Methods of Separation of the Major Histological Components of Rice and Characterization of Their Proteins by Amino Acid Analysis[1]

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ABSTRACT

Two methods, developed for the clean separation from rice of aleurone with attached grain coats, involved soaking de-embryonated grains in water or in formic acid plus sucrose and then scraping off the starchy endosperm. The aleurone cell fine structure was retained in the water treatment but destroyed in the formic acid treatment, which also ruptured aleurone cell walls. Grain coat walls were obtained by ultrasonication of rice grains in decane. Analyses of these components as well as of embryo, starchy endosperm, and the whole grain allowed a satisfactory quantitative balance to be made of the amino acid and protein content of the grain and its major histological components. The embryo, aleurone cells, and grain coat together amount to about 9% of the grain by weight, yet they contain 17% of the protein and 23% of the lysine. Because these histological components (which also contain the bulk of the vitamins in rice) are removed as bran during milling, the promotion of increased consumption of brown rice rather than of milled rice seems desirable even though protein from brown rice is less digestible than that from milled rice.

Rice is primarily the cereal grain of the developing countries and therefore has been less well studied than the major cereals of industrial countries, grains such as wheat, barley, and corn. Although the compositions of brown rice, milled rice, and the bran fractions are known (Juliano 1972), information on the chemistry of the major histological components of rice is lacking, due to a lack of methods for the preparation of clean components.

Milling procedures, using even the mildest conditions available in the laboratory, are not selective and cause an appreciable amount of grain breakage. The germ (embryo) can be readily removed by dissection of the grain, but the clean removal of the outer layers of the caryopsis (grain coat and aleurone) by hand dissection is much more difficult (Chrispeels and Varner 1967; Hinton 1947, 1948; Jacobsen and Knox 1974). We have studied these and other methods such as ultrasonication, which has been useful in the selective disruption of keratin fibers (Bradbury 1973, Bradbury and Chapman 1964), in order to develop reliable methods for the separation of the major histological components of rice.

A second objective of this study has been the amino acid analysis of the protein in these components, to obtain the distribution of protein throughout the grain. Such information should be useful in documenting the nutritional advantages of the consumption of brown rice rather than of polished rice, because bran layers are known to contain more protein than does the starchy endosperm. A detailed comparison of the protein contents of the histological components of normal rice and of a high protein rice developed at the International Rice Research Institute (IRRI) will be reported subsequently.

MATERIALS AND METHODS

Materials

The brown rice (*Oryza sativa,* L.) used in this work was IRRI variety IR32 and contained 1.31% nitrogen. Chemicals and solvents were reagent grade and were normally used without further purification. Constant-boiling-point 6*M* hydrochloric acid was obtained by distillation of BDH analytical, reagent grade, concentrated hydrochloric acid.

Light and Electron Microscopy

The various components of rice were examined routinely by phase contrast microscopy. For more detailed examination, pieces of the components (about 1 mm square) and pellets of cell wall fractions (processed in Beckman microfuge tubes) were fixed in glutaraldehyde and osmium tetroxide and embedded in epoxy resin as previously described for barley (Jacobsen et al 1971). Thick (1.5-μm) sections were stained in 0.05% toluidine blue (pH 9) at 50° C for 3 min and examined in a Carl Zeiss Ultraphot II light microscope. For transmission electron microscopy, silver to gold sections were post-stained with uranyl acetate and lead citrate by standard procedures (Jacobsen et al 1971). Sections were cut with a Reichert OmU2 ultramicrotome, and thin sections were examined in a Hitachi HU 12 electron microscope.

