358. *Gallotannins. Part I. Introduction: and the Fractionation of Tannase.*[1]

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Earlier work on the chemistry of the gallotannins is summarised and a description is given of the separation of the enzyme tannase into fractions with specific properties. In particular a galloyl esterase, valuable in the investigation of the gallotannins, has been isolated, which lacks significant carbohydrase activity but hydrolyses galloyl esters to gallic acid and the corresponding alcohol.

The vegetable tannins are usually classified into (1) the condensed or non-hydrolysable tannins, including extracts of Quebracho (*Schinopsis lorentzii* or *balansae*) and wattle (*Acacia mollissima*), which contain little if any carbohydrate material and are converted into insoluble, amorphous phlobaphenes by the action of mineral acids, and (2) the hydrolysable tannins which yield carbohydrates, usually glucose, and one or more phenolic acids by reaction with acids, alkalis, or enzymes.

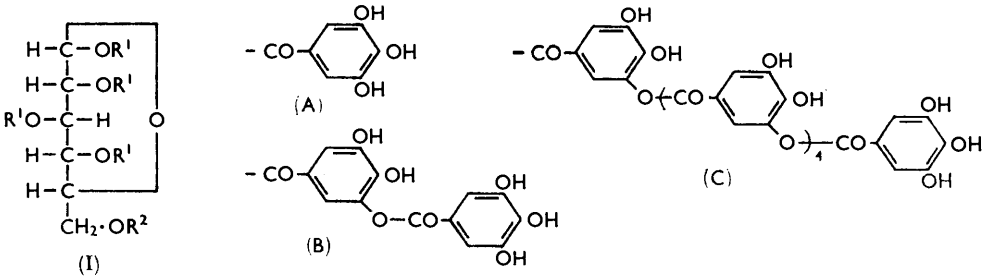
The hydrolysable tannins are subdivided into (2a) the gallotannins, including Turkish (*Quercus infectoria*, galls) and Chinese (*Rhus semialata*, galls) gallotannins and sumach tannins (*Rhus coriaria* and *typhina*), yielding glucose and phenolic acids of which gallic acid predominates on hydrolysis; and (2b) the ellagitannins, including myrobalans (*Terminilia chebula*), algarobilla (*Caesalpinia brevifolia*), divi-divi (*Caesalpinia coriaria*), and valonea (*Quercus valonea*) extracts, which give, on hydrolysis, glucose and ellagic acid together with gallic acid and frequently with other acids structurally related to gallic acid. This division of the hydrolysable tannins is not entirely satisfactory. Chebulinic acid,[2] a crystalline constituent of myrobalans, is usually included in the gallotannin group, although it has close structural relations with the ellagitannins, and the main component of Tara tannin (*Caesalpinia spinosa*) (see Part III of this series) yields quinic and gallic acid, but no glucose on hydrolysis.

The non-hydrolysable tannins are complex products of unknown constitution which probably result from the condensation [3] and/or the oxidation [4] of flavanoid compounds such as catechin or the leucoanthocyanins, although the work of Grassmann, Endres, and Pauchner [5] on the tannin of spruce bark suggests that other structural units may be involved. Remarkable advances have been made in recent years in ellagitannin chemistry, particularly by Schmidt and his colleagues [6] at Heidelberg, who have suggested attractive formulæ for corilagin and chebulagic acid (from myrabalans and divi-divi), and brevifolincarboxylic acid and brevilagin (from algarobilla).

The chemistry of the gallotannins, which is the main concern of this and subsequent papers, formed the subject of the classical work of Emil Fischer.[7] As a result of (*a*) analytical work including the amounts of glucose and gallic acid liberated by hydrolysis, and (*b*) the yields of 3,4-di-*O*-methyl- and 3,4,5-tri-*O*-methyl-gallic acid obtained by hydrolysis of the methylated tannin, Fischer [7] and Freudenberg [8] concluded that the amorphous Chinese gallotannin was a mixture of isomers and closely related compounds whose average composition corresponded to that of a β-penta-*m*-digalloylglucose (I; $R^1 = R^2 = B$).

