composition, what suggests that they are homologous to each other, as found for wheat gliadins (Bietz et al., 1977).

As well as the amino acid composition of low molecular weight gliadins and A hordeins, the amino acid composition of A secalins (M_r 16 000; N terminal is Leu) is characterized by high Tyr and sulfur amino acid contents and a lack of Lys and His.

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Polyphenolic Changes in Ripening Bird-Resistant Sorghums

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Three chemical assays (vanillin–H_2SO_4, Folin–Denis, and cyanidin coloration), three biochemical assays (protein precipitation, α-amylase inhibition, and hemanalysis), and a paired preference assay on *Quelea quelea* were used to evaluate eight bird-resistant sorghum varieties in the milk, light dough, firm dough, and mature stages of grain development. Each assay showed an increase in the respective polyphenolic activity that peaked in the dough stages (usually firm dough) and then dropped sharply in the mature stage. Polyphenol activity in varieties classified as group II tended to peak earlier in grain development and then drop by a greater extent in the ripened grain. Although there is evidence that tannin biosynthesis goes to a higher degree of polymerization in group II than group III sorghums, the synthetic mechanism alone does not fully explain the differences between the two groups. Gel permeation, thin-layer, and paper chromatography analyses indicated that the tannins were procyanidins in both groups. Therefore, further elucidation depended upon differences in grain structure or the influence of other grain components. Three of these factors are discussed.

Sorghum is grown on 44 million hectares throughout the world (FAO, 1978), many of which are susceptible to extensive bird damage. Nearly all the sorghum grown in this country is used for livestock feed, but it is a staple food crop and the basic source of nourishment for the human population in many developing countries. Because of climatic conditions, farming practices, or marketing conditions, many farmers can grow only sorghums. Often, bird depredation further restricts the farmer's choices to brown, tannin-containing, bird-resistant (BR) varieties. Therein lies the dilemma; most brown sorghums available to farmers have unfavorable flavor and nutritional qualities. The U.S. standards for sorghums recognize them according to four classes: yellow, white, brown, and mixed ("Official United States Standards for Grain", 1974). Most of the

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world follows a similar classification for marketing, and lower prices are received for brown sorghum classes.

Most of the polyphenolic properties of BR sorghums are presently attributed to procyanidin tannin oligomers (Strumeyer and Malin, 1975; Gupta and Haslam, 1978, 1980) located in the grain testa. The familiar biochemical properties of these condensed tannins (i.e., astringency, enzyme inhibition, protection of seeds from weathering, deleterious nutritional effects, tanning of hides, etc.) are all related to their ability to bind with proteins (Bullard and Elias, 1980). Hydrogen bond formation between the hydrogen of phenolic hydroxyl groups of the tannin molecule and the oxygen of the keto–imide peptide bond of two or more suitably oriented protein molecules is often suggested as a binding mechanism (Gustavson, 1956; Joslyn and Goldstein, 1964; Van Buren and Robinson, 1969). Hydrophobic (Hagerman and Butler, 1980a), ionic, and covalent bonds have also been suggested (Haslam, 1979). The capacity of tannins to form strong cross-links with proteins is broadly related to molecular size, structure, and shape (Goldstein and Swain, 1963; Quesnel, 1968) but more specifically to the number of separate sites in the molecule able to associate with the protein (Haslam, 1979). Below the triflavan level, molecules are too small to be effective (Goldstein and Swain, 1963; Roux, 1972), and highly polymerized tannins (>10 flavan monomers) either are too insoluble, have too few reactive sites, or are too large to fit the protein orientation for cross-linking (Joslyn and Goldstein, 1964). Protein binding activity seems to increase with size and peaks somewhere between 3 and 10 monomers (Goldstein and Swain, 1963; Roux, 1972).

Astringency, that contracting or dry feeling in the mouth caused by precipitation of proteins in saliva and on mucosal surfaces (Joslyn and Goldstein, 1964; Singleton and Noble, 1976), is considered to be the principal mode of repellency in BR sorghums (Bullard and Elias, 1980). Fruits such as bananas, peaches, plums, or persimmons are astringent in the immature stages of development and then lose this quality during ripening (Hillis and Swain, 1959; Goldstein and Swain, 1963). The same pattern exists in carobs (Tamir and Alumot, 1970). These changes in astringency have been linked primarily to polymerization processes, leading to decreased reactivity and extractability of the tannin molecules or attachment to skeletal tissues in the plant material (Goldstein and Swain, 1963; Price et al., 1979; Gupta and Haslam, 1980). Apparently the polymerization process and associated changes in other tissue components accelerate during ripening.

The literature is not clear as to whether the same phenomenon occurs during grain development in BR sorghums. Gupta and Haslam (1980) observed that the grain of NK-300 is formed initially in a sheath and at the etiolated stage no procyanidins can be detected. As chlorophyll develops in the pericarp, rapid proanthocyanidin synthesis occurs, but as the grain ripens, the monomeric and dimeric flavan-3-ol species rapidly decrease in concentration to leave principally polymeric procyanidin. This laboratory (Jaques et al., 1977; Gupta and Haslam, 1978) proposes the biosynthesis to be a nonenzymatic process involving spontaneous condensation via a carbocation. Glennie (1981), also with NK-300, found polyphenol oxidase and peroxidase activity to decline as the grain developed and to be nondetectable in mature grain.

Others have reported an increase in tannin concentration during seed development that continues (Johari et al., 1977), plateaus (Tipton et al., 1970), or decreases during ripening (Mabbayad and Tipton, 1975; Price et al., 1979: David and Hoseney, 1980; Rooney et al., 1980; Glennie, 1981). None of these have dealt adequately with the mechanisms of tannin production or the many variations found among BR sorghums.

