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POLYPHENOLS IN CEREALS AND LEGUMES

Proceedings of a symposium held during the 36th annual meeting of the Institute of Food Technologists, St. Louis, Missouri, 10 – 13 June 1979

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Contents

Vegetable Tannins — Structure and Biosynthesis

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One of the most distinctive groups of higher plant secondary metabolites is the vegetable tannins. These are polyphenols (molecular weight 500–3000) whose ability to complex and precipitate proteins is germane not only to considerations of higher plant metabolism but also to numerous aspects of food technology. Their structure, biosynthesis, and interaction with proteins will be reviewed with particular reference to the tannin — a procyanidin — elaborated in the seed coat of sorghum.

Evolution and function lie almost synonymously at the heart of biology, and with the flowering of biochemistry in the twentieth century it was apparent that to many natural products, isolated by chemists in the eighteenth and nineteenth centuries, a distinctive role in the life of organisms could now be assigned. Thus citric acid and L-malic acid are essential to carbohydrate metabolism as key intermediates of the ubiquitous tricarboxylic acid cycle; adenosine is a constituent of nucleic acids; the various α-amino acids are the building units of proteins; fatty acids function as integral parts of complex lipid structures; and lanosterol (isolated from sheep's wool) is a key intermediate in steroid biosynthesis. These substances are classified as primary metabolites and, moreover, they are found in broadly similar patterns in most, if not all, living organisms. In contrast, there was an infinitely greater body of substances, each of which had a sporadic distribution in living matter, and to which no specific function could be assigned. Attention was first drawn to this group by plant physiologists, and they are now commonly referred to as secondary metabolites. As Bu'Lock (1965) has remarked, they express the individuality of a species in chemical terms.

While ignorance of their biochemical function and significance prevails at the experimental level, not surprisingly, theories and speculation abound at the philosophical level.

Opposing viewpoints have developed. One theory propounds that they are waste products or "accidents of metabolism" (Muller 1969), notwithstanding the fact that many are toxic to the plant or microorganism unless dissipated into the environment (e.g. volatile monoterpenes), or are harmlessly sequestered in the plant itself (e.g. phenolic glycosides). On the other hand, a contrary opinion maintains that these substances possess (or possessed) pertinent biological functions (e.g. Fraenkel 1959), and the example of the vegetable tannins is frequently cited in support of this view. The importance of vegetable tannins to the plant lies, it is believed, in their effectiveness as repellents to predators whether animal or microbial. According to Bate-Smith (1954), the relevant property is *astringency*, which for animals renders the plant tissue unpalatable by precipitation of salivary proteins and for parasitic organisms impedes the invasion of the plant tissue by immobilizing extracellular enzymes. It is persuasively argued that this strong association with proteinaceous materials is a primary function that has been of considerable evolutionary significance in the plant kingdom.

After the early encouragement that emerged from Emil Fischer's outstanding contributions to the chemistry of vegetable tannins (Fischer 1919), chemists were slow to recognize the complexity of the problems these substances posed, and over the next 40–50 years it became one of the untidy corners of organic chemistry. Nonetheless, the recently obtained knowledge of the structure, molecular shape, biosynthesis, and chemical reactivity of the principal vegetable tannins (Haslam 1966 and 1977; Mayer 1973), now makes possible, for the first time, a systematic examination of their biochemical and biological properties. This must surely be the aim of future work.

It is not possible to give a concise definition of the word "tannin" and the inability to do so has led to numerous misunderstandings in the literature. Tanning is a process whereby an animal skin is turned into leather, and its essence is to bring about cross-linking of the collagen chains in the skin and thus to protect the protein fibres from microbial attack and give the skin greater resis-

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tance to water, heat, and abrasion. During this process, which uses vegetable extracts, the skin may adsorb up to half its weight of "tannins." In this sense the implication of the word "tannin," and indeed its original use by Seguin (1796), clearly indicates a substance that produces leather from hide. In plant extracts these substances are polyphenols of varying molecular size and complexity. Invariably they constitute only a limited proportion of the total polyphenols in a plant tissue, and the failure to make this critical distinction has led one writer (White 1957) to suggest that "much of the botanical data concerning the occurrence of tannins in plants is of doubtful validity since it is based on tests which are insufficiently specific." In a nutshell, the general criteria for phenols (colour tests etc.) are quite inadequate to determine the presence of vegetable tannins.

Bate-Smith and Swain (1962) have adopted the earlier ideas of White (1957) to formulate a definition of vegetable tannins that, with present knowledge, is the most useful one to follow. These authors defined vegetable tannins as "water-soluble phenolic compounds having molecular weights between 500 and 3,000 and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins." This is the definition used here with the added proviso that the phenols are normal metabolic products and are not in vitro transformation products formed by chemical or other means. Nonetheless, it is well to note that this definition derives from considerations of the ways in which polyphenols tan protein fibres of animal skins and as such it groups together a series of phenolic compounds that possess this common characteristic. Clearly this property may be a purely fortuitous one and from the point of view of plant metabolism and plant biochemistry as a whole it may, in the final analysis, be a quite misleading one.