[1] For a preliminary account see Haworth, Jones, and Rogers, *Proc. Chem. Soc.*, 1958, 8.
[2] Fischer and Freudenberg, *Ber.*, 1912, **45**, 915; Fischer and Bergmann, *Ber.*, 1918, **51**, 298; Freudenberg, *Ber.*, 1919, **52**, 1238; Freudenberg and Fick, *Ber.*, 1920, **53**, 1728; Freudenberg, *Annalen*, 1927, **452**, 303; Schmidt, *Fortschr. Chem. org. Naturstoffe*, 1956, 72.
[3] Freudenberg and Weinges, *Annalen*, 1954, **590**, 140.
[4] Hathway and Seakins, *J.*, 1957, 1562; *Biochem. J.*, 1957, **65**, 32P; 1957, **67**, 239.
[5] Grassmann, Endres, and Pauckner, *Ber.*, 1958, **91**, 136, 141.
[6] Schmidt, ref. 2; *Z. Naturforsch.*, 1957, **12**, 262.
[7] Fischer, *Ber.*, 1913, **46**, 3253; 1919, **52**, 809.
[8] Freudenberg, "Tannin, Cellulose, and Lignin," Verlag Chemie, Berlin, 1933, p. 38.

Comparison of Chinese gallotannin with the amorphous synthetic β-penta-*m*-digalloylglucose [9] revealed very many similarities, although differences in water solubility and optical rotatory values were recorded. Karrer, Salomon, and Payer [10] separated Chinese gallotannin by precipitation with aluminium hydroxide into fractions of varying optical rotatory values, which yielded, on treatment with acetic acid and hydrobromic acid, varying amounts of tetra-*O*-galloylglucosyl bromide, thus supporting the basic structure advanced by Fischer. The latter, however, envisaged the possibility of the presence of tri- or even tetra-galloyl chains in the tannin, and Freudenberg [11] emphasised the complexity of the problem by pointing out that many arrangements, varying from penta-*m*-digalloylglucose to a compound (I) where R^1 = A and R^2 = C, were consistent with the experimental observations. The claim by Nierenstein and his colleagues [12] to have isolated tetra-*O*-methylglucose by hydrolysis of diazomethane-methylated tannin has been adequately criticised,[13] but the absence of alcoholic hydroxyl groups has never been convincingly established.



Similar analyses of Turkish gallotannin by Fischer and Freudenberg [14] indicated an average composition of a β-pentagalloylglucose (I; $R^1 = R^2$ = A), and the amorphous tannin displayed many similarities to the synthetic product.[9] The isolation, however, of small amounts of 3,4-di-*O*-methylgallic acid from hydrolysis of the methylated tannin showed that some of the gallic acid was linked in the form of a depside, and Freudenberg [8] concluded that, on average, one of the five glucose hydroxyl groups is free, one is esterified with *m*-digallic acid, and the remaining three with gallic acid. Karrer, Widmer, and Staub [15] separated Turkish gallotannin by precipitation with aluminium hydroxide into fractions with varying optical rotatory values, and like Fischer and Freudenberg [14] they reported the presence of significant amounts of ellagic acid in Turkish gallotannin.

More recent work, usually involving chromatographic methods, has revealed certain anomalies with earlier results. Thus Asquith [16] has shown by paper-chromatographic techniques that synthetic β-pentagalloylglucose, prepared by Fischer's method, is impure, and White,[17] although confirming the presence of appreciable amounts of ellagic acid in Turkish gallotannin, states that pentagalloylglucose " is of little if any significance " in the extract. Kirby, Knowles, and White,[18] and White,[17] have demonstrated that Fischer's Chinese gallotannin was contaminated with gallic and *m*-digallic acid, and traces of two other compounds which they considered were pentagalloylglucose and a trigallic acid respectively. The main gallotannin constituent, isolated by ethyl acetate

[9] Fischer and Bergmann, *Ber.*, 1918, **51**, 1760.
[10] Karrer, Salomon, and Payer, *Helv. Chim. Acta*, 1923, **6**, 17.
[11] Freudenberg, " Die Chemie der naturlichen Gerbstoffe," Verlag Chemie, Berlin, 1920, p. 101.
[12] Nierenstein, Spiers, and Geake, *J.*, 1921, **119**, 284.
[13] Schmidt, *Annalen*, 1934, **479**, 1.
[14] Fischer and Freudenberg, *Ber.*, 1912, **45**, 915, 2709; 1914, **47**, 2485.
[15] Karrer, Widmer, and Staub, *Annalen*, 1923, **433**, 288.
[16] Asquith, *Nature*, 1951, **168**, 738—739.
[17] White, " The Chemistry of the Vegetable Tannins," Society of Leather Trades' Chemists, Croydon, 1956, p. 13.
[18] Kirby, Knowles, and White, *J. Soc. Leather Trades' Chemists*, 1951, **35**, 338; 1952, **36**, 148.