In earlier studies we had observed large differences in the molecular composition of polyphenols in the mature seeds of 15 BR sorghums (Bullard et al., 1980). Price et al. (1978) classified sorghum into three groups according to polyphenol composition. We were particularly interested in tannin-containing varieties classified as group II which are nutritionally similar to nontannin varieties (Oswalt, 1975). There is enough divergence in the polyphenolic properties of BR sorghums to postulate that the expression of tannin activity varies according to genotype. The delineation of these properties and their genetic sources could lead to varieties which have both adequate BR qualities in the immature stages and a ripened grain of good taste and nutritional quality.

An examination of this proposition was the basis for this experiment. Samples from the milk, light dough, firm dough, and mature stages of eight BR varieties were evaluated in bird preference, Folin–Denis, vanillin, cyanidin, α-amylase inhibition, hemanalysis, and protein binding assays. These were to be used as tools to help us better understand the general ripening process and determine the nature and intensity of variations that occur in developing and mature BR sorghum seeds.

EXPERIMENTAL SECTION

Sorghum heads in four stages of maturity (milk = 12–19 days; light dough = 20–29 days; firm dough = 30–40 days; mature ≥ 50 days after half anthesis) were gathered from eight varieties grown at the University of Arkansas, Fayetteville, experimental farm. These genotypes (Hegari = $B_1B_1B_2B_2$ssRRyyiizz; TAM 2566 = $B_1B_1B_2B_2$SSrryyiiZZ; IS 2266C = $B_1B_1B_2B_2$ssrrYYiizz; IS 2801c = $B_1B_1B_2B_2$SSRRyyiizz; IS 2403c = $B_1B_1B_2B_2$SSRRYYIIzz; AR 3005 = $B_1B_1B_2B_2$SSrrYYiizz; BR 54 and Funks G516-BR = $B_1B_1B_2B_2$SSRRYYiiZZ) were to represent a variety of characteristics that occur in BR sorghums. All immature heads were cut with 12–18-in. stems, frozen, transported by air under dry ice to Denver, and stored at −30 °C until used in testing.

Special precautions were taken during sample preparation to minimize enzyme-mediated changes in polyphenolic composition. The heads were removed from the freezer, immersed in liquid nitrogen, and then rubbed and agitated on 6–10-mesh sieves to free and clean the immature seeds. Meanwhile a small Wiley mill had been cooled with liquid nitrogen for grinding under cryogenic conditions. The ground material (17 g) was immediately weighed and placed in 50 mL of acetone in a 250-mL capped centrifuge bottle to inhibit enzyme activity. Samples were then extracted by shaking for 2 h with the 50 mL of acetone, then methanol (1×), and finally 90% methanol (2×). The water in the last extraction was added so that adsorption by plant material could be minimized without changing polarity of the extraction medium to the extent that high molecular weight polymers would be extracted. After each extraction the bottle was centrifuged for 5 min at 1800 rpm. The combined supernates were concentrated to near dryness by rotary evaporation and brought to 50 mL with methanol. Aliquots of each extract were placed in capped culture tubes, reduced to dryness, and held at −15 °C until the respective analysis.

An additional sample of frozen grain (~20 g) was weighed, dried for 3 days in an 80 °C forced-air oven, and reweighed to determine percent moisture. The respective percent moisture value was used to correct all tannin assay data except for the α-amylase and bird preference assays.

Bird Preference Tests. *Quelea quelea* (quelea), a red-billed weaver finch, were trapped in Tanzania, flown to the Denver Wildlife Research Center, and held for 90 days quarantine and acclimatization. All birds were held in a large 2.4 × 4.8 × 2.1 m aviary and allowed free access to water, grit, and a maintenance ration of whole grain sorghum, proso millet, and Purina Game Bird Startena.

The test procedure has been discussed in detail elsewhere (Bullard and Shumake, 1979). Birds were transferred from the aviary to 44 × 25 × 20 cm test cages held on a rodent cage stand. Each cage was divided by wire mesh for testing pairs (one bird on each side of the divider). Five male birds were each offered a choice between hulled proso millet coated with a BR sorghum extract (from 10% by weight ground sample) and the same millet treated with only the solvent. The coating was accomplished by stirring the concentrated sample extract on hulled proso millet (inside a fume hood) while the solvent (essentially methanol) evaporated. Coated seeds were then spread on a sheet of aluminum foil and held for 2 h under good airflow to remove all traces of the solvent. On each test day the two foods were placed in separate cups 5 cm apart at the front of each cage, and cup positions were alternated daily to decrease any position habit bias. Weighed amounts of each food (5 g) were placed in 5-cm diameter × 45 cm high heavy glass dishes at the beginning of each test day (12 h of fluorescent lighting and then 12 h off), and the unconsumed remainder was weighed the following morning for each of 6 days. The difference, corrected for spillage, was recorded as the quantity consumed. Preference was defined as the percentage of total consumption that consisted of test food according to the formula

$$\text{preference} = \frac{\text{test food consumed (g)}}{\text{test + control food consumed (g)}} \times 100$$

Chemical Assays. Because of our desire to compare the tannin changes in ripening sorghum with those in fruits, we followed the investigational process of Goldstein and Swain (1963) and Swain and Hillis (1959) as closely as possible. A Beckman DK-2A ratio recording spectrophotometer (1-cm cell) was used for spectrophotometric analyses and all samples were analyzed in duplicate.

Folin–Denis Assay. Small quantities (usually 10 μL) of the sample aliquot were added to 1 mL of water and vortexed for 1 min to assure complete dissolution. One milliliter of Folin–Denis reagent was added, the mixture was vortexed for 5 s, and then exactly 3 min later, 1 mL of 1 N sodium carbonate was added and this mixture was vortexed. After 1 h the sample A_{725} was determined with a reagent blank in the reference beam. Results were expressed as catechin equivalents (CE) by using standard curves prepared daily for the conditions used in the analysis, from fresh solutions of commercial D-catechin.