Amino Acid Analysis

About 5 mg of material was dried in vacuo at 70° C for 5 hr except for samples of aleurone cells plus grain coat, which were air dried. Samples were weighed with an accuracy of $>1\%$. They were transferred to a small hydrolysis tube; about 1 ml of 6*M* HCl was added, and the tube was sealed in vacuo. Tubes were heated in an oven at 110° C for 22 hr, cooled, and opened. The HCl was evaporated to dryness without loss of material, and the residue was dissolved in the starting buffer used for the amino acid analyzer. The insoluble black humin was removed by filtration and the solution made up to 5 ml in a volumetric flask. An aliquot was applied to a standard 70-cm ion exchange column of a Technicon amino acid analyzer, and the amino acids were eluted in 5 hr with a buffer gradient from the nine-chamber autograd. Separation of the small cystine peak from the large valine peak was achieved by making the pH in chambers No. 3, 4, and 5 slightly lower than recommended to ensure a lower gradient of pH during the elution of cystine and valine. The system was calibrated using a Beckman standard mixture of amino acids. Hydroxyproline was eluted before aspartic acid. The reproducibility of the system was $\pm$ 5%. Results, recorded as residues per 100 amino acid residues, were the mean of three or four analyses obtained on two hydrolysates. Tryptophan was not determined, due to its decomposition during acid hydrolysis. No corrections were made for possible losses of other amino acids by decomposition during hydrolysis. These losses are accentuated by the presence of large amounts of carbohydrate. The percentage of protein in the sample was calculated from the known amount of material loaded on the column and the recovery of amino acids from the analyzer.

Methods of Preparation of Histological Components

Aleurone Cells Plus Grain Coat. After a considerable amount of preliminary work, two methods were used. In the first, both ends of the rice grain were cut off to remove the embryo and to facilitate penetration of water, and the cut grain was just covered by distilled water for three days at room temperature. The water was then decanted off, filtered, and the solution lyophilized to obtain any material extracted from the rice grains by the water. The external layer was then slit with a scalpel and the softened, starchy

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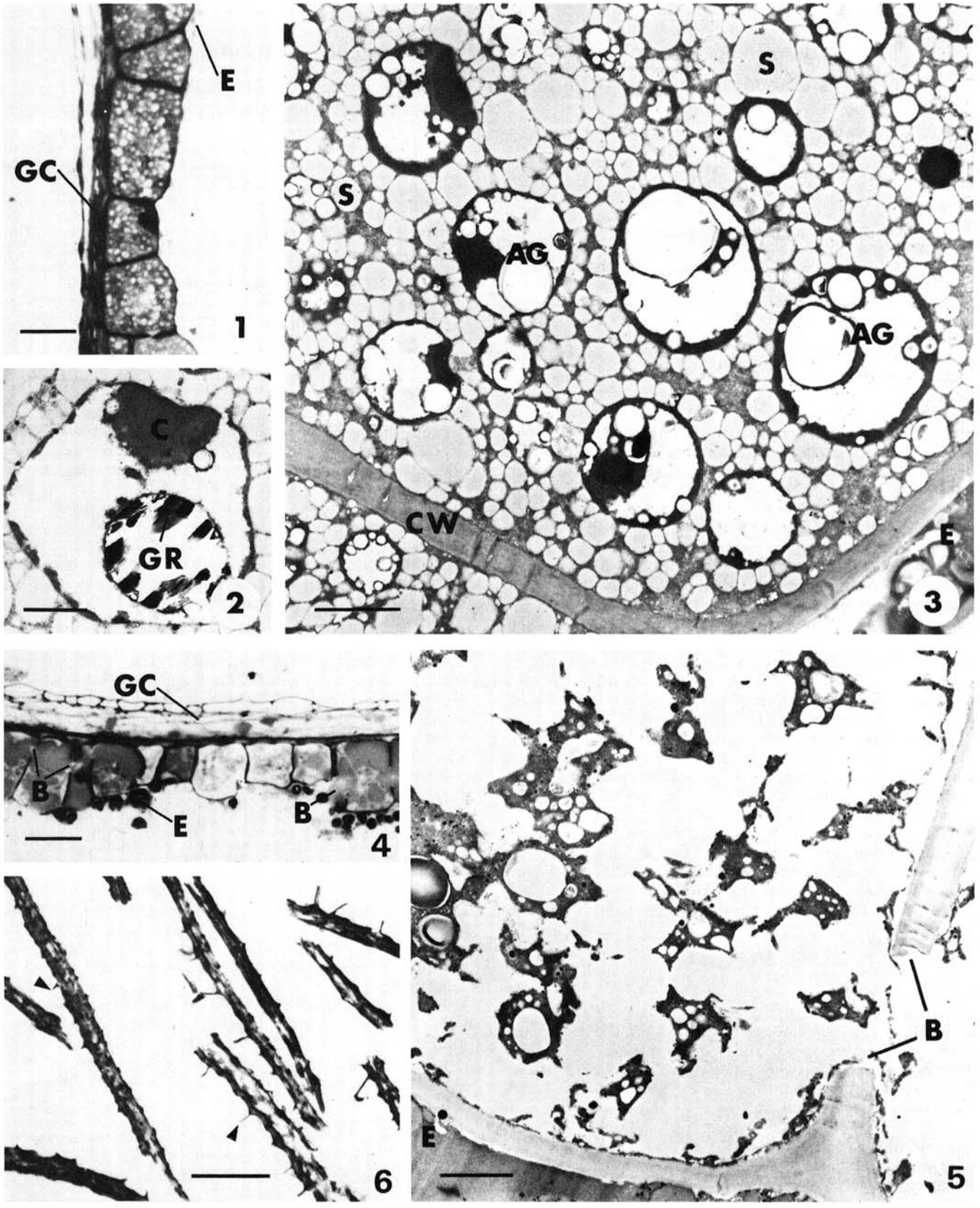