from an aqueous-alcoholic extract buffered to pH 6·5 by means of sodium hydrogen carbonate, was an amorphous powder giving analytical results inconsistent with Fischer's formulation. As a result of new methods of analysis it was claimed that (1) gallotannin contained 4—5 galloyl groups per hexose molecule, (2) hydrolysis of the methylated product, which still contained acetylable hydroxyl groups, gave a high yield of 3,4,5-tri-*O*-methylgallic acid but very little 3,4-di-*O*-methylgallic acid, and (3) the chromatographic evidence and the analytical results suggested a trisaccharide and not a glucose core. The suggested polygalloylated trisaccharide structure,[17] which is quite inconsistent with our results, was subsequently withdrawn by King and White.[19]

Lowe [20] suggested that Sicilian sumach (*Rhus coriaria*) extract was identical with Chinese gallotannin, but Karrer, Widmer, and Staub [15] suggested a close resemblance with Turkish gallotannin. Catravas and Kirby [21] later showed that two-way paper chromatograms of Sicilian sumach and Chinese gallotannin were indistinguishable, but claimed that the tannin contained 16·8—17·7% of glucose instead of the 4—8% reported by the earlier workers. More recently Grassmann, Stiefenhofer, and Endres [22] have claimed that the tannin of Stagshorn sumach (*Rhus typhina*) is a polygalloylated tetrasaccharide, composed of arabinose, rhamnose, and two molecules of glucose, but again the claims are quite inconsistent with the results reported in the present series of papers.

This brief review indicates that further work is necessary, and that the following are some (but by no means all) points which require attention: (1) the homogeneity of the gallotannins, (2) the nature of the carbohydrate core, (3) the presence of polygalloyl chains, (4) the extent of esterification of the carbohydrate core.

In this paper an attempt has been made to develop a method which would differentiate between the conflicting views of Fischer,[7] White,[17] and Grassmann *et al.*[22] on the nature of the carbohydrate nuclei in the gallotannins and in the sumach tannins. It is agreed by all that the ultimate products of hydrolysis by acids and by the enzyme tannase are gallic acid and glucose, but White [17] and Grassmann *et al.*[22] claim that intermediate polysaccharides are produced by hydrolysis with alkali. The enzyme tannase, which has been frequently used in the analysis of tannins, is produced by the growth of *Aspergillus niger* on solutions of commercial myrobalans [23] or gallotannin.[24] This mould is known to contain specific carbohydrases and we have investigated these activities in tannase, although the main objective behind these experiments was the separation, if possible, of specific galloyl esterases from the carbohydrases of the enzyme.

It has been shown that a specific maltase active against maltose but not against methyl α-glucoside,[25] a trehalase active against trehalose,[26] and β-glucosidases active against cellobiose and salicin,[27] were present in tannase, but the presence of previously reported β-rhamnosidase,[28] activive against quercetrin, was not confirmed. Some, though not all, preparations of tannase hydrolysed melibiose, turanose, starch, and inulin. The hydrolysis of sucrose by tannase was shown to be due to a β-fructoinvertase and not to the alternative α-glucoinvertase, since raffinose was readily degraded to glucose, galactose, and melibiose whereas melizitose gave only traces of glucose with the enzyme. Tannase also hydrolysed methyl gallate, protocatechuate, and 3,5-dihydroxybenzoate, but not methyl salicylate, 2,4-dihydroxybenzoate, or 2,5-dihydroxybenzoate, and these results are in agreement with the previous observations by Dyckerhoff and Armbruster.[29] The finding of these authors