Cyanidin Coloration Assay. This has often been called the leucoanthocyanin (LA) assay. Simple aliquots (usually 200 μL) were added to a 50-mL capped culture tube containing 3 mL of 5% HCl in butanol. This mixture was vortexed to assure complete dissolution, placed in a mineral oil bath to a depth slightly above the liquid level of the tube, and refluxed for 2 h at 97 °C. The tube was then removed and cooled under running tap water for 5 min, and the A_{565} determined with a butanol–HCl blank in the reference beam. Concentrations were extrapolated from a standard curve of cyanidin–HCl dilutions in 5% butanol–HCl.

Vanillin–H_2SO_4 Assay. Small aliquots (usually 10 μL) were added to 25-mL glass tubes and processed exactly according to the spectrophotometric method of Swain and Hillis (1959). We expressed results as CE using standard curves prepared daily for the conditions used in the analysis, from fresh solutions of commercial D-catechin.

Biochemical Assays. The following assays were selected so that biochemically we could more directly assess the binding properties of BR tannins. All samples were analyzed in duplicate.

α-Amylase Assay. The method used for this assay was a combination of procedures by Davis and Hoseney (1980) and Barnes and Blakeney (1974). Sample aliquots (usually 100 μL) were added to 25-mL Erlenmeyer flasks and brought just to dryness by allowing them to stand a few minutes at room temperature. Then 4 mL of distilled water was added and the mixture ultrasonicated until the extract completely dissolved. A Phadebas tablet (cross-linked starch polymers containing dye; Pharmacea Diagnostics, Piscataway, NJ) was added and the flask held at 30 °C for 30 min in a Dubnoff shaking incubator. Then 1 mL of enzyme solution (0.003% porcine α-amylase; Sigma Chemical Co., St. Louis, MO; in 0.05 M maleate buffer containing 0.2 g/L calcium chloride) was added and the mixture agitated for exactly 10 min at 30 °C. The reaction was stopped by adding 1 mL of 0.5 N NaOH and vortexing. After dilution with 10 mL of water and filtration, the average A_{620} of duplicate samples was determined with reagent blanks in the reference beam. Percent inhibition was calculated from enzyme activity (IU/1000 mL) by using the formula

$$\%\ \text{inhibition} = \frac{\text{distilled } H_2O \text{ control IU/L} - \text{sample IU/L}}{\text{distilled } H_2O \text{ control IU/L}} \times 100$$

Protein Precipitation Assay. Sample aliquots (usually 100 μL) were processed according to the protein precipitation method of Hagerman and Butler (1978) except activity was related to the concentration of tannic acid instead of BR-54 tannin. The average A_{510} of duplicate samples was determined with a $NaDodSO_4$–triethanolamine solution plus ferric chloride reagent in the reference beam. Results were expressed as tannic acid equivalents (TAE) by using standard curves prepared daily for the conditions used in the analysis.

Hemanalysis. We used a modification of a method by Bate-Smith (1973a,b). Sample aliquots (usually 100 μL) were taken to dryness in a 15-mL centrifuge tube. Then 1 mL of distilled water was added, and the mixture ultrasonicated or vortexed until the extract completely dissolved. Then 1 mL of hemoglobin reagent (2 mL of heparanized white rat blood in 98 mL of distilled water) was added, and the mixture vortexed for 30 s and centrifuged at 5000*g*. The average A_{578} was determined with a reagent blank in the reference beam. Results were expressed at tannic acid equivalents (TAE) by using standard curves prepared daily for the conditions used in the analysis.

Chromatography. The structural characteristics of polyphenols in each sample extract were determined by a modification of the Sephadex LH-20 gel permeation chromatography procedure of Strumeyer and Malin (1975). Sephadex LH-20 separates compounds on the basis of their molecular weight and adsorptive characteristics. Sample extracts equivalent to 7.35 g of sorghum were concentrated to 250 μL and injected on a 1 × 30 cm Sephadex LH-20 column and eluted with 60 mL of absolute ethanol, followed by 60 mL of 95% ethanol, 30 mL of 50% methanol, and then 50% acetone until all tannins were removed. The column was then flushed with absolute ethanol and held

Table I. Means for Chemical Assays of Tannin Activity in Eight Developing and Ripening Bird-Resistant (BR) Sorghum Varieties[a-c]

variety	group	stage	vanillin-H_2SO_4, CE	Folin-Denis, CE	cyanidin, CYE	V/FD × 100	V/LA × 100
Hegari	II	1	0.44c	0.78d	0.78c	56.41	56.41
		2	0.42	0.88	0.69	47.72	60.87
		3	0.25	0.53	0.32	47.17	78.12
		4	0.06	0.08	0.01	75.00	600.00
TAM 2566	II	1	0.20bc	1.18d	0.68bc	16.95	29.41
		2	0.60	1.02	0.98	58.82	61.22
		3	1.07	0.86	1.02	124.41	105.90
		4	0.10	0.21	0.02	47.62	500.00
IS 2266c	II	1	0.56c	1.39bcd	1.44bc	40.29	38.89
		2	0.54	1.39	1.78	38.84	30.34
		3	0.20	0.61	0.27	32.78	74.07
		4	0.10	0.24	0.01	41.66	1000.00
IS 2801c	III	1	0.64bc	1.94ab	1.29abc	32.98	49.61
		2	0.68	1.85	1.45	36.76	46.89
		3	0.77	1.81	1.13	42.54	68.14
		4	0.12	0.45	0.28	26.66	42.86
IS 2403c	III	1	1.06a	1.50ab	0.91ab	70.67	116.48
		2	1.24	2.68	2.08	46.26	59.61
		3	1.14	2.50	1.77	45.60	64.41
		4	0.28	0.86	0.69	32.56	40.58

variety	group	stage	vanillin-H_2SO_4, CE	Folin-Denis, CE	cyanidin, CYE	V/FD × 100	V/CY × 100
AR 3005	III	1	0.82ab	1.76ab	0.88ab	46.59	93.18
		2	0.72	1.93	1.51	37.30	47.68
		3	0.83	1.78	2.04	46.63	40.68
		4	0.54	1.02	1.38	52.94	39.13
BR-54	III	1	0.36bc	1.39bc	0.85abc	25.90	42.35
		2	0.62	1.90	1.38	32.63	44.93
		3	0.81	1.83	1.90	44.26	42.63
		4	0.19	0.67	0.84	28.36	22.62
Funks G516-BR	III	1	0.41ab	1.38a	0.66a	29.71	62.12
		2	1.28	3.08	2.91	41.56	43.98
		3	1.27	2.90	2.77	43.79	45.85
		4	0.18	0.93	1.05	19.35	17.14

