An Examination of the Brown Pigments from Barley Leaves[1]

KENNETH A. CLAUSSEN[2] and **EVAN H. PEPPER**,[3]
North Dakota State University, Fargo

ABSTRACT

Brown pigments were extracted from barley leaves infected by *Helminthosporium sativum*, barley leaves with nonparasitic spots, and leaves of black, Abyssinian barley. These pigments were subjected to chemical and physical tests for identification of melanin together with a commercial melanin, dopa melanin, tyrosine melanin, potato melanin, and the brown pigment from human hair. Histochemical tests for melanin were also made with leaf tissue sections of the pigmented barleys. The results suggest that the brown barley pigments from infected and physiologically spotted leaves are not true melanins. The brown pigment obtained from the Abyssinian barley more closely resembled the synthetic melanins. The behavior of the test materials supports the hypothesis that there are different forms of melanin, each varying according to its source. The methods for extracting melanin and the tests for identification of melanin were inadequate for detailed study. Chemical procedures were more useful than the UV and IR spectrophotometric analyses used in this study.

The dark brown or black pigments formed by plants and animals are commonly termed "melanins." They are produced by disease, insect injury, senescence, and genetic-environmental interactions (1). Barley (*Hordeum vulgare* L.) often shows both parasitic and nonparasitic brown pigmentation (2). Pigmentation may occur in all parts of the barley plant. Nonparasitic browning of barley kernels may be an undesirable quality factor, often confused with "kernel blight" or "weathering." The effect of these brown pigments on yield is unknown in barley and most other crops. There is some evidence that the production of brown pigments is associated with resistance to certain plant pathogens (3).

The relation of brown plant pigments to those of animal origin is unknown (4). Similarly, it is not known whether the brown pigments of higher plants are true melanins (4). While there is no universally acceptable definition of a "true melanin," most workers agree that the melanins are brown-black pigments of high molecular weight, formed by the oxidation and polymerization of phenolic materials, and containing a minimum of 8–9% nitrogen (4). This study was initiated to determine the chemical nature of some brown barley pigments originating from both parasitic and nonparasitic causes. These barley pigments were also compared with animal and synthetic melanins.

MATERIALS AND METHODS

Brown-black pigments were extracted from nonparasitic spots on barley leaves; barley leaves infected by *Helminthosporium sativum* Pammel, King, and Bakke; leaves from a black, Abyssinian barley (CI 967); pericarps of black-pigmented barley kernels (Var. Jet); potato tubers; and human hair.

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[2]Formerly graduate research assistant, Dept. of Plant Pathology, North Dakota State Univ., Fargo; now Bacteriologist, Pet Milk Co., Greenville, Ill.

[3]Formerly Assistant Professor, Dept. of Plant Pathology, North Dakota State Univ., Fargo; now Associate Professor, Dept. of Botany, Brandon Univ., Brandon, Manitoba, Canada.

Green barley leaves, without brown pigmentation, were used in each series as a control. No extraction procedures have been reported for the removal of "melanins" from pigmented plant materials; therefore, several extraction methods used for animal pigments were adapted for the removal of the plant pigments.

All dried leaf material was ground in a Wiley mill with a 60-mesh screen prior to extraction. Pigmented hulls and pericarps, as preparation for extraction, were first removed from the kernels with a pearling machine and then ground in the Wiley mill. Potato melanin was prepared according to Van Middelem's procedure (5).

Human hair, a known source of animal melanin (6), was washed to remove hair oils and dust, rinsed repeatedly in distilled water, and air-dried. To remove fat, the hair was rinsed in petroleum ether for 4 days.

Synthetic melanins were prepared from both tyrosine and dopa (dihydroxyphenyl L-alanine). The tyrosine melanin was prepared by suspending 600 mg. of L-tyrosine in 300 ml. of phosphate buffer (pH 7.0), to which was added 300 mg. of lyophilized tyrosinase (Nutritional Biochemical Co.). The substrate-enzyme mixture was agitated with a magnetic stirrer for 12 hr. at 25°C. and centrifuged for 20 min. at 2,350 $\times$ *g*. The supernatant liquid was evaporated to dryness, and the precipitate was stored in a desiccator. Dopa melanin was prepared nonenzymatically according to Arnow's method (7). A commercially prepared synthetic melanin was also used in the comparative tests (K and K Laboratories, Plainview, N.Y.).