The ability to complex with proteins makes vegetable tannins distinctive metabolic products because formation of substantial quantities of free tannin molecules within the cytoplasm would presumably cause precipitation of structural and catalytic proteins. In this respect they resemble several antibiotics that are more toxic to the producing organism than are their precursors. The presence of vegetable tannins in plant tissues nevertheless has several important practical consequences. The interaction of the tannins with the salivary proteins and glycoproteins in the mouth renders the tissue astringent to the taste and this characteristic may determine one's enjoyment of particular fruit (e.g. blackberry, strawberry, cran-

berry, and apple). Firmly established in the biochemical literature (Howes 1953; Goldstein and Swain 1963) is the belief that changes in the palatability of many fruits that occur on ripening are associated with concomitant changes in the concentration of tannins present in the fruit. A widely expressed view is that the astringency of green immature fruit is due to the presence of tannins but that on ripening these are much reduced in quantity or are modified in some unspecified way.

Structure of Vegetable Tannins

The most acceptable major division of vegetable tannins is one first suggested by Freudenberg (1920) and is based on structural types. It separates the tannins into two classes, the hydrolyzable and the nonhydrolyzable or condensed. Simple treatment of hydrolyzable tannins with acid or alkali and in certain cases hydrolytic enzymes (tannase) splits them into sugars and some recognizable phenolic carboxylic acid (e.g. gallic acid or ellagic acid). Condensed tannins do not readily break down in this way, nor do sugars contribute to their overall structure. From the point of view of the association with proteins and other natural macromolecules, the two classes simply illustrate the principal means whereby plants can elaborate polyphenolic molecules with sufficient phenolic groups to form multiple hydrogen bonds with the substrate. Typical examples are Chinese gallotannin (syn. tannic acid, galls-*Rhus semialata*) and the condensed procyanidin that forms the major polyphenol from sorghum grain.

The histological reaction for vegetable tannins in plants is most commonly due to the presence of proanthocyanidins (referred to in earlier papers as leucoanthocyanidins and synonymous with condensed tannins), and they are present, often in substantial quantities, in many dicotyledons and in the most primitive of vascular plants such as ferns and gymnosperms. Their appearance in plants appears to be associated phylogenetically with the ability to form lignified tissues and with the development of a vascular character. Of the various proanthocyanidins those which form cyanidin on acid treatment — the *procyanidins* — are most widely distributed in plants. Invariably they co-occur alongside one or both of the flavan-3-ols: (+)-catechin or (-)-epicatechin.

Ideas on the biosynthesis of the plant procyanidins and associated flavan-3-ols are based on a range of structural observations and biosynthetic experiments (Haslam 1977). The results of these experiments are summarized in Fig. 1 in a path-
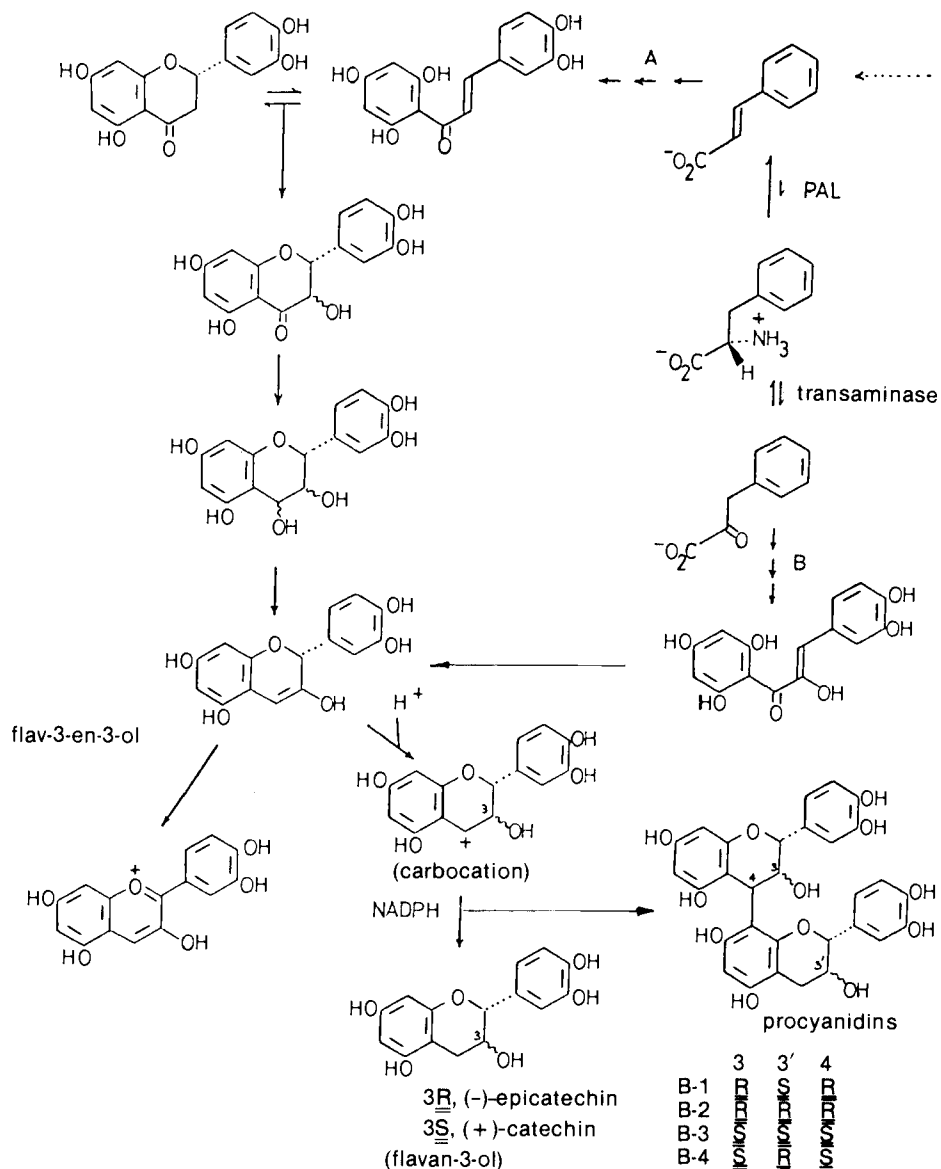
Fig. 1. Biosynthesis of procyanidins.