[a] Aliquots of combined acetone, methanol, and 90% methanol extracts of samples from eight BR sorghum varieties in four stages of maturity. All values have been corrected for moisture content and are listed on a dry weight basis. [b] Assays of extract aliquots are conducted by the respective procedures of Hillis and Swain (1959), expressed in catechin equivalents (CE) for vanillin-H_2SO_4 (V) and Folin-Denis (FD) assays and cyanidin equivalents (CYE) for the cyanidin coloration (CY) assay. [c] All means followed by the same letter are not significantly different.

overnight for the gels to return to their original size before the next test.

Fractions (3 mL) of eluate were read at 340 nm on a Bausch & Lomb Spectronic 20 and plotted according to transmittance. The 3-mL round cuvettes used for this instrument were the correct size for a Buchler Fractomette 20 fraction collector. While the 340-nm wavelength is not at the peak of the absorption curve for proanthocyanins, it is on the trailing edge and this is a good chromatography "detector". The chromatograms were divided into five regions (four nontannin and one tannin), and the area of each region was determined in cm^2 by planimetry.

Thin-layer and paper chromatography analyses were used in examining the proanthocyanidin composition of Funks G516-Br (group III) and TAM 2566 (group II) samples in each stage of maturition. Extracts were concentrated to 20% volume and spotted on activated Eastman silica gel (without fluorescent indicator) thin-layer chromatofilm. Chromatograms were developed with absolute methanol and examined under visible or UV light. (A Sephadex LH-20 tannin reference did not migrate from the origin under these conditions.) Three reagents (bis-diazotized benzidine, vanillin–toluene-*p*-sulfonic acid, and toluene-*p*-sulfonic acid) which give specific colors for various proanthocyanidins were also sprayed on the chromatograms (Roux and Maihs, 1960).

Paper chromatography was used in the analysis of reaction products from the cyanidin coloration assay. Reaction solutions were concentrated to 20% volume and spotted on Whatman No. 1 chromatography paper, and the chromatograms developed with Forestal solvent (HOAc–HCl–H_2O at 30:3:10). Flavylium salts are readily separated under these conditions (Ribereau-Gayon, 1972).

Statistical Analyses. We computed a two-factor analysis of variance (eight BR sorghums × four states of development) to assess differences ($P \leq 0.05$) in tannin activity and then compared treatment means by using the Duncan Multiple Range Test (Duncan, 1957).

RESULTS AND DISCUSSION

Tables I and II summarize the results of the chemical, biochemical, and preference assays by variety. Table III compares the results by group and stage of development. Statistical analyses indicate that significant ($P \leq 0.05$) differences exist among varieties and stages by all three of the chemical assays and by the protein binding and enzyme inhibition assays. A significant difference among varieties but not stages was determined by the quelea preference assay.

Each of the assays showed an increase in the respective polyphenolic activity that peaked in the dough stages (usually firm dough) and then dropped sharply in the mature stage. Table III indicates that significant changes took place during ripening. In the five assays where differences by stage of maturity occurred, the mature stage was consistently different from the other three. The degree

Table II. Means for Biological Assays of Tannin Activity in Eight Developing and Ripening Bird-Resistant (BR) Sorghum Varieties[a]

variety	group	stage	biochemical assays[b]: protein precipitation, TAE	hemanalysis, TAE	α-amylase inhibition, %	quelea preference, %[c]
Hegari	II	1	3.09c	4.28	14.60cd	54.00b
		2	2.71		26.79	31.00
		3	1.93		20.48	24.30
		4	1.03		3.52	55.60
TAM 2566	II	1	2.76c	4.35	19.27bc	44.00b
		2	2.37		23.15	36.80
		3	1.91		32.60	32.20
		4	1.67		0.00	44.10
IS 2266c	II	1	4.11bc		25.33bc	44.00b
		2	3.50		29.21	36.20
		3	2.04		12.24	45.60
		4	1.36		5.94	36.90
IS 2801c	III	1	4.32bc	1.71	22.91ab	44.30b
		2	2.34	1.81	21.94	41.30
		3	2.11	2.27	24.12	49.10
		4	1.40	0.49	17.09	26.10
IS 2403c	III	1	2.59bc	0.81	24.36a	28.00ab
		2	3.63	1.95	31.63	42.40
		3	2.79	2.85	35.54	29.50
		4	1.58	0.49	21.90	38.70
AR 3005	III	1	3.27abc	4.30	25.82ab	20.00ab
		2	3.21	0.07	27.27	32.20
		3	3.11	0.55	29.09	34.60
		4	3.02	0.70	24.85	25.40
BR-54	III	1	3.39ab	1.61	7.39d	30.60a
		2	3.80	1.85	8.36	22.80
		3	3.54	1.78	13.94	8.50
		4	2.52	0.31	3.51	32.70
Funks G516-BR	III	1	3.38a	3.66	19.27	40.90ab
		2	5.66	5.08	21.94	30.70
		3	4.23	3.77	25.82	22.70
		4	2.75	0.21	18.06	40.30

[a] All means followed by the same letter are not significantly different. [b] Biochemical assays of extract aliquots were conducted by the following procedures: α-amylase inhibition by Davis and Hoseney (1980) and Barnes and Blakeney (1974); protein precipitation in tannic acid equivalents (TAE) by Hagerman and Butler (1978); hemanalysis in TAE by Bate-Smith (1973a). [c] Preference response of quelea when given a choice between millet having a surface-coated extract and control millet: percent preference = (test food consumed)/(test + control food consumed) × 100.