Brown-black pigments were extracted from the plant and animal tissues according to the procedures of Filson and Hope (8), Bonner and Duncan (9), and Einsele (10). The extraction procedures of Lea (6) and Einsele (10) were used for the removal of pigments from hair.

A series of physical and chemical diagnostic tests were made on the natural pigments and the synthetic melanins (4,9,11,12). Solubility tests were made with the following solvents: 10% NaOH, H_2SO_4 (concentrated and 0.02*N*), chloroethanol, pyridine, diethyl ether, warm acetone, and warm chloroform. Solubility was determined visually by a darkening of the fluid in which the tissues had been placed.

Bleaching tests were conducted with 1% bromine water (4), 10% H_2O_2 (13), and 0.1% $KMnO_4$ (9), all reportedly diagnostic procedures for the presence of melanin. Suspensions were allowed to remain in the bleaching agent for 24 to 48 hr.

Nitrogen contents of the pigmented materials and the synthetic melanins were determined colorimetrically according to a modification of Johnson's method (14).

The tissue extracts and the synthetic melanins were tested for their ability to reduce ammoniacal silver nitrate, a characteristic of melanins and tannins (4). The "melanic" materials were also subjected to degradation roasting, presumptive evidence for the presence of melanin, according to Fox and Kuchnow (11). Another test for melanins (fusion in KOH) described by Fox and Kuchnow was also used (11).

Infrared and ultraviolet spectra were determined for the natural pigments

and the synthetic melanins. A grating infrared spectrophotometer (Perkin-Elmer, Model 337) was used for the determination of infrared spectra, and the curves were compared with those reported by Bonner and Duncan for animal melanins (9). Ultraviolet spectra were determined with a Beckman DK-2 ratio recording UV spectrophotometer.

A method reported by Thomas (4) for identification of melanin was used with the natural and synthetic test materials. In this technique black pigmentation is reduced to a colorless state, with subsequent reoxidation to the colored state when potassium ferricyanide is added.

Histochemical procedures have been described for the detection of melanins in animal tissues. These procedures were adapted and used with barley leaf sections. The fresh leaf material was killed and fixed in CRAF-1 (15). The leaves were dehydrated in dioxane (15) and infiltrated according to a method of Venning's (16). After infiltration, leaf tissues were embedded in Tissuemat (Fisher Scientific Co., 52°–54°C.). Tissue sections were cut on a rotary microtome at a thickness of 11–13 μ.

Three staining methods were used with both infected and noninfected pigmented barley leaves. Two of these procedures, developed by Lillie, are specific for melanin in animal tissue. In one method (17) Nile blue was used as a differential stain for melanin and lipofuchsins. The highly selective Turnbull's blue staining of melanin was used in the other method (18). The third method used was devised by Sadik and Minges (19) to detect necrotic areas in plant tissue with orange G.

EXPERIMENTAL RESULTS

The results of the chemical tests for identification of melanin are summarized in Tables I to III. The melanins are reported to be soluble in dilute

TABLE I

CHEMICAL TESTS FOR THE PRESENCE OF PLANT AND ANIMAL MELANINS (OXIDATION)

MATERIAL TESTED	OXIDIZING AGENT USED[a] 1% Bromine Water	10% H_2O_2	0.1% $KMnO_4$	MATERIAL TESTED	OXIDIZING AGENT USED[a] 1% Bromine Water	10% H_2O_2	0.1% $KMnO_4$
Tyrosine melanin	+	+	0	Pigmented barley leaf	+	+	0
Dopa melanin	0	+	+	Excised physiologic spots	+	+	0
Potato melanin	+	+	0	Barley leaf lesion (infected)	+	+	0
Hair pigment	+	+	0	Green barley leaf	+	+	0
Commercial melanin	+	+	+	Tyrosine	−	−	−
Black barley leaf pigment	+	0	0				

[a] + = Bleached, 0 = unbleached.

NaOH (4,9,12); and all samples tested were soluble in 10% NaOH, though solubilities were variable among samples. Most of the samples were at least slightly soluble in concentrated H_2SO_4, whereas only a few of the samples were soluble in the dilute acid. In general, the synthetic melanins were more soluble in organic solvents than were the natural pigments.

