361. *Gallotannins. Part IV.*[1] *The Biosynthesis of Gallic Acid.*

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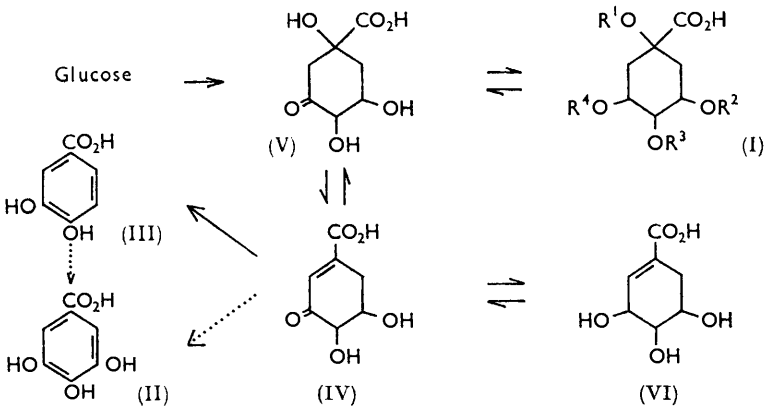
The biosynthesis of gallic and protocatechuic acid from glucose in the mould *Phycomyces blakesleeanus* has been confirmed but it is unlikely that the former arises by oxidation of the latter acid. Qualitative and quantitative results indicate that the gallic acid is produced from glucose by a scheme similar to that suggested by Davis for the biosynthesis of aromatic amino-acids, but with 5-dehydroshikimic acid as the immediate precursor.

Our interest in the biosynthesis of gallic acid was stimulated by observations[1] on the tannin from the pods of *Caesalpinia spinosa* which showed it to have a galloylated quinic acid structure (I; R^1, R^2, R^3, R^4 = galloyl or polygalloyl), instead of the more usual galloylated glucose structure of the gallotannins from Chinese and Turkish galls and sumach leaves.[2] The co-occurrence of quinic (I; R's = H), and gallic acid (II) in the form of a gallotannin suggested that the former might be the biological precursor of gallic acid, and accordingly we have traced the route of biosynthesis of gallic acid in the mould *Phycomyces blakesleeanus* as a preliminary to the study of its biosynthesis and that of gallotannins in plants.

Several workers[3,4] have reported the formation of gallic acid by *Phycomyces blakesleeanus* when grown on glucose media, and Albrecht and Bernard[3] have isolated both this acid and protocatechuic acid (III) from old cultures of the mould. These reports have been confirmed, and the presence of gallic acid in the culture media was demonstrated by paper chromatography after 6—8 days' growth of the mould on glucose media; quantitative measurements indicated at this stage a concentration of 17 μg. of gallic acid per c.c. Both gallic and protocatechuic acid were isolated by counter-current distribution from cultures grown for 56 days, and the presence also of pyrogallol and catechol in these cultures was shown by paper chromatography, though these were not isolated.



Two biosynthetic pathways were considered for the formation of gallic acid; the first of these was the oxidation (III ⟶ (II), and the second the dehydrogenation (IV ⟶ II). The former, which has some chemical analogies,[5] would be similar to the formation of

[1] Part III, preceding paper.
[2] Freudenberg, "Tannin, Cellulose, Lignin," Verlag Chemie, Berlin, 1933.
[3] Albrecht and Bernard, *Helv. Chim. Acta*, 1947, 627.
[4] Brucker, (*a*) *Naturwiss.*, 1954, 309; (*b*) *Flora*, 1956, 159; (*c*) *Planta*, 1957, 627; Schroter, *Kultur pflanze*, 1956, 49; Dennison, *Nature*, 1959, **184**, 2036.
[5] Loudon and Summers, *J.*, 1954, 1134.