Figs. 1–6. 1, Light micrograph of part of an aleurone layer (cells plus grain coat) isolated by scraping from a cut (de-embryonated) rice grain soaked in water for three days, (similar preparation used in Figs. 1–3), **GC** = grain coat adhering to the aleurone layer, **E** = remnants of starchy endosperm tissue, magnification bar = 25 μm; **2,** electron micrograph of an aleurone grain, **GR** = remnants of the globoid inclusion remaining after thin sectioning, **C** = assumed crystalloid, bar = 1 μm; **3,** electron micrograph of part of an aleurone cell, **S** = spherosome, **AG** = aleurone grains, **CW** = cell wall, bar = 2 μm; **4,** light micrograph of an aleurone layer (cells plus grain coat) isolated from a cut (de-embryonated) rice grain immersed in a solution of 0.8 *M* sucrose in formic acid for 3–5 min, (similar preparation used in Fig. 5), **B** = breaks in aleurone cell walls, bar = 25 μm; **5,** electron micrograph of part of two aleurone cells, showing disrupted appearance of cell contents, bar = 2 μm; **6,** light micrograph of grain coats produced by the ultrasonic treatment, showing remnants (arrowheads) of anticlinal walls of aleurone cells, bar = 25 μm.

endosperm separated from the aleurone plus grain coat by scraping (Chrispeels and Varner 1967, Jacobsen and Knox 1974). One-half of the sample of aleurone cells plus grain coat was left to air dry for 24 hr; the other half was washed in distilled water for 1 hr and then in a graded series of alcohol-water mixtures containing 25, 50, 75, and 100% ethyl alcohol, each for 15 min. These washings were combined and evaporated to dryness; the yield of solid material equaled about 5% of the weight of the aleurone cells plus grain coat and contained only about 3% protein. Because of the intact nature of the aleurone cells after soaking, the removal of any protein material from the cells during the three days' soaking in water was considered unlikely (Jacobsen and Knox 1974). In order to confirm this, we examined the water extracted from the soaking treatment.

As a preliminary to the development of the second method, we also studied the swelling of rice grains in various organic liquids. Hexane and acetone did not penetrate the rice grain appreciably in three days; dimethylformamide behaved similarly to water; glacial acetic acid penetrated more rapidly than water; and formic acid penetrated very rapidly. Formic acid caused dissolution of the starchy endosperm in 4 hr. In order to reduce the severity of the formic acid treatment, the length of immersion was reduced to 5 min, and sucrose was used to reduce osmotic shock to the cells.

In the second method, rice cut as before was soaked in formic acid containing 0.8 *M* sucrose for 3–5 min. The aleurone cells plus grain coat were then peeled away from the starchy endosperm. Washed and unwashed samples were obtained in the same manner as in the first method. The formic acid extract was filtered and then lyophilized to remove the formic acid, which gave a residue of sucrose plus material extracted from the rice.

Embryo. This was readily separated from the whole dry grain by hand dissection, using a needle.

