Phytochemistry, 1968, Vol. 7, pp. 2147 to 2156. Pergamon Press. Printed in England

THE GALLOYL GLUCOSE COMPOUNDS IN GREEN CAROB PODS (*CERATONIA SILIQUA*)

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(*Received* 19 *October* 1967, *revised version received* 28 *March* 1968)

Abstract—The phenolic compounds present in green carob pods were separated by solvent extraction into five fractions. In addition to leucodelphinidin, nine hydrolysable tannins were found as major phenols. Among them, two galloylglucoses were isolated and crystallized. These were characterized on the basis of decomposition and detection of products by *Penicillium* tannase, elementary analysis of the crystalline compounds and their acetylated and methylated derivatives, u.v. and i.r. spectra, methanolysis, determination of gallic acid and glucose content and molecular-weight determination. The two compounds were identified as β-D-glucogallin and β-D-1,6-di-*O*-galloylglucose.

INTRODUCTION

NACHTOMI and Alumot[1] reported that extracts from green carobs contained several catechins and leucoanthocyanins which may be regarded as possible precursors of condensed tannins. They also found gallic acid in greater amounts in ripe than in green carobs. We reported on the occurrence of leucoanthocyanins and related phenolics of green and ripe carob pods.[2] We found hydrolysable tannins in green carob pods in addition to leucodelphinidin as the main phenolic. Hydrolysable tannins of known structure consist of polyhydric alcohols esterified with gallic acid or derivatives of gallic acid. The work of Haworth and his collabortors[3,4] has shown that the gallotannins can be classified on the basis of the esterified core present. In this paper we describe investigations of the structure of two crystalline hydrolysable tannins isolated from pods of green carob (*Ceratonia siliqua*).

RESULTS AND DISCUSSION

The major phenolics present in methanol extracts of green carob tissue were recovered as a yellowish-brown ethyl acetate soluble preparation, EtoAcP-H, a brown butanol-soluble preparation BuOHP-H and butanol insoluble white powder BuOH-P. The phenolics in the residual butanol extract precipitable by ethanol were isolated as a brown highly astringent preparation Res.P-Alc. The alcoholic solution after concentration and storage at 0° formed a crystalline or amorphous white precipitate, isolated as Res.-P or Res.-cryst.

These five preparations when chromatographed in 6 per cent acetic acid and in isobutanol-acetic acid-water (14:1:5) variously revealed the presence of 13 phenolics (Table 1). The vanillin reactive phenolics occurred only rarely and most of the phenolics were hydrolyzable by purified tannase. D-catechin, gallic acid and leucodelphinidin were readily identified.

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[1] E. NACHTOMI and E. ALUMOT, *J. Sci. Food Agr.* **14**, 464 (1963).
[2] M. A. JOSLYN, H. NISHIRA and S. ITO, *J. Sci. Food Agr.*, in press.
[3] R. D. HAWORTH, *Proc. Chem. Soc.* 401 (1961).
[4] E. HASLAM, *Chemistry of Vegetable Tannins*, p. 98, Academic Press, London (1966).