protocatechuic acid (III) itself from *p*-hydroxybenzoic acid as observed in *pseudomonas*.[6] However, it is unlikely that this mechanism is responsible for the biogenesis of gallic acid in *Phycomyces blakesleeanus* as the growth of the mould on a glucose medium containing small amounts of [^{14}C]protocatechuic acid did not give any isotopically labelled gallic acid. (The generally labelled [^{14}C]protocatechuic acid was prepared from [^{14}C]quinic acid obtained by the growth of roses in an atmosphere of [^{14}C]carbon dioxide, as described by Weinstein, Porter, and Laurencot.[7] Oxidation of the quinic acid with platinum and oxygen gave good yields of 5-dehydroquinic acid (V) which was quantitatively converted into protocatechuic acid with hydrochloric acid.[8])

Experiments with the widely distributed plant enzyme tyrosinase, which it is believed possesses the ability to bring about the insertion of a new hydroxyl group in the *ortho*-position to the one already present in a monohydric phenol,[9] indicated its inability to effect the conversion of protocatechuic acid into gallic acid and it is therefore concluded that oxidation of protocatechuic acid is not the mode of biogenesis of gallic acid.

The second route of biosynthesis involved the dehydrogenation of 5-dehydroshikimic acid (IV), one of the intermediate products in Davis's scheme [10] for the biosynthesis

FIG. 1. *Production of gallic acid from media containing* (A) *glucose, or glucose plus* (B) *shikimic acid,* (C) *5-dehydroquinic acid, or* (D) *5-dehydroshikimic acid.*

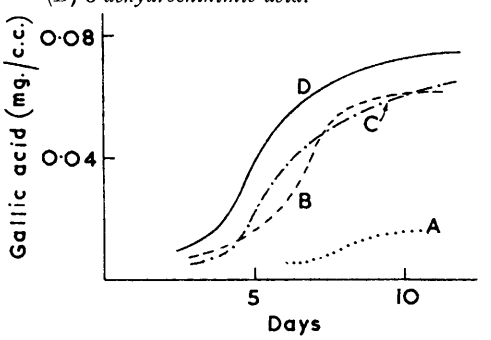
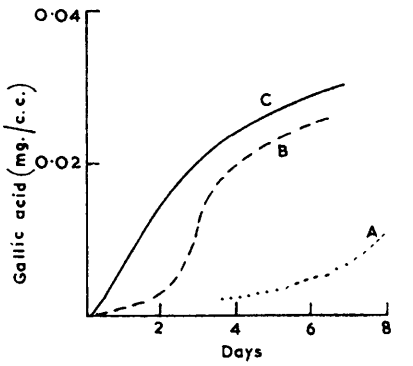


FIG. 2. *Production of gallic acid in replacement experiments from media containing* (A) *glucose,* (B) *5-dehydroquinic acid, or* (C) *dehydroshikimic acid.*



of aromatic amino-acids. Several workers [11,12] have shown that protocatechuic acid is formed in micro-organisms by dehydration of 5-dehydroshikimic acid (IV) and Gross,[11] using isotopically labelled precursors, has shown that this proceeds by elimination of the 3-hydroxyl group. The derivation of gallic acid by dehydrogenation of the same intermediate product (IV), followed by enolisation, was equally plausible. In support, 5-dehydroshikimic acid (IV) is oxidised to gallic acid by aeration in alkaline solution or, in good yield, by the action of Fehling's solution. In addition both 5-dehydroshikimic (IV) and shikimic acid (VI) were isolated from old cultures of *Phycomyces blakesleeanus* by ion-exchange chromatography, thus showing that this route of biosynthesis was operative in the mould. Final proof that 5-dehydroshikimic acid is the immediate precursor of gallic acid in *Phycomyces blakesleeanus* was derived from growth experiments. The mould was grown on glucose media which had been separately supplemented with one of the possible precursors quinic (I; R's = H), shikimic (VI), 5-dehydroquinic (V), and

[6] Evans, *Biochem. J.*, 1947, **41**, 373.
[7] Weinstein, Porter, and Laurencot, *Contrib. Boyce Thompson Inst.*, 1959, **20**, 121.
[8] Davis and Salomon, *J. Amer. Chem. Soc.*, 1953, **75**, 5567.
[9] Dawson and Tarpley, "The Enzymes," Academic Press Inc., New York, 1951, Vol. II, p. 460.
[10] Davis, "A Symposium on Amino-acid Metabolism," Johns Hopkins, Baltimore, 1956.
[11] Gross, *J. Biol. Chem.*, 1958, **233**, 1146.
[12] Tatum, *Proc. Nat. Acad. Science* (*U.S.*), 1954, **40**, 271.