way of procyanidin metabolism that accords in broad outline with the tenets of flavonoid biosynthesis outlined by previous workers, most notably Grisebach (1979) — namely that a $C_6 \cdot C_3$ fragment (cinnamate) is combined with three acetate-derived units to form the $C_6 \cdot C_3 \cdot C_6$ carbon skeleton of the flavonoids. The chalcone ⇌ flavanone pair is the first formed intermediate, but the sequence of chemical changes in the C_3 unit that results in the formation of the individual

flavonoids themselves remains poorly defined. In Fig. 1 a plausible scheme from the chalcone ⇌ flavanone pair to the flavan-3-ols [(+)-catechin and (-)-epicatechin] is shown. A key intermediate is believed to be the flav-3-en-3-ol and a two-step sequence (stereospecific protonation followed by reduction) is postulated to yield the flavan-3-ols.

The procyanidins are thought to arise as byproducts in this two-stage process when the

Polymeric	Trimeric	Dimeric procyanidins	
procyanidins	Tetrameric	B-1 and/or	Monomeric flavan-3-ols
	Pentameric	B-2 and/or	(+) - catechin
	Hexameric etc.	B-3 and/or	and/or
	procyanidins	B-4 and/or	(-) - epicatechin

←—Difficultly soluble ————————→ ←——————— Soluble in methanol ————————————→

M.Wt ∼ 2500

Fig. 2. Procyanidins in plant tissues.

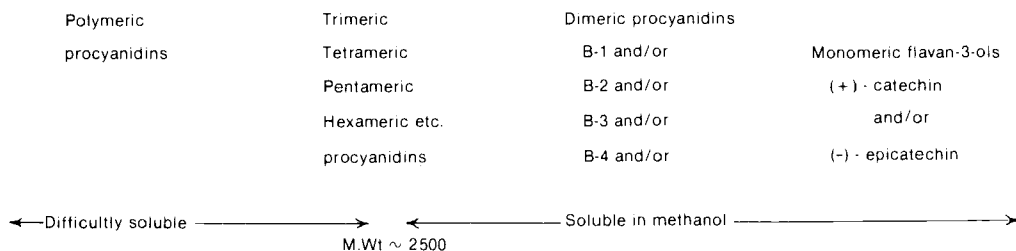supply of biological reductant (NADPH) is rate limiting. The various distinctive patterns of procyanidins found in plants are then thought to arise by reaction of one or both of the intermediate carbocations that escape from the active site of the enzyme with one or both of the flavan-3-ols utilizing their nucleophilic reactivity at carbon atom C-6 or C-8. The dimeric forms are first produced, but these react similarly with further carbocations to produce trimers, tetramers, and higher oligomers. Thus any plant produces a range of procyanidins of differing molecular weight as shown in Fig. 2. Only those flavan-3-ols with molecular weights up to about 3000 are soluble, and it is not known whether those of higher molecular weight are simply insoluble or are in fact attached in some way to skeletal tissues of the plant. It should, however, be emphasized that these difficultly soluble materials frequently represent the major fraction of procyanidin materials in a plant tissue. The question of whether polyphenol metabolism of this type continues over a long period with turnover of the products is somewhat contentious, but our belief is that it does not. Our view is that there is a relatively short "burst" of procyanidin synthesis after which the level of product remains approximately the same in the plant tissue until senescence occurs.