TABLE 1. PAPER-CHROMATOGRAPHIC PATTERN OF THE PHENOLIC PREPARATIONS FROM GREEN CAROB PODS

Spot No.	Substance	R_f Solvents: 6% HOAc	R_f Solvents: iso BuOH—HOAc	Preparation*: EtoAcP—H A	EtoAcP—H B	EtoAcP—H C	EtoAcP—H D	BuOH—P A	BuOH—P B	BuOH—P C	BuOH—P D	BuOHP—H A	BuOHP—H B	BuOHP—H C	BuOHP—H D	Hydrolysis by tannase
1	Unknown hydrolysable tannin	0·14	0·46	+++	+++	+++	−	−	−	−	−	++	++	++	−	Positive
2	Unknown hydrolysable tannin	0·27	0·45	++	++	+++	−	−	−	−	−	++	++	++	−	Positive
3	Digalloylglucose	0·35	0·31	++	++	++	−	++	++	++	−	+++	+++	+++	−	Positive
4	Unknown hydrolysable tannin	0·42	0·42	++	++	++	−	−	−	−	−	−	−	−	−	Positive
5	Unknown hydrolysable tannin	0·56	0·37	++	++	++	−	−	−	−	−	++	++	++	−	Positive
6	Monogalloylglucose	0·66	0·29	+++	+++	+++	−	+++	+++	+++	−	+++	+++	+++	−	Positive
7	Unknown hydrolysable tannin	0·74	0·52	++	++	++	−	−	−	−	−	++	++	++	−	Positive
8	Gallic acid and D-catechin	0·44	0·65	+++	+++	+++	+++	+	+	+	+	++	++	++	++	Negative
9	Unknown hydrolysable tannin	0·38	0·72	+	+	+	−	−	−	−	−	−	−	−	−	Positive
10	Unknown hydrolysable tannin	0·28	0·63	+	+	+	−	−	−	−	−	−	−	−	−	Positive
11	Unknown phenolic	0·44	0·29	+	+	+	+	−	−	−	−	+	+	+	+	Negative
12	Unknown phenolic	0·42	0·12	+	+	+	+	−	−	−	−	+	+	+	+	Negative
13	Leucodelphinidin	Tailing and immobile		+	+	+	++	+	+	+	+	+	+	+	+	Negative

* Detection by: A, $FeCl_3$—$K_3Fe(CN)_6$; B, diazotized p-nitro-aniline; C, $AgNO_3$—NH_4OH; D, vanillin-HCl. Preparation Res.P.-Alc. contained only spot no. 13 and Res.-P. only spot no. 6. +, weak; ++, medium; +++, strong; −, negative (absent).

Two crystalline preparations (Fig. 1) were obtained and investigated in detail, preparation 21B from the butanol-ethanol fraction, corresponding in chromatographic mobility to spot 3 in Table 1, and BuOH-P-6, corresponding to spot 6 in Table 1.

Both 21B and BuOHP-6 were decomposed by tannase to yield only gallic acid and glucose. The R_f values of spots Nos. 1 and 2 in Fig. 2 were those of gallic acid and D-glucose and their identities were proven by co-chromatography. No phenolics colored with vanillin-HCl reagent were observed. From their chromatographic mobility in solvent systems tested,[4] 21B and BuOHP-6 resembled monogalloylglucose and digalloylglucose, respectively.

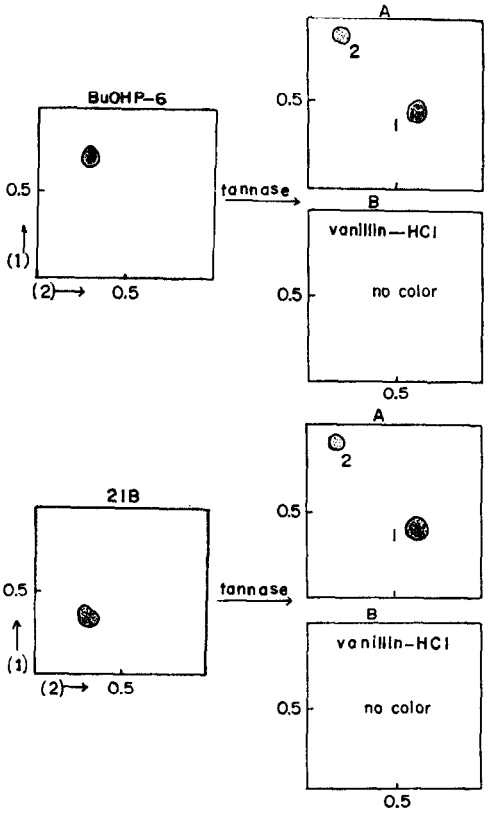

FIG. 2. PAPER CHROMATOGRAM SHOWING DECOMPOSITION OF TWO GALLOYLGLUCOSES BY PENICILLIUM TANNASE.