5-dehydroshikimic acid (IV), and the production of gallic acid followed quantitatively in each medium. When formation of gallic acid by the mould grown on glucose was taken as standard, increased production was observed (Fig. 1) in glucose media supplemented with shikimic, 5-dehydroquinic, or 5-dehydroshikimic acid, that with the last being greatest. The mould did not incorporate quinic acid. The pH of the media during these experiments was in the range 3—4 and the possibility of chemical transformation of the intermediates under these conditions was excluded by finding that no gallic acid was formed with media not subject to the action of the mould. The differing rates of formation of gallic acid in the different media were not attributable to differing rates of growth of the mould since the dry weights of mycelia grown in these ways were the same.

Further growth experiments were made by the medium-replacement technique,[13] since cell-free extracts of the enzymes in the mould could not be obtained. *Phycomyces blakesleeanus* was grown on glucose media for 12 days, whereby formation of gallic acid was established, and the media were then replaced, with little change in pH, by glucose media to which were added one of the possible precursors quinic, shikimic, 5-dehydroquinic, and 5-dehydroshikimic acid. Immediate resumption of the production of gallic acid was observed (Fig. 2) with media containing 5-dehydroshikimic acid, but with the other intermediates a period of induction (36 hr. for 5-dehydroquinic acid and 5 days for glucose alone) was observed before the formation of gallic acid was again detected, thus confirming the rôle of 5-dehydroshikimic acid as the direct precursor of gallic acid, and of shikimic and 5-dehydroquinic acids as near but not immediate precursors. Qualitative paper-chromatographic analysis of the replaced media after 3 days, when gallic acid was being produced, gave interesting results; in media replaced with 5-dehydroshikimic acid no other acid was detected, but on replacement with 5-dehydroquinic acid the presence of 5-dehydroshikimic acid was discerned. This further substantiates the rôles allotted to these acids in the biogenesis of gallic acid, since it indicates the probable necessity of the transformation of 5-dehydroquinic into 5-dehydroshikimic acid before conversion into gallic acid.

In these experiments, quantitative measurement of gallic acid in the presence of protocatechuic acid was made by a new method based on the colour developed by gallic acid with potassium iodate. Interference by protocatechuic acid was small and was limited by the use of an equimolar mixture of the two acids as the colorimetric standard: quantitative measures of the gallic acid present in 30—70% mixtures of the two acids were obtained.

Experimental

Paper Chromatography.—(1) *Phenols.* Whatman No. 1 paper was employed with the solvent systems (A) butan-1-ol–acetic acid–water (4 : 1 : 5), (B) aqueous acetic acid (6%), and (C) butan-2-ol–acetic acid–water (14 : 1 : 5). Two-dimensional chromatograms were prepared in solvent systems B and C. A ferric chloride–potassium ferricyanide spray [14] revealed the *o*-dihydroxyphenols as blue spots on a white background. The R_F values of the components in the solvent systems A, B, and C were: gallic acid 0·68, 0·52, 0·63; protocatechuic acid 0·85, 0·61, 0·75; pyrogallol 0·77, 0·76, 0·76; and catechol 0·91, 0·85, 0·91.

(2) *Cyclohexane acids.* Whatman No. 1 paper was employed with the solvent, benzyl alcohol–t-butyl alcohol–propan-2-ol–water (3 : 1 : 1 : 1) containing 2% of 90% formic acid. The R_F values of the acids were: quinic 0·22, shikimic 0·40, 5-dehydroquinic 0·25, and 5-dehydroshikimic 0·52. The acids were indicated with sprays of (*a*) Bromocresol Green, (*b*) sodium metaperiodate, sodium nitroprusside, and piperazine [15] or (*c*) sodium metaperiodate and aniline.[16] The colours developed with these reagents (*a*), (*b*), (*c*) were: quinic yellow, yellow, pink; shikimic yellow, yellow, red; 5-dehydroquinic yellow, brown, yellow; and 5-dehydroshikimic yellow, brown, yellow-green.

