 10 minutes at 120°, further supplemented with 0.5% glucose (separately autoclaved), inoculated with 1 ml. of a washed 24-hour culture of *E. coli* mutant 170-27,[4c] and incubated with shaking for 2–3 days at 37°.

The light yellow liquid was separated from the bacterial cells in a Sharples centrifuge, acidified to *p*H 3.5 with HCl, and refrigerated until use; alternatively, the culture may be acidified, the cells allowed to settle, and the supernatant clarified by filtration through Celite. The filtrates so obtained were found by bioassay to contain about 200 γ/ml. of III, estimated[5] in comparison with I as a standard.

Bioassay.—Aerobacter mutant A170-143S1 was used as assay organism throughout this work.[5] For limitations of this assay see ref. 6.

(a) **Plate Assay.**—This assay is performed on plates containing about 10^5 organisms in 20 ml. of medium A (see above) supplemented with 20 γ of L-tyrosine and 40 γ of DL-phenylalanine per ml., and solidified with 1.5% agar. One hundredth to one-tenth ml. of the solution to be tested is applied to filter paper disks on the agar; after 18 hours of incubation at 37° the area of growth around each disk is compared with that similarly obtained with known amounts (2–10 γ) of I.

(b) **Tube Assay.**—The solution to be assayed, in volumes up to 0.3 ml., is placed in sterile colorimeter tubes together with an equal volume of 0.2 *N* HCl. The mixture

(18) This bioassay rests on the observation, made during this work, that *A. aerogenes* mutant A170-143S1 responds as well to IV as to III. In contrast, no *E. coli* mutant can respond to IV, and no mutant of either species has been observed to accumulate it. This and other evidence that IV may not be a normal intermediate in aromatic biosynthesis is discussed elsewhere.[5]

(19) R. Robinson, *J. Chem. Soc.*, **111**, 876 (1917).

(20) B. D. Davis and E. S. Mingioli, *J. Bact.*, **60**, 17 (1950). This medium contains only glucose, citrate and inorganic salts. The citrate had been found earlier to increase accumulation of shikimic acid,[21] but it had no influence on the accumulation of III and might have been omitted. It was also found, subsequent to this work, that some other carbon sources (*e.g.*, D-xylose) give somewhat better accumulations than glucose.

is sterilized by heating at 65° for 10 minutes; no appreciable conversion of III to II takes place under those conditions. (As an alternative, the solution may be sterilized by filtration.) The volume is brought to 10 ml. with medium A containing per ml. 20 γ of L-tyrosine, 40 γ of DL-phenylalanine and 5×10^{-4} ml. of a 24-hour culture of the assay organism. After incubation at 37° for 18 hours turbidity is measured with an Evelyn photoelectric colorimeter (540 mμ filter) and compared with that given by 2–20 γ of I.

This bioassay responds to I, II or IV as well as III. Differential bioassays can be carried out, by the same procedure, with *E. coli* mutant 83-1, which can utilize I or II but not III or IV. Further differentiation can be achieved on the basis of alkali stability: heating the sample at 100° for 10 minutes with an equal volume of 0.2 *M* Na_2CO_3 before bioassay destroys II or III but not I or IV. Such tests showed that many preparations of III contained detectable quantities of II, no doubt formed from III during the isolation procedures.

A comparison of growth responses to pure I, II, III and IV is presented elsewhere.[5] The methods of paper chromatography and bioautography used have been described previously.[4b,21]

Isolation of III.—All steps in the isolation procedure were controlled by bioassay. Four liters of culture filtrate (containing about 800 mg. of III) was passed through a column (diameter 70 mm.) of 150 g. of charcoal (Darco G60) under slight pressure of nitrogen. The effluents were inactive. The column was next washed with 3 × 500 ml. of water, followed by 3 × 500 ml. of 1% ethanol. The latter solvent usually removes a small amount of active material. These effluents were discarded. Subsequent washing with 500-ml. portions of 2.5% ethanol usually eluted the bulk of the recoverable activity. Elution was continued until the activity of the effluents dropped well below the maximum observed; about 10 portions were needed. On subsequent washing with 5 and 10% ethanol, appreciable further quantities of III could usually be obtained; occasionally more of the active material came out with 5% than with 2.5% ethanol. These solutions were worked up as described below for the 2.5% ethanol fractions. The total activity recovered amounted to about 25% of that originally present. Slightly better results were obtained with charcoal washed with hot HCl followed by hot ethanol, benzene and acetic acid.