The soluble monomeric, dimeric etc. procyanidins thus represent only the "tip of the iceberg" for the range of procyanidins found in any given plant species. Two-dimensional paper chromatography of these soluble forms does, however, give readily recognizable patterns (fingerprints) that permit plants to be readily categorized (Fig. 3). Thus, for example, *Malus* sp., *Prunus* sp., and *Crataegus* sp. metabolize (-)-epicatechin, procyanidin B-2 etc.; *Salix* sp. and *Fragaria × ananasa* produce (+)-catechin, procyanidin B-3 etc. The most characteristic reaction of procyanidins is their acid-catalyzed degradation to give the pigment cyanidin by rupture of the interflavan bond, and this degradation proceeds by initial formation of the appropriate carbocation from the

"upper half" of the dimeric procyanidin (Fig. 4). The carbocation is normally converted by proton loss and oxidation to give cyanidin, but it may also be intercepted under appropriate conditions to give flavanyl-4-thioethers, and this reaction has proved to be of great importance in subsequent identifications.

Sorghum Procyanidins

"There are no applied Sciences . . . there are only applications of Science and this is a very different matter . . . . The study of the application of Science is very easy to anyone who is master of the theory of it."

Louis Pasteur

Sorghum is an important food grain whose nutritional quality is considerably diminished in many hybrids by the presence of "tannins." Methanolic extracts of "very high-tannin" sorghums were analyzed initially by paper chromatography and these immediately showed one of the characteristic procyanidin "fingerprints" noted above. In particular the flavan-3-ol (+)-catechin and the procyanidin dimer B-1 were readily identified. Subsequently, large-scale extraction of sorghum cultivars gave extracts in which only polymeric procyanidins were present and in which the dimer B-1 and (+)-catechin were detected in only trace amounts. Later work revealed some of the possible reasons for this apparent change in the spectrum of phenols present in different cultivars. The sorghum grain is formed initially in a sheath, and at the etiolated stage no procyanidins can be detected. As chlorophyll develops in the seed coat there is an apparently rapid synthesis of polyphenols and both B-1 and (+)-catechin are present. However, as the seed ripens (to a red-brown appearance) these monomeric and dimeric flavan-3-ol species appear to decline rapidly in concentration to leave the polymeric procyanidin as the principal and in many cases the sole procyanidin in the seed coat.
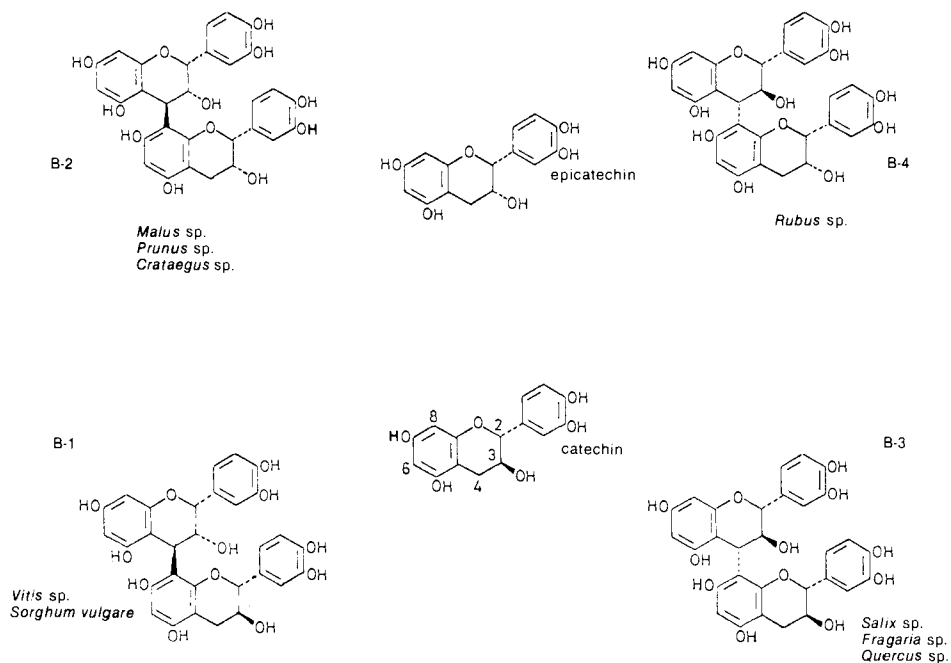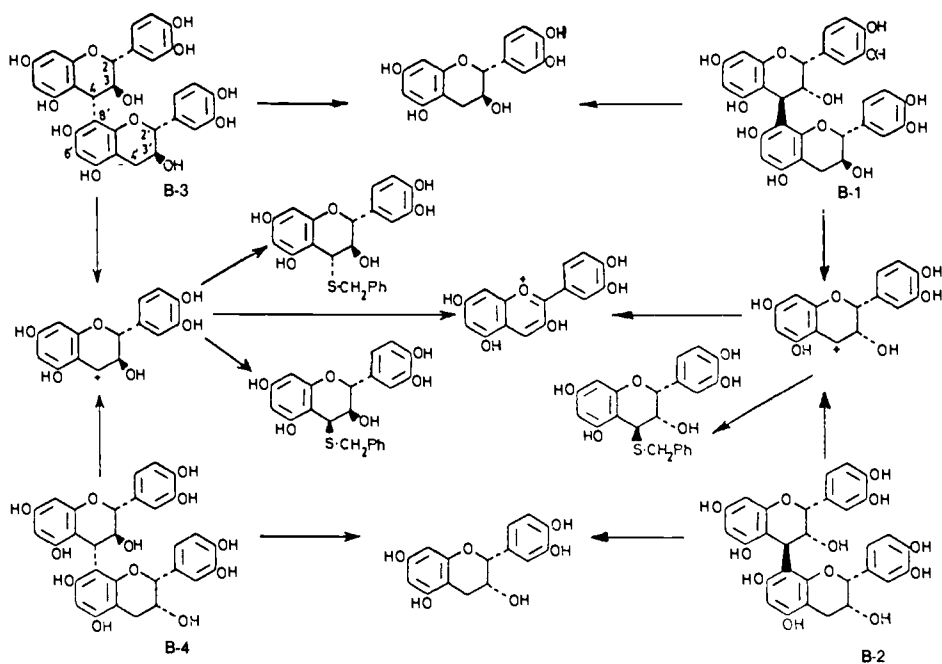
Fig. 3. Procyanidins: natural sources.