Bromine water bleached all the pigments except dopa melanin; H_2O_2 bleached all samples except the Abyssinian barley pigment; and $KMnO_4$

TABLE II

CHEMICAL TESTS FOR THE PRESENCE OF PLANT AND ANIMAL MELANINS (SOLUBILITY)

MATERIAL TESTED	SOLVENT USED[a]						
	10% NaOH	Chloro-ethanol	Pyridine	H_2SO_4 (Conc.)	H_2SO_4 (Dil.)	Ethanol (Abs.)	Acetone (Warm)
Tyrosine melanin	+++	0	0	++	0	0	0
Dopa melanin	++++	+++	++	++++	+	++	0
Potato melanin	+++	0	0	+	0	0	0
Hair pigment	+++	0	0	++	0	0	0
Commercial melanin	++++	++++	++++	++++	++	++	++
Black barley leaf pigment	++++	++	0	++++	0	0	++
Pigmented barley leaf	++++	++	+	++++	0	+	+
Excised physiologic spots	+++	++	0	+++	0	0	0
Barley leaf lesion (infection)	++	0	0	+++	0	0	0
Green barley leaf	++	0	0	++++	0	0	0
Tyrosine	–	–	–	–	–	–	–

[a] ++++ = Very soluble; +++ = soluble; ++ = slightly soluble; + = very slightly soluble; 0 = insoluble.

TABLE III

CHEMICAL TESTS FOR THE PRESENCE OF PLANT AND ANIMAL MELANINS (MISCELLANEOUS)

MATERIAL TESTED	TEST PERFORMED			
	Reduction of[a] Ammoniacal $AgNO_3$	Fusion in KOH[a]	Degradation Roasting[b]	Nitrogen Content[c]
				%
Tyrosine melanin	0	0	++++	6.57
Dopa melanin	+	0	+	5.25
Potato melanin	0	0	++++	8.12
Hair pigment	0	+	++	1.05
Commercial melanin	+	+	++++	6.09
Black barley leaf pigment	+	+	+	1.58
Pigmented barley leaf	0	+	+++	0.58
Excised physiologic spots	0	0	+	0.95
Barley leaf lesion (infection)	0	0	No Sample	1.50
Green barley leaf	0	0	+	0.73
Tyrosine	–	–	–	8.22

[a] + = Reduction; 0 = no reduction.

[b] ++++ = Intense reddening; +++ = some reddening; ++ = slight reddening; + = very slight reddening.

[c] Average of three replications.

bleached only the dopa and commercial melanins. Thomas (4) has suggested that true melanins contain a minimum nitrogen level of 8%. Only potato melanin met this requirement with a nitrogen content of 8.12%. The synthetic melanins ranged from 5 to 7% nitrogen; the hair pigment and the plant pigments contained 1.5% or less.

Dopa melanin, commercial melanin, and the Abyssinian barley pigment were the only materials that reduced ammoniacal silver nitrate. All materials tested by degradation roasting gave a positive reaction, indicating the presence of pyrrole. The most intense reaction was produced by tyrosine-, potato-, and commercial melanin. Dopa melanin and the plant pigments showed only a slight pyrrole reaction.

Ultraviolet and infrared spectra were recorded for the synthetic melanins and the hair and plant pigments (Figs. 1–5). Infrared spectra of some animal melanins were taken from a report by Bonner and Duncan (9) and used for comparative purposes (Fig. 6). Ultraviolet spectral curves, in most cases, showed no significant peaks over the range of 220–340 mμ. Tyrosine,

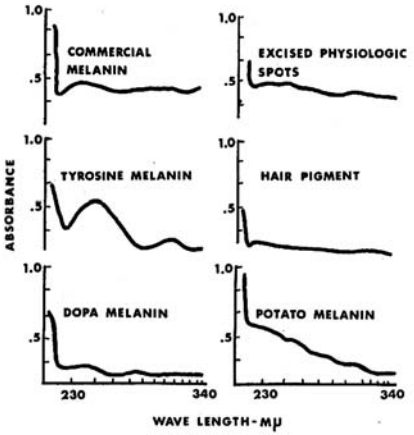


Fig. 1 (left). Ultraviolet spectra of commercial, plant, and animal pigments.

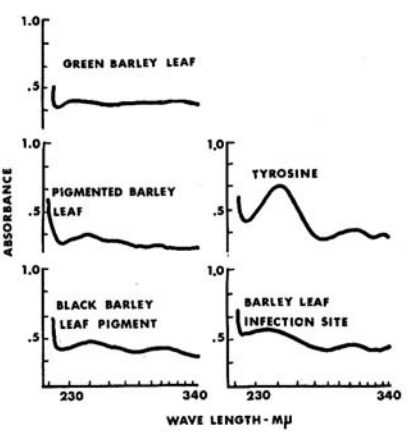


Fig. 2 (right). Ultraviolet spectra of extracted barley leaf pigments and of tyrosine.