The first 2.5% ethanol eluate was discarded, since it often contained an unidentified, inactive contaminant giving a water-insoluble brucine salt in the subsequent step; no III could be obtained from the filtrate of this brucine salt. The other 2.5% alcoholic eluates were combined and evaporated *in vacuo* at 37°. The residual glass, colorless to light yellow, weighed about 1 g. and had an activity corresponding to about 350 mg. of I.

This residue was taken up in 25 ml. of ethanol, the cloudy solution treated with charcoal and filtered, and the filter washed with ethanol. A solution of 2 g. of brucine in 10 ml. of ethanol was added. The liquid became milky and on chilling deposited the brucine salt of III in spherical aggregates, often contaminated with some resinous material. The mixture was kept in a freezer for 1–2 days. The precipitate was next filtered with suction, washed with ice-cold ethanol, and dried *in vacuo*. The total activity recovered amounted to about 90% of that present in the 2.5% ethanol eluates used; the purity of the product varied between 50 and 100%. Since brucine does not influence the bioassay of III, it was possible to control the yields and purity of the brucine salt directly. Attempts to purify the salt by recrystallization were unsuccessful.

The brucine salt was next taken up in 20 ml. of water, the solution was treated with charcoal if necessary, and a slight excess of a saturated aqueous solution of picric acid was added in small portions. A yellow precipitate of brucine picrate formed, which soon turned reddish-orange. After storage in a refrigerator for several hours the precipitate was filtered off, washed with water, and discarded. The aqueous filtrate was extracted continuously with ether for 6 hours to remove the excess picric acid and, presumably, inactive impurities. Evaporation *in vacuo* of the aqueous layer yielded a colorless glassy residue which contained practically all the activity present in the brucine salt.

(21) B. D. Davis and E. S. Mingioli, *J. Bact.*, **66**, 129 (1953).

The product was covered with ether. Slow crystallization took place in the refrigerator; it could be accelerated by seeding. After about one day the ether was decanted and replaced by fresh solvent. After another 1–2 days the crystallizate, which adhered strongly to the container, was scraped off, transferred to a filter, and washed well with ether. After drying *in vacuo* the compound formed a white to yellowish, microcrystalline, non-hygroscopic powder, about 60–100% as active as I; yield 630 mg. Various samples melted between about 132 and 140° after previous sintering; on further heating, the melt resolidified around 150° and melted again at 195–200° (*cf.* formation of protocatechuic acid described below).

Purification.—Two hundred seventy-five mg. of the crude material was dissolved at room temperature in 10 ml. of acetone. Charcoal was added, the solution filtered, and the filter rinsed with 2 ml. of acetone. Ten ml. of chloroform was added slowly and the mixture was quickly filtered to remove a small quantity of a flocculent precipitate. To the clear colorless filtrate 6 ml. of chloroform was added and the slightly cloudy solution was chilled and seeded. On scratching the walls of the container granular crystals appeared; these were filtered off after 1–2 days, washed with ice-cold acetone–chloroform mixture, and dried to constant weight at room temperature *in vacuo* over P_2O_5; yield 167 mg.

The compound may also be recrystallized from a large volume of boiling ethyl acetate; however, crystals appeared only after considerable concentration of the solution *in vacuo*, and the product obtained had a less satisfactory m.p. than samples obtained from acetone–chloroform.

Pure III melts at 140–142° after sintering at 138°; on further heating the melt resolidifies at about 154–160° and remelts at 195–200°. A mixture with II (m.p. 150–152°[21]) melts at 123–135°.

Anal.[22] Calcd. for $C_7H_{10}O_6$: C, 44.21; H, 5.30. Found for a sample from acetone–chloroform: C, 44.19; H, 5.33; for a sample from ethyl acetate: C, 44.17; H, 5.28.

Properties of 5-Dehydroquinic Acid.—III is very soluble in water, methanol, ethanol, acetone, dioxane and acetic acid; little in hot ethyl acetate; and not appreciably in the other common organic solvents; $[\alpha]^{28}D$ −82.4° (*c* 0.71 in anhydrous ethanol); λ_{max} 269 mμ, ϵ 43.8 (0.6% solution in anhydrous ethanol). The presence of a peak at shorter wave lengths (λ_{max} 230 mμ, ϵ 97.4) points to contamination of the sample used with about 1% II, which was also observed microbiologically[23]; as was mentioned before, preparations entirely free of II were not reproducibly obtainable, owing to the ease of its formation from III.