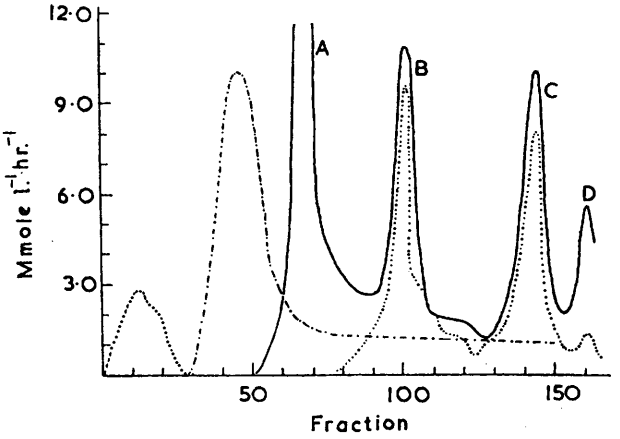
[19] King and White, *Chem. and Ind.*, 1958, 683.
[20] Löwe, *Z. analyt. Chem.*, 1873, **12**, 128.
[21] Catravas and Kirby, *J. Soc. Leather Trades' Chemists*, 1948, **32**, 155.
[22] Grassmann, Stiefenhofer, and Endres, *Chem. Ber.*, 1956, **89**, 454.
[23] Freudenberg, Blümmel, and Frank, *Z. physiol. Chem.*, 1927, **164**, 262.
[24] Knudsen, *J. Biol. Chem.*, 1913, **14**, 159.
[25] Bourquelot, *Compt. rend.*, 1883, **97**, 1322.
[26] Bourquelot, *Compt. rend.*, 1893, **116**, 826.
[27] Hofmann, *Biochem. Z.*, 1934—1935, **275**, 320.
[28] Freudenberg and Walpulski, *Ber.*, 1921, **54**, 1659.
[29] Dyckerhoff and Armbruster, *Z. physiol. Chem.*, 1933, **219**, 38.

that methyl *m*- and *p*-hydroxybenzoate, and ethyl and phenyl acetate, acted as substrates for tannase was, however, not confirmed.

Quantitative measurements of the β-glucosidase activity were made by using salicin,[30] of the β-fructoinvertase by using sucrose,[31] of the maltase by estimation of the glucose after hydrolysis of maltose, and of the galloyl esterase by hydrolysis of methyl gallate by a modification of the method described by Schmidt.[32] The hydrolysis of methyl gallate was shown to follow zero-order kinetics, whereas Dyckerhoff and Armbruster [29] report the reaction to be of first-order; but these authors used long reaction times during which the liberated gallic acid might inhibit the enzyme or cause an alteration in the buffer pH. The pH optimum of the galloyl esterase, however, lay in the range 5·0—6·5 as recorded by these workers.[29]

Preliminary attempts at the fractionation of tannase were made by heat treatment, by the action of cupric and mercuric ions, and by precipitation with ammonium sulphate. Some denaturation of the carbohydrases was observed on heating and the invertase was inhibited by mercuric ions in concentrations greater than 10^{-5}M, but no separation was

Fractional elution of tannase ingredients.



—— *Esterase*; – – – *maltase*; . . . *β-glucosidase*; – · — · — *invertase*

observed with ammonium sulphate. Column electrophoresis [33] gave a partial separation and also indicated that the enzymes were acidic proteins having isoelectric points below 5, but the methods were not of practical value in separation of the esterases from the carbohydrases of the enzyme.

Separation of the galloyl esterase was eventually achieved by ion-exchange chromatography on the basic resin Dowex " 2," gradient elution with a buffer of decreasing pH being used. Qualitative tests on the substrates mentioned above showed that the maltase was eluted initially, followed by the invertase and the esterase, but much of the latter was overlapped by β-glucosidases. No melibiase was detected in the eluate, although the starting material was active. Quantitative determinations of the enzyme activities gave the more complete picture illustrated in the Figure. Attempts to correlate the enzymic activities with the protein content of the eluates were unsuccessful and this may be due to the presence of much inert protein in the original tannase.

The galloyl esterase was eluted in four peaks (A,B,C,D), three (B,C,D) of which were

[30] Bray, Thorpe, and White, *Biochem. J.*, 1950, **46**, 275.
[31] Park and Johnson, *J. Biol. Chem.*, 1949, **181**, 150.
[32] Schmidt, " Die Methoden der Fermentforschung," Georg. Thieme, Leipzig, 1941.
[33] Fonss-Beck and Li, *J. Biol. Chem.*, 1954, **207**, 175.

largely contaminated with the β-glucosidase. The main esterase peak (A) was inactive towards salicin, maltose, raffinose, melizitose, nigerose, isomaltose, gentiobiose, and maltotetrose, but showed a slight activity towards sucrose which was obviously due to the tailing of the invertase peak. Attempts to free the esterase (A) from the small invertase activity were made by further chromatography of the esterase and by chromatography of tannase which had undergone preliminary purification by precipitation of inert material at pH 9·0 and autolysis at pH 5·0; both these methods, however, resulted in the loss of esterase activity.