Table III. Means for Chemical and Biological Assays Overall and by Sorghum Classification[a,b]

classification and stage	chemical assays: vanillin-H_2SO_4, CE	Folin-Denis, CE	cyanidin, CYE	biological assays: protein precipitation, TAE	α-amylase inhibition, %	quelea preference, %	V/FD × 100	V/LA × 100
overall (n = 8)								
milk	0.56a	1.41b	0.94b	3.36a	19.88a	38.22	39.72	59.57
light dough	0.76a	1.84a	1.60ab	3.40a	23.78a	34.17	41.30	47.50
firm dough	0.79a	1.60ab	1.40a	2.62a	24.22a	30.81	49.37	56.43
mature	0.19b	0.56c	0.54c	1.92b	11.85b	37.47	33.93	35.18
group II (n = 3)								
milk	0.40	1.12	0.97	3.32	19.76	47.33	35.71	41.24
light dough	0.52	1.10	1.15	2.86	26.38	34.67	47.27	45.22
firm dough	0.51	0.67	0.54	1.73	21.77	34.03	76.12	94.44
mature	0.09	0.18	0.01	1.35	3.15	45.53	50.00	900.00
group III (n = 5)								
milk	0.66	1.59	0.92	3.39	19.95	32.76	41.51	71.74
light dough	0.91	2.29	1.87	3.73	22.23	33.88	39.74	48.66
firm dough	0.96	2.16	1.92	3.16	25.70	28.88	44.44	50.00
mature	0.26	0.79	0.85	2.25	17.08	32.64	32.91	30.58

[a] Overall = group II + group III varieties; group II = Hegari, TAM 2566, and IS 2266c; group III = IS 2801c, IS 2403c, AR 3005, BR 54, and Funks G516-BR. [b] All means followed by the same letter are not significantly different.

of difference apparently depends on whether a variety is group II or III by the classification method of Price et al. (1978). Group II sorghums tended to be different from the higher tannin-containing group III sorghums such as Funks G516-BR, BR-54, and AR 3005 as evidenced by both the chemical and biological assays.

A general net synthesis of polyphenols was indicated from the milk through the dough stages in all three chemical assays, although a different chemical characteristic was being measured by each. Vanillin reagent (V) reacts in an approximately stoichiometric manner with unsubstituted phloriglucinol groups in tannins, cyanidin

(CY) reagent converts flavans to their flavylium salts, and Folin–Denis (FD) reagent reacts nonstoichiometrically with hydroxyl groups. Therefore, the ratios can be used as indicators of molecular size. Although not significantly different, the increasing V/FD values (Table III) from the milk stage through the firm dough stage are consistent with ongoing synthesis of vanillin-reactive molecules from smaller phenols. The Folin–Denis reagent would react with all intermediates whereas vanillin could not react until proanthocyanidin molecules had been synthesized (Bullard and Elias, 1980). The decrease in ratio values from the firm dough to the mature stage indicates that the mean size of extracted tannins increases after ripening begins. The V/CY values indicated the same trend for group III sorghums, but group II values for the mature stages were incongruous. Although the CY reaction is desirable from the standpoint of being specific for flavanols and their cogeners, it is low in yield because of unpredictable side reactions, especially the major polymerization side reaction of amorphous phlobaphen formation (Haslam, 1966; Lewak, 1968; Mathew, 1969; Ribereau-Gayon, 1972). Lewak (1968) indicates that if differences in simple phenols are not a problem, then the V/FD ratio gives much more reliable results than the V/CY coefficient. We believe this to be true for sorghum nontannin phenols (discussed below).

All of the biological tests (Tables II and III) followed the same activity pattern shown by the chemical tests. The TAE for protein binding in mature samples are somewhat elevated because reagent reactive nontannin flavanoid pigments such as luteolinidin (Gupta and Haslam, 1980) were trapped in the protein–tannin pellet and elevated the A_{510} value. We subsequently eliminated this problem by adding a step to the procedure where the pellet is vortexed in 4 mL of absolute methanol and again centrifuged. The protein precipitation assay, with this modification, is the most direct, simple, and accurate method of measuring protein binding activity of tannins and has emerged as our choice for screening purposes.

Significant differences ($P \leq 0.05$) were found among both varieties and stages by the α-amylase inhibition test, and the activity paralleled that of the other assays. However, we found it necessary to analyze an enzyme control with each set of samples because enzyme activity changed almost hourly. Similar problems with variability have been reported for the α-amylase method at Texas A&M (Rooney et al., 1980; Earp et al., 1981) and for the β-glucosidase method (Gupta and Haslam, 1980). This variability, combined with the high cost of materials and time, makes this method an undesirable one for analysis of large numbers of samples.

Hemanalysis gave variable and incomplete results and can be considered no more than an indicator test. Hence, summary data have not been included in Table III. However, this assay did reveal dramatic differences between group II and III sorghums. Hemoglobin–tannin precipitates could be separated from the supernatant at 5000g centrifugation for group III but not group II samples.

The quelea preference response (Table II) followed the same trend as the chemical and biochemical tests. This indicates that the tannins were eliciting astringent reactions in the mouth and the birds were responding. Among-animal variations usually make it more difficult to measure differences in behavorial than in chemical or biochemical assays. Consequently, this was the only test listed in Table III which did not have significant among-stage differences by ANOVA.