Solvent systems: (1), 6 per cent HOAc; (2), iso BuOH—HOAc.

The gallic acid contents in the two crystalline preparations and in "Baker Analyzed Reagent Tannic Acid" purified by the method of Armitage *et al.*[5] were BuOHP-6, 45·4 per cent (calc. for monogalloylglucose, 51·2 per cent); 21B, 65·5 per cent (calc. for digalloylglucose dihydrate 69·2 per cent); tannic acid 93·0 per cent (calc. for $C_{62}H_{44}O_{38}$,[6] 92·3 per cent). The glucose contents as determined by a modification of the method used by Armitage

[5] R. ARMITAGE, G. S. BAYLISS, J. W. GRAMSHAW, E. HASLAM, R. D. HAWORTH, K. JONES, H. J. ROGERS and T. SEARLE, *J. Chem. Soc.* 1842 (1961).

[6] R. ARMITAGE, E. HASLAM, R. D. HAWORTH and T. SEARLE, *J. Chem. Soc.* 3808 (1962).

TABLE 2. CHROMATOGRAPHY OF METHANOLYSIS PRODUCTS FROM BuOHP-6 AND 21B

Phenolic	Methanolysis product detected				
	Gallic acid (0·41, 0·67)*	Methyl gallate (0·51, 0·80)*	Other phenolics detected		
Gallotannin	+ + + + +	+ + + + +	Unaffected gallotannin (0·20, 0·34)* + + + + +	*m*-Digallic acid (0·29, 0·70)* + + +	Methyl-*m*-digallic acid (0·34, 0·80)* + + +
BuOHP-6	+ + + + +	+	Unaffected BuOHP-6 (0·67, 0·29)* + + + + +		
21B	+	+ + +	Unaffected 21B (0·40, 0·23)* + + + + +	Unknown phenolic (0·57, 0·22)* + + +	
6-Galloylglucose(?) from 21B and β-glucosidase*	+	+ + + + +	Unaffected phenolic (0·53, 0·20)* + + +	1-monogalloylglucose(?) (0·63, 0·27)* +	

( )* Respective R_f values in 6 per cent HOAc and in iso BuOH—HOAc—H_2O. + + + + +, intense; + + +, medium; +, weak.

et al.[5] were tannic acid, 18·0; BuOH-P-6, 48·5 ($C_{13}H_{16}O_{10}$ requires 54·2); 21B, 34·8 ($C_{20}H_{24}O_{16}\cdot 2H_2O$ requires 34·6).

Methanolysis by the method of Haslam and Stangroom[7] yielded products with the R_f values shown in Table 2. It is evident that the two compounds differ markedly from poly-galloyl esters of D-glucose in their paper chromatographic mobility and in their resistance to methanolysis. BuOHP-6 on methanolysis yielded mainly gallic acid and unchanged phenolic together with a trace of methyl gallate. 21B, however, yielded methyl gallate, traces of gallic acid and two galloylated glucoses, one of which was unchanged 21B and the other tentatively identified as 6-*O*-galloylglucose. Unlike tannic acid, methyl-*m*-digallate and *m*-digallic acid were not detected after methanolysis of either substance.