Consequently we have used the component corresponding to the main specific galloyl esterase peak (A) to investigate the nature of the carbohydrate core of Chinese and Turkish gallotannins, Sicilian and Stagshorn sumach and of other tannins. As this specific galloyl esterase lacks significant carbohydrase activities, it would be expected to hydrolyse the tannins to gallic acid and the carbohydrate core. It is important to appreciate that the small residual invertase activity will not invalidate in any way the conclusions derived from the action of the enzyme on the tannin, since earlier work [7] and the more recent chromatographic evidence of Grassmann, Stiefenhofer, and Endres [22] and ourselves has demonstrated that the tannins investigated do not contain fructose. The detailed results of the action of this esterase on the gallotannins are described in later papers, but in all cases examined the products of hydrolysis were glucose and gallic acid. No indication of other carbohydrates was detected and it is concluded that Turkish and Chinese gallotannins, and Sicilian and Stagshorn sumach tannins, are galloylated glucoses, as suggested originally by Fischer,[7] and not galloylated polysaccharides, as suggested by later workers.[17,22]

Experimental

Preparation of Tannase.—A sample of *Aspergillus niger* " 106 " was kindly supplied by Professor S. R. Elsden and used throughout this work. The mould was grown on the 2% tannic acid solution described by Knudsen [24] adjusted to pH 6·5 with 2N-sodium hydroxide. Incubation was carried out at 30° for 6 days, then the mycelium was collected and converted into the " acetone powder." The latter (48 g.) was extracted by shaking with 0·2% sodium chloride solution (1 l.) at 2° for 24 hr. and the mixture was then filtered through a pad of Hyflo-supercel to give a clear brown solution. The latter was dialysed for 18 hr. at 2° against 0·001N-acetate buffer (pH 5·0) and after freeze-drying gave tannase (0·970 g.).

Enzymic Activities of Tannase.—(1) *Qualitative methods.* (*a*) Carbohydrases. Sucrose, cellobiose, maltose, lactose, raffinose, melibiose, turanose, trehalose, salicin, methyl α-glucoside, and starch were used as 0·5% solutions in 0·1N-acetate buffer (pH 5·0). Inulin was used as a saturated solution in the same buffer, and quercetrin as a saturated solution containing 10% ethanol. The substrates (0·5 c.c.) were incubated overnight at 37° with 0·1% tannase solution (0·1 c.c.), and the hydrolysis products identified by paper chromatography on Whatman No. 1 paper in the solvent system butan-2-ol–acetic acid–water (14 : 1 : 5) with known carbohydrates as markers. The sugars were detected by spraying with aniline hydrogen phthalate [34] or silver nitrate.[35] The results are discussed on p. 1831.

(*b*) Esterase. The substrates were tested as 0·5% solutions in 0·1N-acetate buffer (pH 5·0; 0·5 c.c.) and incubated overnight with 0·1% tannase solution in 0·1N-acetate buffer (pH 5·0; 0·1 c.c.). The solutions were chromatographed on Whatman No. 1 paper in 6% acetic acid, and the products identified with markers. *ortho*- and *para*-Dihydric phenols were detected with a ferric chloride–potassium ferricyanide [36] spray, and other phenols with a spray of diazotised sulphanilic acid.[37] The results are discussed on p. 1831.

Ethyl and phenyl acetate were used as 0·1% solutions in aqueous acetone (5%) adjusted to pH 5 with 0·1N-sodium hydroxide. 0·1% Tannase solution (0·1 c.c.) was added and the reaction followed on an autotitrator. No alkali was consumed for 1 hr.

[34] Hough, *J.*, 1950, 1702.
[35] Trevelyan, Porter, and Harrison, *Nature*, 1950, **166**, 144.
[36] Kirby, Knowles, and White, *J. Soc. Leather Trades' Chemists*, 1953, **37**, 283.
[37] Ames and Mitchell, *J. Amer. Chem. Soc.*, 1952, **74**, 252.

(2) *Qualitative methods.* The unit of enzyme activity was defined as 1 litre of enzyme solution which in 1 hr. at 37° will hydrolyse 1 millimole of substrate.