All the chemical and biochemical tests indicated that tannin changes similar to those reported for ripening fruits (Goldstein and Swain, 1963) occur in sorghums. Our results, and those recently reported by Glennie (1981), are consistent with the model of Gupta and Haslam (1978, 1980) that is discussed above for sorghums. We assume that the 1700–2000 molecular weight range they found for NK-300 (a group III variety) generally applies for our five group III candidates. However, the data indicate that tannin properties for our group II's would be quite different, especially in the mature stage.

In an experiment involving mature grain samples (Bullard and Elias, 1980), V/FD and V/CV ratios indicated that the mean sizes of oligomers in the extracts of group II varieties were larger than those for group III. This did not account for differences in the unextracted tannins that were insoluble because of size, suggesting the polymerization reaction goes to a higher degree of polymerization in group II than group III sorghums. That size influences the activity of even extracted tannins was shown in another experiment; the methanol extract (smaller oligomers) of a commercial tannin product was significantly less preferred than the nonextractables in a quelea preference study (Zeinelabdin, 1980).

In another experiment, however, molecular size was shown to not be the only factor involved in group II and III differences (Bullard and Elias, 1980). One speculation, proanthocyanidin differences, arose when we observed distinctions in color between cyanidin assay reaction products of mature grain from the two groups. We have since examined the extracts of Funks G516-BR (group III) and TAM 2566 (group II) grains in four stages of maturity by paper and thin-layer chromatography. Thin-layer analyses indicated there were no significant flavolan differences in extracts from the milk, light dough, or firm dough stages and that proanthocyanidins hydroxylated in the 5 position predominated.

Paper chromatography analyses of the cyanidin coloration assay products provided further elucidation of flavolan composition. A major red spot with an R_f of 0.5 and a minor spot with an R_f of 0.62 were found for all four stages of Funks G516-BR and for the milk, light dough, and firm dough stages of TAM 2566. The major spot coincided with the cyanidin reference spot and literature reference values (Ribereau-Gayon, 1972), but we did not find a purple spot with $R_f = 0.32$ for delphinidin. Orange spots were found at $R_f = 0.66$ for both varieties in all four stages of maturity which probably represented luteolinidin ($R_f = 0.61$), a nontannin pigment reported to be present in sorghums (Bullard and Elias, 1980). These particular investigations were not exhaustive, but the results convinced us that both group II and group III tannins are composed of procyanidins.

This finding helps explain the differences in color mentioned earlier between reaction products of the leucoanthocyanin (called cyanidin coloration here) assay on mature group II and III samples (Bullard and Elias, 1980). Both groups had an orange or light brown color from pelargonidin, luteolinidin, or other plant pigments, but in group III varieties enough tannins were extracted for the scarlet reaction product to dominate the appearance of the solution.

The thin-layer chromatograms helped us to better understand the procyanidin properties of the two varieties. Nonmigrating tannin oligomers were present in all milk, light dough, and firm dough samples. In Funks G516-BR the concentration of migrating oligomers increased from the milk through the light dough stage and was barely

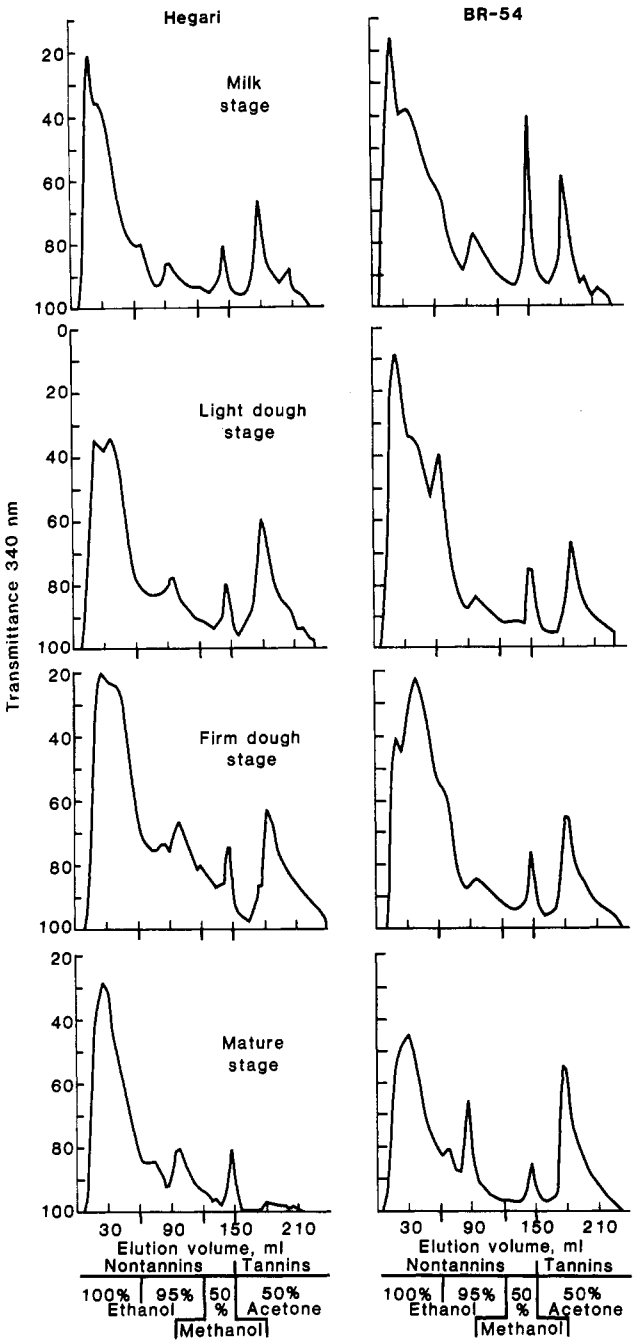

Figure 1. Sephadex LH-20 gel permeation chromatograms of extracts from two tannin-containing sorghums in four stages of maturity. Hegari is a group II sorghum and BR-54 a group III sorghum. Nontannins were removed with 100% ethanol, 95% ethanol, and 50% methanol, followed by tannins which eluted with 50% acetone.

detectable in the mature stage. This indicates that some ongoing synthesis takes place throughout the immature stages but these are incorporated into larger polymers during the final ripening stage. TAM 2566 chromatograms were not as intense but had the same pattern for mobile oligomers (R_f = 0.03, 0.33, and 0.48) in the milk and light dough stages, but only one spot of lowered intensity (R_f = 0.33) appeared in the firm dough stage, indicating this variety loses these flavolans earlier. Only a trace of tannin was detectable in the spot remaining at the origin for the mature TAM 2566 sample.