[13] Evans, *Ann. Reports*, 1956, **53**, 282.
[14] Kirby, Knowles, and White, *J. Soc. Leather Trades' Chemists*, 1953, **37**, 283.
[15] Cartwright and Roberts, *Chem. and Ind.*, 1955, 231.
[16] Yoshida and Hasegawa, *Arch. Biochem.*, 1957, **70**, 377.

Culture of Phycomyces blakesleeanus.—*Phycomyces blakesleeanus* was grown at 25° in full light. The culture medium recommended by Schöpfer [17] was used and contained glucose 10%, L-asparagine 0·2%, magnesium sulphate 0·05%, potassium dihydrogen phosphate 0·15%, and aneurin 0·025%.

Isolation from Culture Media.—(1) *Gallic and protocatechuic acid. Phycomyces blakesleeanus* was grown on Schöpfer medium (10 l.) for 56 days in full light, then the culture was separated from the mycelia by filtration and the mycelia were crushed with water (2 l.) in a Kenwood mixer. The combined aqueous extract and culture medium were reduced to 1 l. by rotary evaporation at 30° and passed through ZeoKarb 215 (40 × 4 cm.) to remove cations. Extraction with ether (10 × 250 c.c.) and evaporation gave a gum (0·94 g.) which paper chromatography showed to contain pyrogallol, catechol, and gallic and protocatechuic acid. The gum was subjected to a 75-tube counter-current distribution between ether and water. Concentration of fractions 18—24 gave gallic acid (0·12 g.) which crystallised from water in needles, m. p. and mixed m. p. 234—236°. Concentration of fractions 39—47 gave protocatechuic acid (0·08 g.) which crystallised from water in prisms, m. p. and mixed m. p. 195—196°.

(2) *Shikimic and 5-dehydroshikimic acid.* The aqueous solution obtained on removal of the phenolic acids by ether was concentrated at 30° to 500 c.c. and the water-soluble acids were removed by passage down Amberlite C.G. 400 (40 × 6 cm.; acetate form). The column was washed with water (10 × 1 l.) to remove traces of non-acidic material, and the acids were then eluted with 6N-acetic acid (3 × 1 l.). The acetic acid was removed from the eluate by evaporation at 30°, and the acids were re-adsorbed on a further column of Amberlite C.G. 400 (20 × 6 cm.) and eluted by gradient elution from a reservoir containing 6N-acetic acid (2 l.) and a mixing chamber containing 0·5N-acetic acid (500 c.c.). 150 Fractions of 10 c.c. were collected at the rate of 4 an hour and every fifth fraction was analysed by paper chromatography. Concentration of fractions 20—30 gave shikimic acid (0·20 g.) as a gum (methyl ester, m. p. and mixed m. p. 116—117°). 5-Dehydroshikimic acid was isolated from fractions 76—87 as a gum (0·10 g.) which after freeze-drying crystallised in needles (from ethyl acetate), m. p. and mixed m. p. 139—141° (Found: C, 49·0; H, 4·9. Calc. for $C_7H_8O_5$: C, 48·8; H, 4·7%), whose infrared spectrum was identical with that of authentic 5-dehydroshikimic acid.[18]

Oxidation of 5-Dehydroshikimic Acid.—Fehling's solution was prepared with sodium acetate sufficient to give pH 7. 5-Dehydroshikimic acid (0·10 g.) was dissolved in 0·2M-phosphate buffer (pH 8), and Fehling's solution added until no further precipitate of cuprous oxide appeared (2·0 c.c.). The solution was then acidified with 2N-hydrochloric acid and extracted with ethyl acetate (4 × 20 c.c.), and the extract dried ($CaSO_4$). Paper chromatography indicated the presence of large amounts of gallic and traces of protocatechuic acid. The former was isolated by counter-current distribution between ether and water as described above, and obtained as needles (0·075 g.), m. p. and mixed m. p. 236—240°.

Oxidations with Tyrosinase.—Samples of tyrosinase were prepared by the method of Mallette *et al.*[19] from the expressed juices of potato. The enzyme activity was tested with *p*-cresol and catechol.