(*a*) Invertase. 0·1% Tannase solution (1·0 c.c.) was incubated at 37° with sucrose solution (3·0 c.c.; $1{\cdot}33 \times 10^{-2}$M) in 0·1N-acetate buffer (pH 5·0). Samples were withdrawn every 10 min. and the glucose was determined by the method of Park and Johnson.[31]

(*b*) β-Glucosidase. 0·1% Tannase (1·0 c.c.) was incubated at 37° with salicin solution (3·0 c.c.; $1{\cdot}3 \times 10^{-2}$M) in 0·1N-acetate buffer (pH 5·0). Samples were withdrawn every 10 min. and analysed for saligenin by the Folin reagent.[30]

(*c*) Maltase. An approximate measure of maltase activity was obtained by incubation of 0·1% tannase solution (0·25 c.c.) with maltose solution ($1{\cdot}3 \times 10^{-2}$M) in 0·01N-acetate buffer (pH 5·0; 0·75 c.c.). Samples (0·01 c.c.) were chromatographed on Whatman No. 1 paper together with standard glucose solutions in butan-2-ol–acetic acid–water (14 : 1 : 5); the glucose spots on the chromatograms were cut out and eluted with water (4·0 c.c.) for 4 hr. After filtration, aliquot parts (3·0 c.c.) were estimated for glucose.[31]

(*d*) Galloyl esterase. 0·5% Methyl gallate solution (0·9 c.c.) was adjusted to pH 5·0 with 0·01N-sodium hydroxide, and 0·1% tannase solution (0·1 c.c.) was added. The reaction was followed by titration of the liberated gallic acid with 0·01N-sodium hydroxide to pH 6·80 on an autotitrator. The consumption of alkali was 0·15, 0·29, 0·44 c.c. after 10, 20, and 30 min. respectively.

pH *Optimum of Galloyl Esterase.*—The activity of the galloyl esterase was determined at pH 4·0, 5·0, 5·5, 6·0, 6·5, and 7·0, the following values of enzymic activity being obtained: 33, 33·5, 43, 52, 52·5, 53, and 49 respectively. The enzymic activities determined in the presence of a 0·1-molar ratio of free gallic acid at pH 4·5, 5·0, 5·5, 6·0, and 6·5 were 21, 37, 42, 47, and 46 respectively.

Fractionation of Tannase.—(a) *Action of heat on tannase.* A 0·1% solution of tannase (2 c.c.) was heated from 30° to 100° during 45 min. and, after dilution to 5 c.c., samples (0·2 c.c.) were tested for activity against methyl gallate, sucrose, and salicin. Comparison with an identical unheated solution showed that the invertase and β-glucosidase activities had been reduced. The esterase was unaffected.

(b) *Action of cupric and mercuric ions on tannase.* To samples of the substrate (sucrose, salicin, or methyl gallate) in 0·1N-acetate buffer (pH 5·0; 0·8 c.c.) were added portions (0·1 c.c.) of cupric sulphate or mercuric chloride solution so as to give final concentrations of these ions in the range 10^{-3}—10^{-6}M. 0·1% Tannase solution (0·1 c.c.) was added to each and the solutions were incubated for 12 hr. at 37°. Solutions were similarly prepared for comparison containing tannase and substrate, and tannase and metal ions. Examination by paper chromatography showed that mercuric ions in excess of 10^{-4}M inactivated the invertase. No effect was observed with the cupric ions.

(c) *Column electrophoresis of tannase.* The apparatus used was similar to that used by Fonss-Beck and Li.[33] Starch was packed as a slurry with the appropriate buffer solution (0·1N-acetate for pH 5·0; 0·1N-phosphate for pH 7·0 or pH 8·0) in a semicylindrical glass trough (50 × 2·5 cm.). Excess of buffer was drained off a slot cut in the middle of the column, and a piece of Whatmann " 3MM " paper, which had previously been soaked in water (0·1 c.c.) containing tannase (3 mg.), was inserted. The column was covered with liquid paraffin to prevent evaporation, and a current of 18 milliamp. at 230 v passed for 24 hr. at 2°. The liquid paraffin was removed by washing with light petroleum (b. p. 60—80°), and the column cut into 1 cm. sections with a thin-bladed scalpel. Each section was extracted by shaking with 0·1N-acetate buffer (pH 5·0; 5·0 c.c.) for 3 hr. and, after centrifuging, samples were tested for esterase, β-glucosidase, and invertase activity. The tabulated results were obtained

	pH of electrophoresis		
	5·0	7·0	8·0
Esterase	Sections 1—5	Sections 3—9	Sections 3—13
Invertase	,, 1—4	,, 1—5	,, 1—9
β-Glucosidase	,, 3—6	None detected	,, 5—13