The results of elementary analysis were as follows: (*a*) Found. BuOHP-6: C, 44·56; H, 4·98 per cent. BuOHP-6 dried over P_2O_5 in vacuum at 50°: C, 46·32, 46·76; H, 4·81, 4·77 per cent. 21B: C, 46·02; H, 4·64 per cent. 21B dried over P_2O_5 in vacuum at 50°: C, 49·14; H, 4·32 per cent. (*b*) Calculated for monogalloyl glucose $C_{13}H_{16}O_{10}$: C, 47·02, H, 4·82; for $C_{13}H_{16}O_{10}\cdot 2H_2O$: C, 44·57; H, 5·14, for digalloyl glucose $C_{20}H_{20}O_{14}$: C, 49·60; H, 4·12, for $C_{20}H_{20}O_{14}\cdot 2H_2O$: C, 46·20; H, 4·60. The purified crystalline acetate obtained from BuOH-P-6 (m.p. 118–120°) contained C, 51·76; H,4·73; acetyl 46·5 (calc. for $C_{27}H_{30}O_{17}$: C, 51·84; H, 4·80; acetyl 48·16 per cent). The acetylated 21B (m.p. 96°) contained C, 52·76, H, 4·38, acetyl 43·6 (calculated for $C_{38}H_{38}O_{23}$: C, 52·90; H, 4·48, acetyl 44·89). The methylated derivative (m.p. 78–80°) obtained from BuOHP-6 contained C, 56·9; H, 6·49; OCH_3, 50·55. $C_{20}H_{30}O_{10}$ (methylated monogalloylglucose) required: C, 55·81; H, 6·97; OCH_3, 50·46 per cent. Methylated 21B (m.p. 76–77°) contained C, 58·11; H, 6·24; OCH_3, 47·94 per cent. $C_{29}H_{38}O_{14}$ (methylated digalloylglucose) required: C, 57·04, H, 6·22, OCH_3, 45·73 per cent.

The u.v. spectral maxima of 0·66 mg. % solution of the phenols in 50 per cent ethanol were as follows: gallic acid 263,215; tannic acid 280 (279·5), 217·5; methyl gallate 275, 218; BuOHP-6 282, 218; and 21B 279, 218 nm. (Fig. 3).

The i.r. spectra of the compounds in KBr were compared with those of gallic acid, methyl gallate and pure tannic acid (Fig. 4) and it can be seen that 21B and BuOHP-6 resemble tannic acid more than gallic acid and methyl gallate. There is a close agreement between 21B and tannic acid, since at least 10 peaks are nearly identical in absorbance and frequency. Differences do appear, notably at frequencies of 1070, 940, 910 and 820 cm^{-1}. Although the pattern of BuOHP-6 is like that of tannic acid, there are also some points of similarity between BuOHP-6 and gallic acid.

The approximate molecular weights found were 401 for BuOHP-6 and 555 for 21B which compare favorably with the calculated molecular weights of 350 for monogalloylglucose and 520 for digalloylglucose. The specific rotations of BuOHP-6 and 21B were $[\alpha]_D^{22°} = -19 \pm 2°$ (water, $c=1$) and $[\alpha]_D^{22°} = -11 \pm 2°$ (95 per cent ethanol, $c=1$), respectively. The melting points of the two compounds were: BuOHP-6, 193∼196°; BuOHP-6 dried over P_2O_5, 209∼211°; 21B, 184∼185°; 21B dried over P_2O_5, 195, 196°; tannic acid, 210∼230° (no constant m.p.); gallic acid, 248∼250°; methyl gallate, 199∼202°. M.p. of anhydrous 1-galloyl-β-D-glucose was recorded as 214∼215° by Nursten[8] or 211–212° by Mayer,[9] and of 3,6-digalloyl glucose as $d > 185°$ by Nursten.[8]

Approximate molecular weights of the acetylated and methylated compounds were:

[7] E. HASLAM and J. E. STANGROOM, *Biochem. J.* **99**, 28 (1966).
[8] CYRIL LONG (editor), *Biochemists' Handbook*, pp. 998–1018, Van Nostrand Company, London (1966).
[9] W. MAYER, Pflanzen-gerbstoffe, *Encyclopedia of Plant Physiology* (edited by W. RUHLAND), Vol. **10**, pp. 354–388, Springer Verlag, Berlin (1958).

acetylated BuOHP-6, 648 (acetate of monogalloylglucose requires 625); methylated BuOHP-6, 410 (methylated monogalloylglucose requires 430); acetylated 21B, 841 (acetate of digalloyl glucose requires 862); methylated 21B, 612 (methylated digalloylglucose requires 610).