Infrared spectra[9] of I, II, III and IV are given in Fig. 3. They were taken with a Perkin–Elmer spectrograph, Model 21; NaCl optics; Nujol mull, resolution 5, response 3–3, gain 8, speed 0.10 μ/mm.

R_f values: *n*-butanol–formic acid I, 0.42; II, 0.45; III, 0.20; IV, 0.18; phenol–water I, 0.43; II, 0.47; III, 0.32. None of these compounds moves in *n*-butanol–3% NH_3.

III reduces Fehling and Tollens reagents at room temperature. On heating with orcinol in *n*-butanol and HCl, separation of the layers, and shaking of the butanol phase with aqueous NaOH,[24] the NaOH phase becomes orange-brown. An aqueous solution gives a strong yellow color on addition of a drop of dilute $FeCl_3$ solution.

Attempts to prepare additional crystalline salts were unsuccessful. No crystalline oxime or thiosemicarbazone could be obtained. Semicarbazide yielded a precipitate consisting of aggregates of short colorless needles, strongly contaminated with light-red material. No way of purifying the compound was found. 2,4-Dinitrophenylhydrazine in aqueous ethanolic H_2SO_4[25] gave a dark, microcrystalline precipitate in poor yield. Attempted purification from dioxane gave a minute quantity of a yellow, crystalline product, m.p. 254–260° with decomposition. The small yields prevented further work.

(22) Analyses by Dr. W. C. Alford, National Institutes of Health, Bethesda, Md.

(23) This concentration of II would not contribute appreciably to the observed peak at 269 mμ.

(24) J. H. Williams, *Nature*, **170**, 894 (1952).

(25) R. L. Shriner and R. C. Fuson, "The Systematic Identification of Organic Compounds," 3rd ed., John Wiley and Sons, Inc., New York, N. Y., 1948, p. 171.

Reaction of 5-Dehydroquinic Acid with Thiosemicarbazide.—Nine and one-half mg. of III (0.05 mmole) was allowed to react with 4.6 mg. of thiosemicarbazide (0.05 mmole) in 6 ml. of water at room temperature for 18 hours, protected from light. The resulting solution showed λ_{max} 230 mμ (ε 9,640) and 266 mμ (ε 17,600).[26] Attempts to isolate the thiosemicarbazone were unsuccessful. Similar treatment of II with a molar equivalent of thiosemicarbazide yielded λ_{max} 236 mμ (ε 15,400) and λ_{max} 314 mμ (ε 12,100)[26] (see Fig. 2). Evans and Gillam[11] found λ_{max} 270–273 mμ (ε 20,000–25,000), and 230 mμ (ε 7,000) for the thiosemicarbazones of saturated carbonyl compounds, while the thiosemicarbazones of α,β-unsaturated carbonyl compounds showed λ_{max} 301.5 ± 2 mμ (ε 20,000–30,000) and 245 ± 4 mμ (ε 10,000). The location of the peak of II-thiosemicarbazone at 314 rather than 302 mμ may well be due to the additional conjugation with the carboxyl group.

Formation of Protocatechuic Acid. (a) **By Pyrolysis.**—A sample of III was melted on a hot stage; on further heating crystals formed at 156°. The melt was kept at this temperature until crystallization appeared to be complete. The material formed was identified as protocatechuic acid by its green color with $FeCl_3$ and by mixed m.p. with authentic material of m.p. 201–202°; found for the mixture, m.p. 202–203° after some sintering at 197°.

(b) **By Treatment with HCl.**—Three mg. of III was boiled for 20 seconds with a few drops of concentrated HCl. On cooling a magma of fine needles resulted, identified as protocatechuic acid by mixed m.p.

Conversion of 5-Dehydroquinic Acid to 5-Dehydroshikimic Acid.—An aqueous solution (10 ml.) of pure III (20 mg.) was kept at 100° for 90 minutes in the presence of a strongly acidic resin, Amberlite IR-120. The filtrate was found by paper chromatography to contain both III and II.[27] On chromatographing the filtrate on a charcoal column and eluting with ethanol of increasing strength II was mainly eluted, in agreement with earlier findings,[1] by 60–75% ethanol. Evaporation *in vacuo* of these eluates yielded crystals, but these could not be separated from contaminating glassy material.