The substance (1 mg.) was dissolved in a mixture of 0·1M-citric acid (2·5 c.c.) and 0·2M-disodium hydrogen phosphate (2·5 c.c.), and tyrosinase solution was added. Solutions were analysed after 1, 2, 3, 6, and 18 hr. After acidification with 2N-hydrochloric acid and extraction with ether, the extract was examined for phenolic material by paper chromatography. *p*-Hydroxybenzoic acid thus treated gave protocatechuic acid, but the latter acting as substrate gave no gallic acid.

Preparation of [^{14}C]*Quinic Acid* (cf. Weinstein, Porter, and Laurencot [7]).—Freshly cut, partially open, roses of the variety Lydia were placed in water in a bell-jar (4 × 1 l.) which was then partially evacuated. [^{14}C]Carbon dioxide was liberated in the bell-jar by the action of lactic acid (1 c.c.) on [^{14}C]barium carbonate (50 mg.). The jar was placed in full light and illuminated during periods of darkness with two 100-w electric bulbs, the temperature being kept below 28°. When the rose blooms had fully opened (3—4 days) the apparatus was evacuated once more, and the leaves and blooms (10 g.) were ground with crushed glass and water (5 × 200 c.c.). The aqueous extract was centrifuged and passed through a column of ZeoKarb 215(H^+), and the water-soluble organic acids were adsorbed on Amberlite C.G. 400 (3 × 4 cm.; acetate

[17] Schöpfer, *Arch. Mikrobiol.*, 1934, **5**, 511.
[18] Grewe and Jeschke, *Chem. Ber.*, 1956, **89**, 2080.
[19] Mallette, Lewis, Ames, Nelson, and Dawson, *Arch. Biochem.*, 1948, **16**, 288.

form). After washing of the column with water (1000 c.c.), the acids were removed by gradient elution from a mixing chamber containing 0·5N-acetic acid (400 c.c.) and a reservoir containing 4N-acetic acid (1 l.). Sixty fractions (10 c.c.) were collected, every third fraction was examined by paper chromatography, and those containing quinic acid were combined and concentrated at 30° to yield a colourless gum which crystallised from ethanol. Further crystallisation from ethanol gave [^{14}C]quinic acid (0·13 g.) as prisms, m. p. and mixed m. p. 173—174°. The specific activity, counted with a gas-flow Geiger–Müller tube, was 7200 counts min.$^{-1}$ mg.$^{-1}$.

[^{14}C]-5-*Dehydroquinic Acid.*—A solution of [^{14}C]quinic acid (0·12 g.) in water (20 c.c.) was adjusted to pH 6 with solid sodium hydrogen carbonate. Platinum (0·1 g.), freshly prepared by hydrogenation of platinum oxide, was added and a brisk stream of oxygen passed through the solution at 40° for 6 hr. The solution was filtered and passed through ZeoKarb 215 and then Amberlite C.G. 400 (10 × 15 cm., acetate form). The acids were removed by gradient elution with acetic acid, and 80 fractions (7·5 c.c.) collected and analysed by paper chromatography. Concentration of the appropriate fractions gave [^{14}C]-5-dehydroquinic acid (0·08 g.) as an oil which crystallised from acetone. Further crystallisation from acetone–chloroform gave needles, m. p. and mixed m. p. 136—138° (Found: C, 44·2; H, 5·7. Calc. for $C_7H_{10}O_6$: C, 44·2; H, 5·3%).

[^{14}C]*Protocatechuic acid.*—[^{14}C]Protocatechuic acid was prepared as described by Davis [8] by heating [^{14}C]-5-dehydroquinic acid with concentrated hydrochloric acid.

Growth of Phycomyces blakesleeanus *with* [^{14}C]*Protocatechuic Acid.*—*Phycomyces blakesleeanus* was grown on Schöpfer media (25 c.c.) containing [^{14}C]protocatechuic acid (8 mg.), and the culture medium was examined after 10 days by paper chromatography. No [^{14}C]gallic acid was detected on the chromatogram by autoradiographic analysis.