The Sephadex LH-20 chromatographic profiles of BR-54, a group III sorghum, and Hegari, a group II sorghum, are given in Figure 1. Our chromatograms indicate that the nontannins are similar for all eight varieties. Several

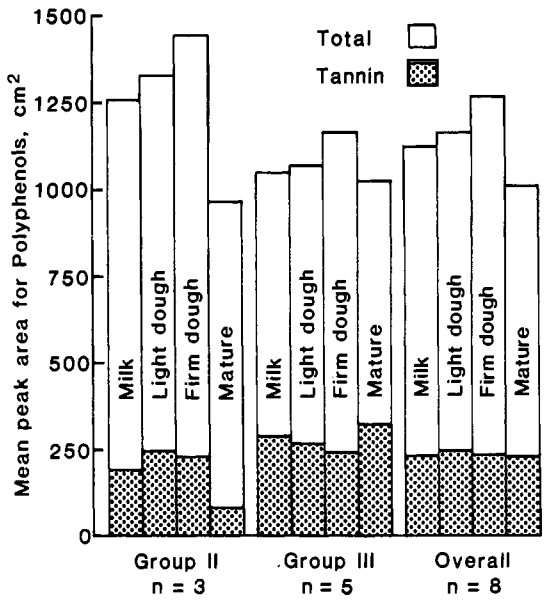

Figure 2. Relative Sephadex LH-20 chromatogram areas (transmittance at 340 nm) in cm² of extracts from sorghum seeds in four stages of maturity. Tannin area is related to the total (nontannin plus tannin) peak area with respect to group II, group III, or overall classification.

nontannin flavanoids have been previously reported in sorghums (Bullard and Elias, 1980). In the chemical assays the FD values correlated well with V (r = 0.86) and CY (r = 0.90), indicating that most of the nontannin phenols were flavanoids. We have collected, purified, and tested seven Sephadex LH-20 nontannin fractions and found five of them to react in both V and CY assays. Spectrophotometrically, they have maximum absorption at wavelengths between 279.5 and 283.5 nm, the same range in which tannin absorption reaches its peak. The FD values (CE) for the other two minor fractions average only 56% (w/w) for the mean of these five fractions.

Although the ANOVA did not indicate a significant difference among varieties or stages for the four nontannin peaks, there were indications that the procyanidin oligomer in the 50% methanol eluting peak is polymerized into higher molecular weight tannins. The tannin peak is present during the three immature stages for both varieties, but with Hegari it nearly disappeared in the mature stage while it grew in BR-54. This observation applied over all varieties and is expressed in Figure 2. Also, we see that the amount of extractable polyphenolics dropped in the mature stage, but most of this was expressed in the group II varieties. This indicates that more of the sorghum polyphenols are left bound or unextracted in the mature group II samples than in those from group III.

Thus, it appears that the main differnces between the two groups are in the tannins available for expression of their activity. All data indicate that a process begins to take place in the firm dough stage of group II sorghums that reduces their chemical and biochemical activity to a much greater degree than in group III sorghums. In addition to the processes of biosynthesis mentioned earlier, investigators have tangible evidence of three other possible sources of these differences: (1) other extractable grain components, (2) other grain tissue components, and (3) structure of the testa.

In the hemanalysis assay the tannin–hemoglobin precipitates from group III were deposited on the sides of the tube at 5000g centrifugation, but we were unable to deposit those from group II even though several modifications were attempted. This indicates that other soluble seed com-

ponents, perhaps saponins (Bate-Smith, 1977), inhibited this process.

Recently we lyophilized and ground immature group II and III samples, left one set dry and added the amount of water lost back to the other set. Both sets were extracted with absolute methanol and analyzed by the protein precipitation assay. Group III sorghums had about 0.33× and group II about 2× the tannin activity in wet samples (corrected) as they did in dry samples. This suggests that hydration effects differ in the tissue components of the two groups. Important differences in starch (Davis and Hoseney, 1980), protein (Hagerman and Butler, 1980b), and cell wall polysaccharides (Goldstein and Swain, 1963) may differentially influence expression of tannin activity. Price et al. (1979) postulate that anatomical tissue differences might be one of the reasons why group III tannin is easily extracted in methanol but group II can be extracted only with acidic methanol in their assays. In our laboratory, sequential methanol (20 min at 30 °C) and 50% methanol–water (3 h at 40 °C; water increases tannin solubility) extracts from ground mature group II seeds yielded significantly less polyphenols than the vanillin–HCl analysis (Bullard and Elias, 1980).

Any cellular disruption during ripening probably contributes to a decrease in tannin activity. Freezing or boiling disruptions in immature grains appear to increase the formation of complexes between tannin and other cellular components (Price et al., 1979). For NK-300 Glennie (1981) suggested a crushing of testa cells by expanding endosperm that would enhance complex formation. In observations of six sorghum varieties with scanning electron and light microscopy (Blakeley et al., 1979; Rooney et al.,1980), differences were found even among kernels of the same variety; some appeared as blocky subunits and others were structureless. Rooney et al. (1980) observed "distinct differences in the structures of the testa among sorghums that were classified as Group II's. The testa of some Group II sorghums appears lighter in color and consists of layers. One of the layers is colorless when viewed with bright field microscopy. The colorless layer varies in thickness and is between the testa and aleurone cells. It is clearly a layer and one that we have not observed before". This layer may have characteristics that contribute to the binding properties observed in group II varieties.