(d) *Chromatography of tannase on Dowex* " 2 ". Dowex " 2 " resin (200—400 mesh) was equilibriated with 0·15N-acetate buffer (pH 4·75) and set up as a column (13 × 3·2 cm.). A solution of tannase (66 mg.) in 0·15N-acetate buffer (pH 4·75; 2·0 c.c.) was adsorbed, and

gradient elution carried out at 2° with 0·2N-acetate buffer in the reservoir and a mixing chamber containing 0·15N-acetate buffer (pH 4·75; 400 c.c.). Fractions (9·0 c.c.) were collected at the rate of 2 per hr.; at fraction 110 the buffer solution in the reservoir was replaced by 0·75N-acetic acid (pH 2·50) and a further 57 fractions collected. Every sixth fraction was dialysed at 2° for 18 hr. against distilled water and qualitative tests were carried out with methyl gallate, sucrose, salicin, cellobiose, and melibiose. Quantitative estimations were also made and the protein content determined by the method of Lowry *et al.*[38] Fractions were combined as shown for further study:

Fractions	2—22	32—50	62—81	92—112	139—153	159—165
			A	B	C	D
Enzyme	Maltase	Invertase	Esterase	Esterase	Esterase	Esterase

The combined fractions were concentrated at 25°, dialysed, and freeze-dried, and solutions in 0·01N-acetate buffer (pH 6·0; 4·0 c.c.) were stored at −10°. The esterase fractions were tested for carbohydrase activity by incubating samples (0·1 c.c.) for 48 hr. with 0·5% solutions of sucrose, maltose, nigerose, isomaltose, gentiobiose, and maltotetrose and 1% solutions of raffinose and melizitose in 0·05N-acetate buffer (pH 6·0; 0·5 c.c.). The solutions were prepared for chromatography by passage down a small column of Dowex " 50 " to remove sodium ions, and paper chromatograms were then prepared on Whatman No. 1 paper in butan-2-ol–acetic acid–water (14 : 1 : 5). The first esterase fraction (A) was found to be slightly active against sucrose, and the remaining esterase fractions (B, C, D) were also active against salicin.

(e) *Attempted rechromatography of the esterase fraction A.* A sample of the esterase fraction (A) was rechromatographed on a small column of Dowex " 2 " as described above. The esterase fractions were collected, but on dialysis were rapidly inactivated.

Preliminary Preparation of Tannase.—(a) *Treatment of tannase at* pH 9·0. A 1% solution of tannase (5·0 c.c.) was cooled in ice whilst N-sodium hydroxide was added dropwise until the pH reached 9·0, a heavy precipitate then appearing. This was removed by centrifugation and the supernatant liquid was found to retain all its original activity towards sucrose, salicin, and methyl gallate. After dialysis the tannase was freed from solution by freeze-drying.

(b) *Autolysis of tannase at* pH 5·0. Tannase (24 mg.) which had been subject to treatment at pH 9·0 was dissolved in 0·1N-acetate buffer (pH 5·0; 5·0 c.c.) and incubated under toluene at 37° for 24 hr. After dialysis the material still retained its activity against sucrose, salicin, and methyl gallate, and the two-dimensional chromatograms on Whatman No. 1 paper run in 6% acetic acid and butan-2-ol–acetic acid–water (14 : 1 : 5) did not respond to sprays of silver nitrate [35] or aniline hydrogen phthalate.[34]

Chromatography of Tannase after Treatment at pH 9·0 *and* pH 5·0.—Partially purified tannase (50 mg.) was chromatographed on Dowex " 2 " as described above. The esterase fractions were detected by their activity towards methyl gallate. Concentration at 25° and dialysis, however, rapidly inactivated the esterase.

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THE UNIVERSITY, SHEFFIELD, 10. [*Received, November 9th*, 1960.]

[38] Lowry, Rosenbrough, and Randall, *J. Biol. Chem.*, 1951, **193**, 265.