Fig. 4. Procyanidin dimers: degradation.

carbocation (1)   (+) catechin

B-1

Procyanidin polymer
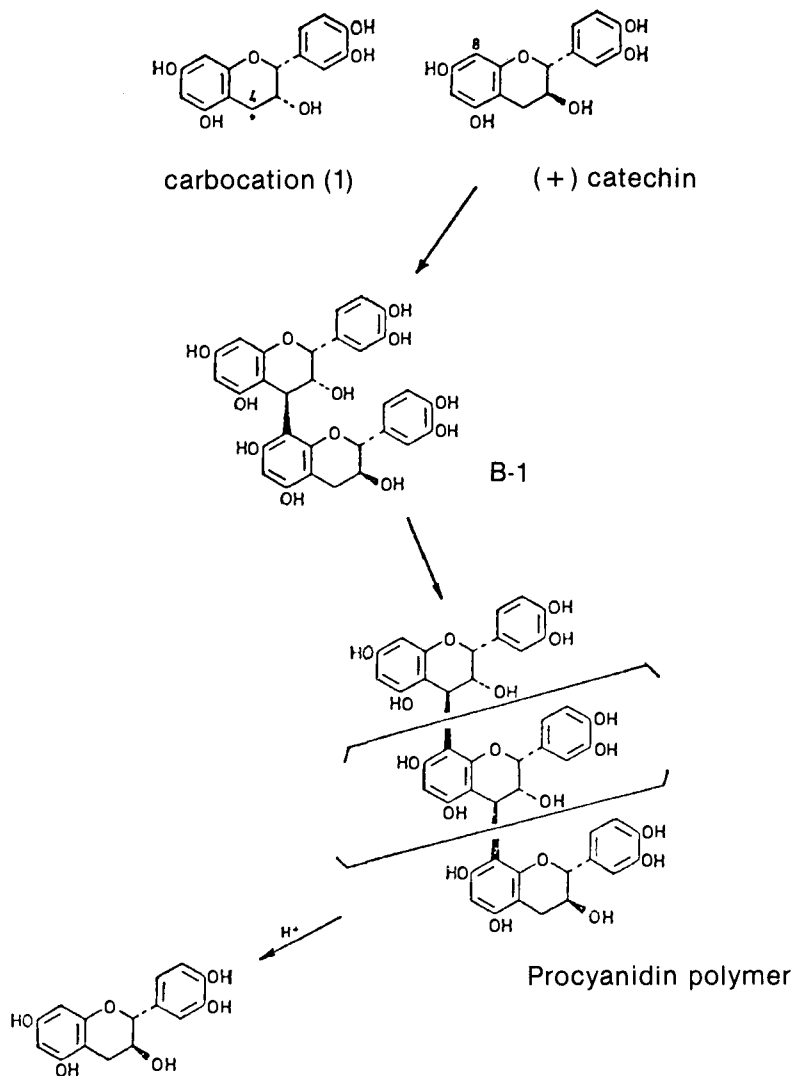
Fig. 5. Structure and biosynthesis of procyanidin polymer from Sorghum NK 300.