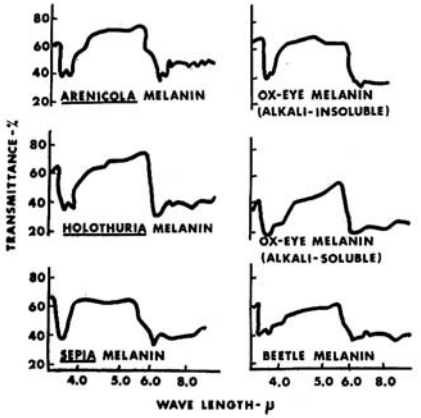


Fig. 3 (left). Infrared spectra of animal melanins. After Bonner and Duncan (9).

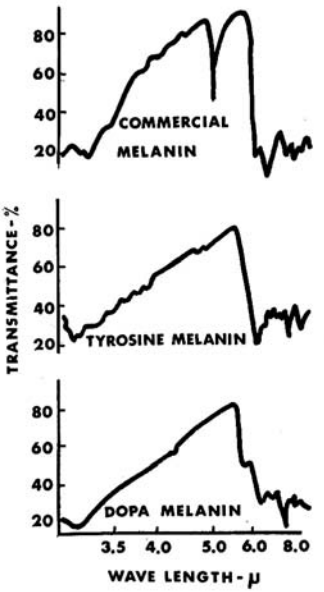
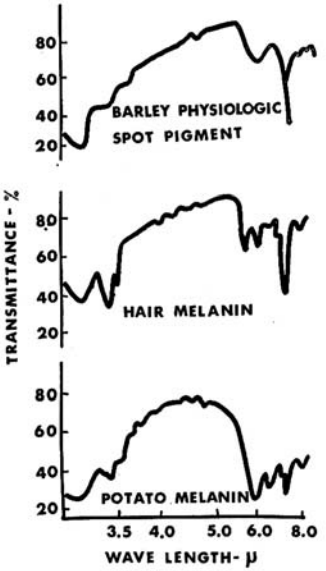


Fig. 4 (right). Infrared spectra of commercial and synthetic melanins.



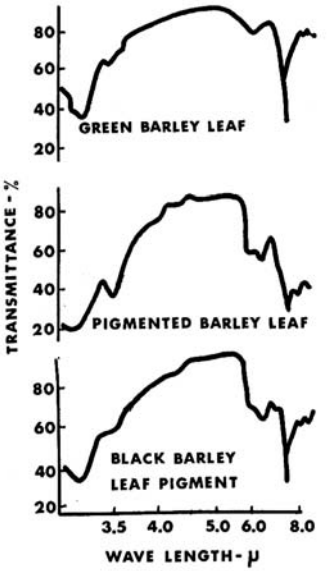


Fig. 5 (left). Infrared spectra of plant and animal pigments.

Fig. 6 (right). Infrared spectra of extracted barley leaf pigments.

tyrosine melanin, and pigments from infected barley leaves all showed peaks at 240 and 295 mμ. All samples showed peaks at 3.4, 6.0, and 7.3–7.5 μ on the infrared absorption curves. Only commercial melanin produced a peak at 4.8 μ. Commercial melanin and hair pigment each showed a small peak at 5.8 μ. All of the plant materials had similar I-R curves. The synthetic melanins resembled each other, but the plant and animal pigments did not. Infrared spectral curves for both plant and animal pigments showed some similarities.

The dark-pigmented areas of black Abyssinian barley leaves stained dark green with Lillie's ferrous iron technique. Nonparasitic brown spots on barley leaves (Var. Feebar) showed the same staining reaction with Lillie's method. Counterstaining with Van Giesen's stain emphasized the dark-green color. Barley leaves, infected with *H. sativum,* showed no such reaction when stained by Lillie's method. The dark-pigmented areas of Abyssinian barley leaves stained dark purple with the Sadik and Minges method, whereas the cell walls of normal tissue stained yellow-orange when counterstained with orange G.