R_f values of the two compounds on the paper chromatograms in 6 per cent HOAc and in isobutanol-acetic acid-water were compared with those of other gallotannins[4] as follows: BuOHP-6, 0·71 and 0·31; 21B, 0·39 and 0·41; β-1-*O*-galloyl-D-glucose (β-glucogallin), 0·75 and 0·30; 3,6-di-*O*-galloyl-D-glucose, 0·45 and 0·42; 3,4,6-tri-*O*-galloyl-D-glucose, 0·33 and 0·38; 2,3,6-tri-*O*-galloyl-D-glucose, 0·25 and 0·56; 1,3,6-tri-*O*-galloyl-D-glucose, 0·12 and 0·32; 2,3,4,6-tetra-*O*-galloylglucose, 0·21 and 0·60; β-penta-*O*-galloyl-D-glucose, 0·08 and 0·58; corilagin, 0·35 and 0·20.

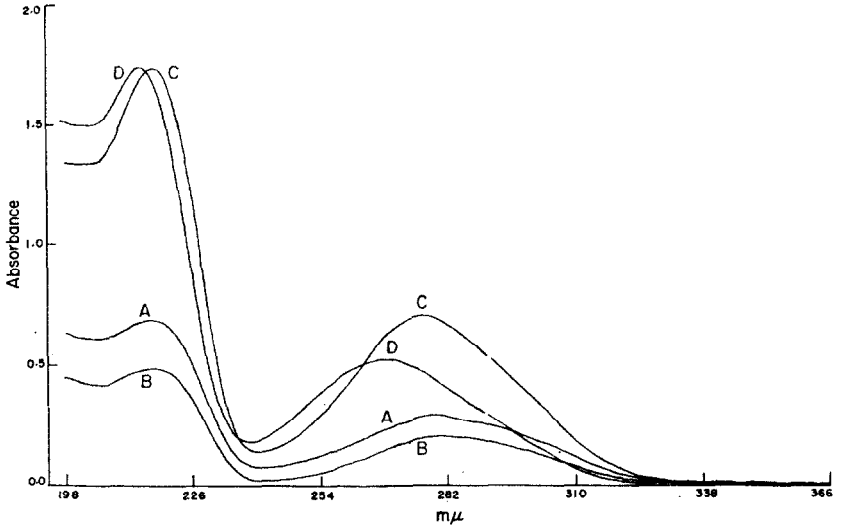

FIG. 4. U.V. SPECTRA OF PRODUCTS OF 21B BY β-GLUCOSIDASE.

A, gallotannin purified by E. Haslam (0·66 mg per cent in 50 per cent ethanol); B, BuOHP-6 (0·66 mg per cent in 50 per cent ethanol); C, 6-galloylglucose derived from 21B crystal by β-glucosidase hydrolysis (50 per cent ethanol solution); D, gallic acid derived from 21B by β-glucosidase hydrolysis (50 per cent ethanol solution). Max. in nm: A, 217·5, 279·5; B, 217, 281; C, 217, 277; D, 215, 268.

Aqueous solutions of BuOHP-6 and 21B did not give insoluble precipitates with gelatin solution containing NaCl. BuOHP-6 was hydrolysable by emulsin, tannase and β-glucosidase, yielding gallic acid and glucose only (Table 3). Hydrolysis of 21B with emulsin and β-glucosidase gave gallic acid and an unknown galloyl derivative, but did not give glucose. This unknown galloyl derivative was further hydrolyzed by tannase into gallic acid and glucose, but not hydrolyzed by emulsin or β-glucosidase.