The polymeric procyanidin (up to ca. 5% of the grain) was isolated after chromatography of sorghum extracts on Sephadex LH-20 and gave analytical figures corresponding to a polytetrahydroxyflavan-3-ol structure. It contained, when pure, no sulfur or nitrogen. Treatment of the polymer with hydrochloric acid in ethanol at 60 °C gave cyanidin; it was therefore subsequently degraded by two acid-catalyzed procedures.

In this way the procyanidin polymer — which is the principal if not sole vegetable tannin of sorghum — was characterized as a hexameric-hepta-

meric polyflavan-3-ol (Fig. 5, n = 4 or 5) with an average molecular weight of 1700–2000 (Haslam and Gupta 1978). On the basis of previous work, and the observations outlined earlier, the polymeric procyanidin in sorghum cultivars may be presumed to be formed from (+)-catechin and the carbocation (1) in a multiple condensation process to give a polymeric structure of the general form shown in Fig. 5 in which the interflavan bonds are formed predominantly but not exclusively between C-4 and C-8 of the various flavan units.

Analysis of Procyanidins in Sorghum

The Vanillin Reaction

The method of quantitative analysis for tannins that has become most widely used for sorghum grain in the laboratory is that of the reaction of the polyphenols present with vanillin and hydrochloric acid. This reaction is not specific for polyphenols that are tannins and the reagent will react with any phenol that has an unsubstituted resorcinol or phloroglucinol nucleus activated toward electrophilic substitution in the molecule. It is therefore specific for a very narrow range of flavan-3-ols, dihydrochalcones, and proanthocyanidins, but it will not discriminate between these on the basis of molecular weight. In the sorghum analysis it will measure the total of flavan-3-ols and higher oligomers (which include the tannins).

The procedures outlined in the literature for the vanillin assay do not, in some cases, adequately define the conditions employed and the parameters that may be varied. Several features of the reaction and the method were therefore examined: these included the concentration of vanillin and hydrochloric acid in the reagent, the temperature at which the reaction was conducted, the choice of appropriate standards for the reaction, and various extraction procedures. The following conditions were found to be most suitable:

(1) Sorghum grain was ground to a fine powder (to pass a 0.5 mm sieve) and a sample (5.0 g) extracted with methanol (total volume 200 ml, 4 × 50 ml) in a high-speed mixer (10 min each extraction). The methanol extracts were filtered free and combined and reduced to a small volume at 30 °C by rotary evaporation. The extract was then transferred to a graduated flask with methanol and the final solution made up to 50 ml with methanol.

(2) The reagent was prepared by mixing *just prior* to use equal volumes of a vanillin solution (4% in methanol) and hydrochloric acid (8% of 12N acid in methanol). All solutions must be prepared as required.

(3) Aliquots of the sorghum grain extract (1.0, 2.0, 3.0, 4.0, and 5.0 ml) were added to graduated flasks and the volumes made up as required to 5.0 ml with additional methanol. From each flask an aliquot (1.0 ml) was removed and added to the reagent solution (5 ml) in a small cuvette at 30 °C and thoroughly mixed. After 30 min the optical density of the solution was measured at 500 nm using the reagent in the blank cell. Each measurement was made in triplicate as described above and a graph of optical density versus concentration constructed.

(4) Sorghum procyanidin polymer (NK 300, 25 mg — extracted as previously described) was dissolved in methanol (50 ml) and aliquots (1.0, 2.0, 3.0, 4.0, and 5.0 ml) taken as above and added to flasks and made up to 5.0 ml in volume. Aliquots (1.0 ml) were taken from each solution and reacted with the reagent solution (5 ml) for 30 min at 30 °C. The optical density of the solutions was measured at 500 nm and a calibration curve constructed from measurements made in triplicate. Comparison of the slopes of the standard and unknown graphs gave the concentration of tannin in the extract.

(5) Some comments on this method are appropriate. The vanillin reaction is a temperature dependent one and for accuracy and reproducibility it was found that the reaction must be conducted at a fixed temperature. Variation in the concentration of vanillin and of concentrated hydrochloric acid also were discovered to cause fairly wide variations (20–30%) in the observed absorbance at 500 nm for catechin, epicatechin, procyanidin dimers B-2 and B-3, and for the polymeric procyanidin (tannin) isolated from Sorghum NK 300. Most surprising, perhaps, has been the observation that the time courses for colour development for the various substrates in the vanillin assay are quite different and it has not yet been possible to decide precisely what the reasons are for these differences. It is clear that the reaction kinetics are markedly different for different substrates and that the reactions occurring in the assay are rather more complex than previously believed.

These observations are broadly responsible for the choice of reaction conditions in the vanillin assay and for the choice of the sorghum procyanidin polymer as standard for the assay. Given these restrictions the vanillin assay appears to be a workable and reproducible procedure. However, it must be borne in mind that the results from this method are generally likely to be high relative to other methods, because the reaction measures low as well as high molecular weight phenols and therefore does not measure tannins alone. Alternative procedures have therefore been examined in an attempt to devise methods more specific for the tannins in sorghum.