Another experiment, in which 15 mg. of III was heated in 10 ml. of 0.1 *N* HCl at 100° for 60 minutes, clearly showed that by-products are formed along with II; the absorption spectrum revealed end absorption with a broad plateau between 235 and 250 mμ, rather than the expected sharp maximum of II at 234 mμ. However, characteristic peaks of III-thiosemicarbazone (λ 264 mμ) and of II-thiosemicarbazone (λ 315 mμ) could be shown (Fig. 2) after this material was evaporated to dryness *in vacuo* and allowed to react for 48 hours at room temperature with 7.2 mg. of thiosemicarbazide in 25 ml. of water.

Catalytic Hydrogenation.—Forty mg. of III was dissolved in 7 ml. of ethanol and saturated with hydrogen in the presence of 25 mg. of PtO_2. The catalyst was filtered off and washed with a small quantity of water. The solution was assayed for III and IV (*i.e.*, alkali-labile and stable growth-factor activity) both before and after hydrogenation. Before hydrogenation the total activity, equal[6] to that of 16 mg. of I, was destroyed by alkali treatment; after hydrogenation the total activity present was alkali-stable but was equivalent to only 8 mg. of I. The loss of activity may indicate formation of some of the unknown, presumably biologically inactive 5-epimer of IV, or hydrogenolysis of part of the material. No II-activity was present at any time.

The solution was evaporated to dryness *in vacuo*. The residue was moistened several times with ethyl acetate, which was distilled off *in vacuo;* finally, a few drops of ethyl acetate were added. On rubbing crystallization took place. After filtering and washing with ethyl acetate the recovered crude material weighed 10 mg., much of the product having adhered to the glass. The product was recrystallized from boiling ethyl acetate; m.p. 168–169°; authentic quinic acid 168–170° (lit. 161–162°)[28]; mixed m.p. 168–169°. The crystals had alkali-stable growth-factor activity under the conditions of bioassay for IV.

For further identification of the IV obtained, a small sample was converted to acetone quinide by the method of Grewe and Nolte.[29] The product was obtained in fine crystals, but the small amount available was not sufficient for recrystallization; however, the material was compared with authentic acetone quinide by chromatography in *n*-butanol–formic acid followed by spraying of the chromatogram with 0.2 *N* HCl and steaming at 100° to liberate IV. Both spots, located by bioautography, had an R_f value of 0.87.

Treatment of Dehydroquinic Acid with Acetone.—Four mg. of III was shaken at 37° with 4 ml. of acetone and a small quantity of the acidic resin Amberlite IR-120. After 30 hours the growth-factor activity of a sample of the filtrate was undiminished. The mixture was filtered, the filtrate evaporated, and the residue recrystallized from acetone–chloroform. The resulting crystals were unchanged III; they melted at 135–139°, resolidified on further heating and remelted at 193–197°. The activity of I and IV in similar experiments had been reduced to a small fraction of the original, owing to formation of the acetonides, which may be isolated.

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(26) The ε values have limited significance since thiosemicarbazone formation is slow and is accompanied by decomposition. In Fig. 2, for instance, which shows the spectra of the thiosemicarbazones formed in very dilute solution, the extinction of the 266 mμ peak of III-thiosemicarbazone is obviously low in comparison with the value given above.

(27) II was demonstrated by its growth-factor activity for mutant 83-1 as well as for A170-143S1. In addition, when viewed under a Mineralight lamp it is easily visible, in contrast to III, as a dark spot on the fluorescing paper.

(28) Beilstein, Vol. X, p. 535. The higher value found here may be due to the use of the Fisher–Johns apparatus; *cf.* the value of 183–184° found (G. Tanret, *Bull. soc. chim.*, [4] **29**, 223 (1921)) in closed capillary or on the Maquenne block, and the m.p. 174° reported by A. Watanabe, *J. Pharm. Soc. Japan*, **56**, 71 (1936); *C. A.*, **31**, 2062 (1937).

(29) R. Grewe and E. Nolte, *Ann.*, **575**, 1 (1952).