DISCUSSION

Several workers (7, 20, 21, 22) agree that there are many types of melanin. Sluis (12) noted that solubility differences among the melanins might be due to differences in the origin of the pigment, the presence of insoluble protein in combination with the melanin, or differences in polymerization or hydration. All the pigments in our study were soluble in 10% NaOH, and most were soluble in chloroethanol. The solubility of the pigments in concentrated acid was expected (23). Dopa melanin and commercial mela-

nin were both soluble in dilute acid and absolute ethanol. The three barley leaf pigment samples were all soluble in pyridine. The relative insolubility of the hair melanin was probably due to contamination by adhering protein (12). The difference in solubility between the pigments from infected barley leaves and those extracted from nonparasitic barley leaf spots suggests that the pigments found in the region of fungal infection were not melanistic, at least at the time of extraction.

The bleaching of brown pigments is presumptive evidence for melanin. Bromine water bleached all of the pigments except dopa melanin. Hydrogen peroxide bleached all of the materials except the pigment from Abyssinian barley leaves. Potassium permanganate bleached only the commercial and dopa melanins. The bleaching tests indicate that the commercial and dopa melanin are similar in nature.

Most authorities fix the minimum nitrogen level of true melanin at 8% (4,20). In our study, only potato melanin met this requirement. The synthetic melanins had a nitrogen content from 5.25 to 6.5%; the natural pigments all contained very small amounts of detectable nitrogen. None of the plant or hair pigments examined in the present study can be classified as true melanins if the 8% figure is a valid criterion.

Only dopa melanin, commercial melanin, and the pigment from black Abyssinian barley leaves reduced ammoniacal silver nitrate. Both dopa and the intermediate product formed from the oxidation of dopa quinone are known to carry out this reduction action (24). It seems likely that black barley leaf pigment contains a structure similar to that found in the two synthetic melanins. The degradation roasting test is a positive test for pyrrole and indole (11). All of the "melanin" materials tested released pyrrole; however, the presence of pyrrolic compounds in barley leaf material may simply mean that chlorophyll has been degraded by heating. The KOH-fusion test (11) also detects pyrrolic and indolic compounds, including melanin, the precursors and breakdown products of melanin, as well as free pyrrole and indole. The response of the synthetic melanins and the natural pigments was erratic. If the positive reaction to the KOH-fusion test by the leaf pigment of black Abyssinian barley is ascribed to the breakdown of chlorophyll, then the other leaf pigments should also have given a positive reaction. Such was not the case, and this would suggest that the black pigment from Abyssinian barley is chemically related to commercial melanin.

The IR spectral curves obtained in our study were similar to those of animal melanins reported by Bonner and Duncan (9). In addition to transmissions at 3.0–3.4 and 6.0–6.3 μ, an additional peak was observed on all curves of plant pigments at 7.3 μ. This peak indicates the presence of nitro compounds, and of hydroxyl, paraffin, methyl, and methylene groups (25). Commercial melanin and hair pigment showed a small peak at 5.8 μ, suggesting that ketones and aldehydes were present in these materials (25).

Until better methods of extraction and purification can be developed it is doubtful if the IR curves in our study or those of Bonner and Duncan are of much value in the identification of melanins for the following reasons: 1) There are only a few broad peaks indicating the presence of a large

amount of material absorbing at or near these wave lengths; many organic and inorganic materials can be made to rotate or vibrate causing absorption peaks at the wave length of the next prominent peak. 2) The chemical formula or formulas of "melanin" are unknown, hence no known compound is available for use as a standard of comparison.

The UV analyses did little to support the other data on the presence of melanin in the plant and animal extracts. The curves for tyrosine, tyrosine melanin, and infected leaf tissue simply indicated the presence of free tyrosine.

The leaves of black Abyssinian and Feebar barley with nonparasitic spotting showed positive histochemical reactions to Lillie's method (18). Tissues from infected barley leaf tissue did not show this reaction. The Abyssinian barley tissue also gave a positive reaction to the test for necrotic tissue (19). Necrosis is manifested by the impregnation of the cell walls with a ligninlike material which has a strong affinity for thionin. It is probable that this ligninlike material may be the same substance that reacts positively to Lillie's staining method, since the same pigmented areas in tissues of Abyssinian barley leaf reacted positively to both tests.

The diagnostic tests used in this study indicate that: 1) the pigments extracted from lesions of barley leaves infected by *H. sativum* are not melanins; 2) the pigment from leaves of black Abyssinian barley is a melaninlike material; 3) there are chemical differences in melanins obtained from different sources; 4) the brown-black plant pigments show some similarity to the animal melanins studied by Bonner and Duncan; and 5) better extraction, purification, and diagnostic procedures are needed for isolation and identification of the "melanins" of both plants and animals.

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