The reaction of the spray reagents towards BuOHP-6, 21B and the products of 21B by emulsin and B-glucosidase together with their behavior in u.v. light are shown in Table 4. Gallic acid and an unknown galloyl derivative present in hydrolyzate of 21B by β-glucosidase were separated and their u.v. spectra were measured (Fig. 4), in comparison with BuOHP-6 and Chinese gallotannin purified by Haslam. The unknown galloyl derivative differs clearly from BuOHP-6 (1-galloylglucose) in R_f value and in its u.v. spectra. This unknown galloyl derivative gives positive reactions with hydrogen phthalate and benzidine tests indicative of the presence of free aldehydic groups. From R_f value (0·47) of the unknown galloyl derivative

TABLE 3. CHROMATOGRAPHIC DETECTION OF PRODUCTS OF ENZYMATIC HYDROLYSIS OF THE GALLOYLGLUCOSES

Products of enzyme	R_f in		BuOHP-6			21B		
	6% HOAc	iso BuOH—HOAc	Emulsin	β-Glucosidase	Tannase	Emulsin	β-Glucosidase	Tannase
Gallic acid	0·43	0·64	+	+	+	+	+	+
Glucose	0·87	0·19	+	+	+	−	−	+
6-Monogallolglucose(?)	0·59	0·24	−	−	−	+	+	−

on paper chromatogram in *n*-butanol-water-acetic acid-ethylene glycol solvent system (Schmidt and Lademann[10]) and colors revealed by spraying with ammonium vanadate (olive, disappears rapidly) and by vanadate followed by 1 N sulfuric acid (blue and fading after a while), this substance is probably 6-mono-*O*-galloyl-D-glucose.

All the above results combine to show that compound BuOHP-6 is β-D-glucogallin and that 21B is β-D-1,6-di-*O*-galloylglucose.

The phenolic corresponding to No. 1 spot (R_f 0·14 and 0·46, Table 1) obtained as a brown-grey powder soluble in methanol, on hydrolysis with tannase gave only gallic acid and glucose. Its u.v. spectrum was very similar to that of purified Chinese gallotannin.

TABLE 4. DETECTION OF BuOHP-6, 21B AND THEIR PRODUCTS BY ENZYMATIC HYDROLYSIS ON THE PAPER CHROMATOGRAMS

Compound*	U.v. light		Spray		
	Alone	Ammonia vapor	Ferric chloride -potassium ferricyanide	Diazotized *p*-nitro aniline	Ammonical silver nitrate
BuOHP-6	Violet or violet-blue	Violet	Blue	Grey after brown-yellow	Olive
21B	Violet or violet-blue	Violet	Blue	Grey-brown after yellow	Violet-grey
Gallic acid	Blue	Violet	Blue	Brown after orange-yellow	Olive
6-Monogalloylglucose(?)	Blue	Violet	Blue	Brown-grey after yellow	Olive

* None of the compounds reacted with vanillin-HCl.

MATERIALS AND METHODS

Paper Chromatography

Phenolics were chromatographed on Whatman No. 1 paper in two dimensions with 6 per cent (v/v) acetic acid and isobutanol-acetic acid-water (14:1:5) at 25 ± 2°.[3,4] Phenolics were revealed by spraying with ammonical silver nitrate,[11] diazotized *p*-nitroaniline,[12] vanillin-HCl[13,14] and ferricyanide ferric chloride mixture.[14] Carbohydrates were chromatographed with above solvent systems and detected by sprays of benzidine or aniline hydrogen phthalate.[15,16]

Preparation of Penicillium Tannase

Penicillium tannase was prepared by purification of the brown powder obtained from "Fusuma Koji" of *Penicillium* sp. No. 80 B strain[17], grown on the bran medium containing tannic acid or gallic acid as an inducer of tannase, followed by fractionation by salting out