Ultraviolet Absorption

In contrast to low molecular weight phenols, the polyphenols of sorghum that constitute the tannin fraction are absorbed from ethanol solution onto Sephadex LH-20 and are only removed by elution with methanol. This observation forms the basis of the second procedure utilized to analyze the tannins in sorghum. The general pro-

cedure is outlined below. It is important that all the solvents used should be redistilled.

An extract of the polyphenols in the sorghum grain (5.0 g) was prepared in methanol (50 ml) as described above in the vanillin assay. An aliquot of this solution (2.0 ml) was applied to a column of Sephadex LH-20 in ethanol (25 × 2.5 cm). The methanol solution was absorbed onto the column and the column was then eluted with ethanol (500 ml) to remove low molecular weight polyphenols. The residue of soluble phenolic materials still retained on the column consists principally of the polymeric procyanidin and this may be eluted with methanol (1000 ml). The methanol eluate was concentrated at 30 °C and finally made up to a volume of 100 ml in a graduated flask. The optical density of this solution was measured at 280 nm and the amount of tannin determined from a standard concentration versus optical density graph prepared from authentic polymeric procyanidin (NK 300). Each analysis was carried out in triplicate and the mean of three values obtained.

This method is simple to operate, but it is also extremely tedious and time consuming, and this is its great disadvantage. Thus, whereas the vanillin assay may permit several samples of grain to be analyzed on the same day, one assay of a sorghum grain (carried out in triplicate) using the ultraviolet absorption method takes from 3 to 4 days to complete. This is due to the very slow elution rate of Sephadex LH-20 columns. The method is also an expensive one in terms of the utilization of solvents and Sephadex LH-20. It is however, we believe, relatively specific for the tannins in sorghum.

The Cyanidin Coloration Method

This procedure makes use of the fundamental characteristic of procyanidins — that is the formation of the red pigment cyanidin when heated with acid. Using this procedure all procyanidins (dimers, trimers, and high oligomers) react and are thus estimated by the chromogenic reaction. Whether dimers and trimers are tannins is in itself questionable, but from our own observations their concentration is very low in all the sorghum samples that we have examined. The procedure is briefly outlined below.

In this method a methanol extract (50 ml) of sorghum seeds (5 g) was prepared as outlined earlier (vanillin assay, above). Aliquots (0.5 ml, 1.0, 1.5, and 2.0 ml) were taken, added to conical flasks (50 ml), and evaporated to dryness. Butan-1-ol (10 ml) containing hydrochloric acid (12N, 30% v/v) was added to each flask and the solutions heated at 115 °C for 2.5 h. After cooling, the

solutions were transferred to graduated flasks and the volumes made up to 25 ml with butan-1-ol. The optical density of each solution was measured at 545 nm, and a graph was plotted of optical density versus concentration. Comparison with a standard calibration graph obtained analogously using the polymeric procyanidin from Sorghum NK 300 then gave the percentage of tannin in the unknown.

Some comments on this procedure are important. The development of cyanidin pigmentation from procyanidins by heating in alcoholic mineral acid is not a straightforward reaction. Side reactions occur with the formation of other coloured products (phlobaphens) and it is important that those interfere as little as possible with the colorimetric estimation. Maximum colour development is dependent both on the temperature at which the reaction is conducted and upon the acid strength of the media. Fairly wide differences (10–50%) were observed upon changing the acid (HCl) content of the reaction medium (10–60%). Some pigment degradation may also occur after long reaction times ($>$ 3 h) but maximum colour development is apparent after 2.5 h in the assay used. The method has been successfully used in this laboratory for samples of sorghum grain with relatively high tannin contents. It is much less reliable in our estimation in cases where the tannin content is low, and this is due to the side reactions noted and to other, as yet, undetermined colour-producing reactions that occur in the procedure.

β-Glucosidase Method

We have finally sought to develop an entirely new procedure for tannin assay in sorghum grain that utilizes the major property of these polyphenols, which may be classified as tannins, that distinguishes them from all other phenols — namely their ability to precipitate and complex with proteins. Various protein solutions have from time to time been proposed as test substances for the purpose of estimating vegetable tannins. Thus both gelatin and casein have been used, and Bate-Smith has utilized the proteins of hemolyzed blood to determine the relative astringency of some vegetable tannins. In this work the association of the vegetable tannins in sorghum grain with the enzyme β-glucosidase (EC 3.2 1.21) has been examined in a quantitative manner to discover if use can be made of this property to estimate the vegetable tannin content of sorghum grain.

β-glucosidase enzyme was extracted from sweet almonds and used as a freeze-dried powder ($\sim$ 4 units of enzyme activity/mg of protein ex-

Table 1. Analysis of tannin content of sorghum seeds.