Determination of Gallic Acid.—The solution investigated (1, 2, 3, or 4 c.c.) was diluted to about 9 c.c., treated with 0·04M-potassium iodate (0·5 c.c.) and made up to 10 c.c. with water. After 4 hr. at room temperature the colour was measured at 500 mμ on a Unicam S.P. 500 spectrophotometer. The results were plotted and compared with those for a standard gallic acid solution (~10^{-3}M). Three different solutions gave (*a*) 8·0, 7·8, (*b*) 10·3, 10·2, and (*c*) 11·8, 12·1, the theoretical values being (*a*) 8·2, (*b*) 10·6, and (*c*) 12·0 mg./c.c. respectively. In the presence of protocatechuic acid, analysis was carried out with a standard solution containing equimolar amounts of gallic and protocatechuic acid. Four synthetic mixtures of gallic and protocatechuic acid, containing 75, 66·6, 33·3, and 25% of gallic acid severally, gave (*a*) 13·0, 12·7, (*b*) 14·0, 13·8, 13·9, (*c*) 11·6, 11·3, 11·9, and (*d*) 10·2, 10·4, and 10·1, and the theoretical values being (*a*) 11·9, (*b*) 13·3, (*c*) 12·0, and (*d*) 11·3 mg./100 c.c. respectively.

Growth of Phycomyces blakesleeanus *on Mixtures of Glucose and the Substrates* (I, IV, V, VI).—Four aqueous solutions (90 c.c.) containing glucose (9·0 g.) and one of the substrates quinic (I; R's = H), 5-dehydroquinic (V), shikimic (VI), and 5-dehydroshikimic acid (VI) (0·9 g.) were sterilised by filtration through " steripads." Standard sterilised Schöpfer medium (90 c.c.) from which the glucose had been omitted was added to each of these solutions and after inoculation with flooded *Phycomyces blakesleeanus* culture (3 c.c.) the mixtures were divided into thirty-six portions (5 c.c.), which were incubated in small flasks (25 c.c.) at 25° in full light. The solutions were analysed for gallic acid after 2, 4, 6, 8, 10, and 12 days, three solutions being taken for each substrate and the average of the values plotted. Mycelia were separated by filtration and washed with water (1 c.c.), and the filtrate and washings were combined and made up to 9·5 c.c. with 0·5M-acetate buffer (pH 4·0). The optical density of this solution was measured at 500 mμ, and 0·04M-potassium iodate (0·5 c.c.) was added. After 4 hr. the optical density was measured and the gallic acid content was determined by comparison with a standard gallic acid solution as indicated above. The results are plotted in Fig. 1. The weights of dried mycelia produced by each solution were determined after 6 and 12 days, but no significant differences were observed for the different substrates. Experiments in which the different media were left for 12 days without inoculation produced no gallic acid.

Replacement Experiments.—(1) *Qualitative analysis.* Four standard Schöpfer media (20 c.c.) were inoculated and incubated at 25°. After 12 days the medium was removed with a sterile pipette, and the mycelium washed with sterile water (2 × 10 c.c.) and placed in contact with a standard Schöpfer medium (20 c.c.) containing one of the substrates quinic, 5-dehydroquinic, shikimic, and 5-dehydroshikimic acid (0·20 g.). Incubation was continued for a further 3 days, then the mycelia were separated and the aqueous filtrate passed down a column of ZeoKarb

215 (1 × 20 cm.). Ether-extracts (4 × 50 c.c.) of the eluates were analysed by paper chromatography for phenolic material. The aqueous layer was concentrated at 30° and analysed similarly by paper chromatography.

(2) *Quantitative analysis.* Standard Schöpfer media (5 c.c.) were incubated at 25° for 12 days, and the media removed under the sterile conditions described above. Replacements were carried out with standard Schöpfer media (5 c.c.) sterilised by filtration and containing one of the substrates quinic, shikimic, 5-dehydroquinic, and 5-dehydroshikimic acid (25 g.). Incubation was continued at 25° and the gallic acid content of the replaced media analysed after 1, 2, 4, 6, and 8 days. The solutions were analysed for each substrate, with the results shown in Fig. 2.

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