[10] O. T. SCHMIDT and RUDOLF LADEMANN, *Liebig Ann.* **571**, 41 (1951).
[11] T. WHITE, K. S. KIRBY and E. KNOWLES, *J. Soc. Leather Trades' Chemistry* **36**, 148 (1952).
[12] T. A. GEISSMAN, (editor), *The Chemistry of Flavonoid Compounds*, Macmillan, New York (1961).
[13] S. ITO, *Nippon Shokuhin Kagaku Zashi* **12**, 295 (1965).
[14] D. E. HATHWAY, *Biochem. J.* **70**, 34 (1958).
[15] H. T. GORDON, W. THORNBERG and L. N. WERUM, *Anal. Chem.* **28**, 849 (1956).
[16] K. YAMAGUCHI, *The Analytical Methods of Phytochemistry*, Vol. **1**, pp. 778, Nanko-do, Tokyo, Japan (1963).
[17] H. NISHIRA, *J. Fermentation Technology (Japan)* **37**, 85, 89 (1959).

with ammonium sulfate and lyophilization.[18–20] The lyophilized preparation obtained was further purified by gel filtration with Sephadex G-100, followed by treatment on DEAE-Cellulose, and found to be homogenous on paper-electrophoresis. Purified white powder was obtained by freeze-drying of enzyme solutions fractionated by the above treatment.[21] The *Penicillium* tannase preparation attacked methyl gallate and purified tannic acids from Chinese, Turkish, and Sumach, but not maltose, cellobiose, salicin, trehalose, melibiose, turanose, lactose, starch, inulin or casein. Very weak sucrose decomposing activity was found.

Extraction of Phenolics and Crystallization of the Galloylglucoses

The phenolics were obtained by extracting sliced green carob tissue with methanol, concentration *in vacuo*, and removing chlorophylls and other ether-solubles by serial extraction with ethyl ether. The phenolics were then separated into ethyl acetate soluble but hexane precipitable fraction (EtOAcP-H) and into butanol-soluble hexane precipitable fraction (BuOHP-H).[22] During the concentration of butanol extract prior to drying and precipitation with hexane some phenolics precipitated and were recovered as BuOH-P. Preparation BuOHP-H was purified by continuous liquid-liquid extraction using ethyl acetate saturated with water.[23,24] For countercurrent extraction, BuOHP-H (500 mg) was dissolved in 30 ml of ethyl acetate saturated with water and extracted in a 60 tube Craig liquid–liquid extraction for 5–6 hr in presence of N_2. On the basis of optical densities at 280 nm, the upper solutions from tube No. 7 to 35 were collected and concentrated to dryness. The powder obtained was dissolved in 10 ml of *n*-butanol, and then precipitated with *n*-hexane followed by washing with hexane and ether. This powder was dissolved in a small quantity of hot water and when cooled and stored for 2–3 days at 0°, long needle-like crystals settled out. Similar crystals were obtained in 12·5 per cent yield by storing concentrated aqueous solutions of BuOHP-H at 0°. These crystals, purified by repeated crystallization from water, constitute preparation 21B. Another crystalline preparation was obtained from BuOH-P by storing concentrated aqueous solutions containing small amounts of butanol at 0° for 2–3 weeks. This preparation was designated as BuOH-P-6.

Methanolysis of BuOHP-6 and 21B

The crystals were refluxed in methanol for 7 days (purified tannic acid, 916 mg in 100 ml methanol; BuOHP-6, 43 mg in 10 ml methanol; 21B, 46 mg in 10 ml methanol).[7] After removal of the solvent, the residue was dissolved in a small amount of 50 per cent methanol. Products of methanolysis in the solution were subjected to paper chromatography and detected with the spray reagent as described above. Spots were identified by comparison with authentic samples.