Overall, the most important observations in our study were (1) the consistency among chemical, biochemical, and quelea preference assays of tannin activity, (2) further elucidation of the polyphenolic changes that occur during ripening, and (3) the differences in maturation characteristics between group II and group III sorghums. Each of these assays has uniquely provided information that helps us better understand polyphenolic properties in BR sorghums and differences that exist between groups II and III. We have evidenced that not only are there differences in the degree of polymerization of group II and III tannins but also there are other differences in grain components and structure which influence tannin activity and the expression of that activity. There is a genuine need for additional multidisciplinary work before we fully understand the bases for the existence of these differences.

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Dihydropergillin: A Fungal Metabolite with Moderate Plant Growth Inhibiting Properties from *Aspergillus ustus*

Horace G. Cutler,* Farrist G. Crumley, James P. Springer, and Richard H. Cox

Dihydropergillin, $C_{15}H_{18}O_4$, is a newly discovered metabolite isolated from cultures of *Aspergillus ustus* found growing on seed of *Pisum sativum* var. *macrocarpon*. It is structurally related to pergillin and significantly inhibited wheat coleoptile growth at 10^{-3} and 10^{-4} M. Structural determination was by single-crystal X-ray diffraction analysis.

We recently characterized and described a fungal metabolite, from *Aspergillus ustus*, that possessed moderate plant growth inhibiting properties, to which was assigned the trivial name pergillin (Cutler et al., 1980). Further studies with extracts of the fungus have led to the isolation of a new metabolite, dihydropergillin, that has greater activity than pergillin in our assays.

We now report the isolation and identification of dihydropergillin (I) (Figure 1) and its effects in wheat coleoptile bioassays.

MATERIALS AND METHODS

Production, Purification, and Isolation of Dihydropergillin. *A. ustus* (Bainier) Thom & Church (ATCC accession no. 38849) was isolated from greenhouse-produced pea seeds *Pisum sativum* var. *macrocarpon* (cv. Oregon Sugarpod) grown in Georgia. The fungus was grown on potato dextrose agar slants for 14 days at 26 °C and further maintained at 5 °C until used. Shredded wheat medium, in Fernbach flasks (2.8 L), consisted of 100 g of shredded wheat, 200 mL of Difco mycological broth (pH 4.8), 2% yeast extract, and 20% sucrose per flask (Kirksey and Cole, 1974). The medium was inoculated with the fungus and incubated at ~26 °C for 12 days. After the addition of 300 mL of acetone, the contents of each flask were macerated with a Super Dispax homogenizer, and the resulting pulp was strained through cheesecloth. The liquid phase was filtered through Whatman No. 1 filter paper on a Buchner funnel and then reduced in volume under vacuum at 50 °C to an aqueous phase. The water phase was extracted twice with an equal volume of ethyl acetate, and the ethyl acetate fractions were combined and dried over anhydrous sodium sulfate. This was followed by distillation of the ethyl acetate fraction, under vacuum, to produce a crude extract. The crude extract was added to the top of a silica gel (70–230 mesh) chromatography column (9.0 × 10 cm) that had been slurry packed in benzene and eluted stepwise with 1.0 L each of benzene, ethyl ether, ethyl acetate, acetone, and methanol. Each solvent was allowed to drain to the top of the silica gel before addition of the next sequential solvent. Each bulk fraction was reduced in volume, under vacuum, and aliquots were bioassayed with etiolated wheat coleoptiles. Since the ethyl ether fraction inhibited wheat coleoptile growth, it was reduced in volume and further fractionated with a silica gel (70–230 mesh) chromatography column (4.0 × 50 cm) that had been slurry packed in hexane, and 800 mL of hexane was percolated through the column and discarded. A linear gradient of hexane to ethyl acetate (1.0 L of hexane and 1.0 L of ethyl acetate) was then used, and 20-mL fractions were collected and evaporated to ~2 mL, and 25-μL aliquots were bioassayed. Tubes 31–60 (1.42–2.00 L of total solvent) were active in the assay system. These were combined, reduced in volume, and again placed on a freshly prepared silica gel (70–230 mesh) column (4.0 × 50 cm) that had been slurry packed in benzene. Six-hundred milliliters of benzene was allowed to percolate through the column and discarded, a linear gradient of benzene to ethyl acetate (1.0 L of benzene and 1.0 L of ethyl acetate) was used, and 20-mL fractions were collected. Since bioassays detected plant growth inhibition in tubes 41–65 (1.42–1.90 L of total solvent used), they were combined, reduced to a suitable volume, and loaded onto an RP2 (silica gel 60 silanized, 70–230 mesh, E. Merck) column (3.5 × 45 cm) that had been slurry packed in acetonitrile–water (1:1 v/v). Of the same solvent, 600 mL was allowed to percolate through the column, and 20-mL fractions were collected. Biological activity was associated with tubes 1–22 (0.62–1.04 L of total solvent), and these were combined and placed on a silica gel (70–230 mesh) chromatography column that had been slurry packed in ethyl acetate–benzene (55:45 v/v). Solvent was allowed to percolate through the silica gel, and the various fluorescent bands were noted at 366 nm. As the first fluorescent band started eluting, 5-mL fractions were collected. Biological activity was obtained in fractions 4–10 (20–50 mL of solvent after the first UV–visible band started exiting the column). These fractions were reduced, under vacuum at 50 °C, to the aqueous phase and partitioned against ethyl acetate. The ethyl acetate was dried over anhydrous sodium sulfate and reduced to a small volume. This fraction was added to the top of a C_{18} reverse-phase (obtained by cutting open a Waters Associates Prep PAK-500 C_{18}) chromatography column (2.5 × 11 cm)

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