Sorghum cultivar	Vanillin assay[1]	Ultra-violet method[1]	Cyanidin method[1]
BR 44	0.76, 0.70, 0.82	0.85, 0.80	0.80, 0.73, 0.71
BR 54	1.61, 1.49, 1.64	1.14, 1.24, 1.29	1.68, 1.62, 1.73
BR 64[2]	2.66, 2.48, 2.68	2.12, 2.20, 2.08	2.09, 2.15, 2.01
NK 300[2]	0.92, 0.88, 0.81	0.70, 0.75, 0.82	0.90, 0.94, 0.79
NK 300[3]	0.11, 0.15	—	0.02, 0.03
IS 2319[4]	0.14, 0.12	—	0.07, 0.06
IS 3648	2.95, 2.82, 2.69	2.38, 2.20, 2.41	2.80, 2.64, 2.73
IS 3924	0.09	—	0.04
IS 4225[2]	0.66, 0.59, 0.69	0.55, 0.50, 0.58	0.85, 0.74, 0.76
IS 9115	0.53, 0.58, 0.59	0.54, 0.49, 0.46	0.71, 0.67, 0.61
IS 9119[5]	0.14	—	0.06
IS 11167[5]	0.20, 0.15, 0.24	—	0.22, 0.20
IS 15991[2]	0.31, 0.28, 0.21	0.10, 0.12	0.16, 0.17
954114[3]	0.04	—	0.05
954206[4]	0.04	—	0.02
RS 671	0.02, 0.08	0.06	0.06

[1]Standard was the polymeric procyanidin isolated from Sorghum NK 300 supplied by Purdue University, Lafayette, Indiana. Figures given are percentage of dry weight of the whole sorghum seed.
[2]Coloured pericarp with coloured testa.
[3]White pericarp with white or no testa.
[4]White pericarp with coloured testa.
[5]Coloured pericarp with white or no testa.

tract). Sorghum grain was extracted into methanol as previously described, the methanol removed at 30 °C, and the residual polyphenols dissolved in 0.2 M acetate buffer, pH 5.0. Aliquots of the phenol solution are then taken and added to a solution of the enzyme ($\sim$ 2 mg per ml) in the same buffer. The precipitated protein is removed from the solution by brief centrifugation and the residual enzyme activity in the aqueous supernatant then determined by abstracting aliquots and adding these to a solution of p-nitrophenyl β-D-glucoside in acetate buffer (0.2 M, pH 5.0). The rate of release of the p-nitrophenol from the substrate was determined by adding aliquots at given time intervals to tris-HCl buffer (pH 8.5) and the extinction at 420 nm recorded. A plot of the p-nitrophenol formed against time gave a progress curve from which the initial reaction rate, and hence the enzyme activity remaining in solution, was determined. In this way the extent of precipitation of the β-glucosidase by the sorghum phenols was evaluated. This was compared to a standard precipitation curve of β-glucosidase prepared using differing concentrations of the procyanidin polymer derived from sorghum NK 300, and from this comparison the percentage of tannin in the unknown sorghum sample determined.

The method is a time-consuming one and, although it is only outlined above, it is very sensitive but requires considerable attention to detail.

We have not been able to achieve the type of consistency that is shown in the other three analytical methods described above over the period in which we have been using the procedure. The inconsistencies are due, we presume, to the numerous stages that have to be carried out and these appear to introduce random errors. However, in so far as the procedure appears to measure only those phenols that precipitate proteins (in this case β-glucosidase), it may be said to be the only procedure that actually measures the concentration of vegetable tannins in the sorghum grain in a specific manner. It may therefore be of interest to note that this procedure gives values that are similar to those shown in the accompanying tables but which are generally lower by some 10–15%.

Extraction of Polyphenols in Sorghum

We have utilized throughout this work one method for the extraction of polyphenolic material from the sorghum grain — namely solubilization in methanol. Some questions may be raised as to whether this is the best method and whether it removes *all* the phenolic material from the grain. Regarding the first question, the answer is that we have tried a variety of solvents (ethanol, propanol, water, acetone, dimethylformamide) and found that methanol has the most useful pro-

Table 2. Analysis of tannin content of sorghum seeds.

Sorghum cultivar	Vanillin assay		Ultra-violet method		Cyanidin method	
	Catechin[1]	Polymer[1]	Catechin[1]	Polymer[1]	B-2[1]	Polymer[1]
IS 3648	11.00	2.95	2.97	2.38	5.8	2.8
IS 4225	2.5	0.66	0.70	0.55	1.6	0.85
IS 9115	1.64	0.53	0.68	0.54	1.33	0.71
BR 44	2.87	0.76	1.2	0.85	1.5	0.8
BR 54	6.0	1.61	1.42	1.14	3.14	1.68

[1]Standards in analytical procedure.

perties so far as subsequent operations are concerned. As to the second question, the answer is that methanol does not remove all the phenolic material, some appears to remain behind, and may well be quite insoluble or attached to carbohydrate or other polymers in the grain.