Hydrolysis

Aliquots of crystalline preparation were hydrolyzed in 0·5 per cent solution in 0·1 N phosphate buffer, pH 5·8 at 40° with purified tannase for 6 hr and then chromatographed. For comparison 20 mg aliquots, dissolved in 5 ml of 0·1 N phosphate buffer at pH 5·8 were

[18] H. NISHIRA, and N. MUGIBAYASHI, *Sci. Rep. Hyogo Univ. Agr.* **4**, 113 (1960).
[19] H. NISHIRA, and N. MUGIBAYASHI, *Leather Chemistry (Japan)* **11**, 93 (1965).
[20] H. NISHIRA, Memoirs of Hyogo Univ. of Agr. No. 16, Chem. Ser. No. 3, pp. 1–33 (1963).
[21] H. HISHIRA and M. A. JOSLYN, Purification of tannase. Results to be published elsewhere.
[22] T. A. GEISSMAN and H. F. K. DITTMAR, *Phytochem.* **4**, 359 (1965).
[23] D. G. ROUX and E. PAULUS, *Biochem. J.* **82**, 320 (1962).
[24] C. HSIA, L. L. CLAYPOOL, J. L. ABERNETHY and PAUL ESAU, *J. Food Sci.* **29**, 723 (1964).

hydrolyzed with 4 mg emulsin (Worthington Biochemical, New Jersey) and β-glucosidase (General Biochemical, Ohio) for 24 hr at 37°. The hydrolyzate were then chromatographed as above.

Acetylation and Methylation

Acetylation was performed by dissolving well-dried crystals in 2·5 ml of dry pyridine, adding 10 ml of acetic anhydride and allowing the mixture to stand for 24 hr at 35°. The acetates were recrystallized by dissolving in ethanol and diluting with an equal volume of water containing a few drops of acetic acid. Methylation was carried out with excess ethereal diazomethane at 5° and the products obtained were purified by recrystallization from methanol or dilution with water.

Determination of Glucose

The method used was a modification of that of Armitage *et al.*[5] The crystals were dissolved in 0·5 N acetate buffer pH 6·0 (2 ml), 0·025 per cent tannase solution (1 ml) and a few drops of toluene were added, and the whole was incubated at 40° for 2 days. The reaction mixture was passed through Dowex-2 resin columns (1 × 3 cm), and then the columns were washed with distilled water. The eluates collected with washed water were combined. Glucose contents were measured by ferricyanide reduction method.[25]

Determination of Gallic Acid

The crystals were dissolved in 0·5 N acetate buffer of pH 6·0 (10 ml), 0·025 per cent *Penicillium* tannase (0·5 ml) added, and the solution incubated for 24 hr at 40°. Aliquot parts (2·0 ml) were diluted to 100 ml and optical density measured at 280 nm using Model DU spectrophotometer.[5]

Molecular Weight

The molecular weights of the preparation tested were determined from vapor pressure depression in a Model 301A Mechrolab Vapor Pressure Osmometer. For Bu OHP-6, water was used as solvent and sucrose as a standard. For 21B, 95 per cent ethanol was used as solvent and D-catechin as standard.

Optical Rotation

Optical rotations were measured in a single wedge Schmidt and Haensch saccharimeter at 22° in semimicro saccharimeter tube 1 dm in length.

Spectrophotometry

Beckman Model DU, Bausch and Lomb Spectronic Model 505 and Cary spectrophotometer Model 15 were used.

Acknowledgements—The technical assistance supplied by Marvin M. Morris, Laura L. Potter and George Hugenberg is gratefully acknowledged. I.r. spectra were supplied through the courtesy of Gunter Zweig and George Mallett, Agricultural Toxicology Department, University of California, Davis, California. We are grateful to the Laboratory of Organic Chemistry, University of California, Berkeley, California, for elementary analysis. We thank Professor Dr. E. Haslam, Department of Chemistry, University of Sheffield, for the gift of purified gallotannin. The investigation was supported by the Public Health Service Research Grant No. U1 EF 00080-05 from National Center for Urban and Industrial Health.

[25] M. A. JOSLYN, *Methods in Food Analysis*, pp. 422–426, Academic Press, New York (1950).