Starchy Endosperm. This material was separated from the aleurone cells and grain coat after three days immersion of the cut grain in water as described in the first method.

Whole Dry Grain. This was crushed to a fine powder to ensure a homogenous sample.

Cell Walls of Grain Coat. The grain coat, the external layer of the rice grain caryopsis, consists largely of cellulosic cell walls (Juliano 1972). Initial unsuccessful attempts were made to remove the grain coat selectively by a mild ultrasonication of rice grains immersed in decane (using a 500-watt Mullard-M.S.E. ultrasonic disintegrator) set at a low power level (Bradbury and Chapman 1964). Even milder methods such as the use of a Vibro-mix stirrer (Bradbury et al 1966) and the shaking of the sample in decane using an automatic flask shaker, failed to produce a clean sample of grain coats. The material dispersed in the liquid always consisted of a mixture of grain coats, aleurone cell walls, and material from the starchy endosperm (starch granules, etc). A wide range of organic solvents and water were studied, and decane was found suitable because a reasonable amount of material was dispersed without evidence of extensive disruption of the starchy endosperm.

Several grams of rice were ultrasonicated for 30 min in decane at a high power level with cooling in an ice bath. The dipersed material was separated from the bulk of the decane by centrifugation and then layered on top of a column of liquid composed of 90% hexane and 10% carbon tetrachloride (v/v). The smaller particles (disrupted material from the starchy endosperm and the aleurone layer) sedimented only slowly, but the larger grain coat cell walls sedimented rapidly and were collected. This material was layered on a solution of 93.8% chloroform and 6.2% hexane (v/v, density 1.45) and centrifuged at 2,000 rpm for 20 min. The grain coat floated and residual starch granules sedimented to the bottom. The process was repeated twice and the top layer collected for microscopy and analysis.

RESULTS AND DISCUSSION

Microscopic Examination of Histological Components

Aleurone cells (still firmly adhering to the grain coat) prepared by water immersion of cut rice for three days followed by scraping are essentially intact (Figs. 1–3). The electron micrographs in Figs. 2 and 3 show the fine structure of rice aleurone cells, containing aleurone grains (with globoid inclusions) and lipid bodies (spherosomes), as recently reported for this cereal grain by Bechtel and Pomeranz (1977). They conducted a detailed ultrastructural examination of the grain coat and aleurone cells of dry rice grain, and these results were used as a morphological standard. The morphology of aleurone cells obtained by us from cut grains that

TABLE I
Amino Acid Analyses[a] of Rice Aleurone Cells Plus Grain Coat[b]

	Aleurone Plus Grain Coat Produced By						
	Soaking in Water		HCOOH/Sucrose Treatment		Aleurone Plus Grain Coat, Mean Value	Water Extracted After Soaking Cut Grains for Three Days	HCOOH/Sucrose Extract After 5-Min Immersion
Amino Acid	With Washing	Without Washing	With Washing	Without Washing			
Alanine	9.9	10.0	10.3	10.5	10.2	12.9	8.6
Arginine	5.9	5.9	5.8	5.6	5.8	3.8	7.1
Aspartic acid	10.2	10.0	10.4	11.4	10.5	10.5	11.5
Cystine	0.40	0.55	0.36	trace[c]	0.44	0.1	0.31
Glutamic acid	12.2	13.6	14.2	13.1	13.2	11.5	15.9
Glycine	9.8	10.6	10.6	10.7	10.4	12.8	9.2
Histidine	2.7	2.9	2.5	2.6	2.7	1.9	2.3
Isoleucine	4.7	4.3	4.4	4.5	4.5	4.3	4.7
Leucine	8.5	8.8	8.1	7.9	8.3	8.0	8.3
Lysine	5.0	5.2	4.4	4.9	4.9	4.8	3.7
Methionine	1.1	0.11[c]	0.64	0.80	0.85	1.5	0.72
Phenylalanine	4.8	4.4	4.3	4.7	4.5	4.6	4.9
Proline	6.5	6.3	6.1	5.6	6.1	4.9	6.0
Serine	3.7	3.7	4.0	4.3	3.9	3.2	3.2
Threonine	4.0	3.6	4.0	3.8	3.9	5.0	3.3
Tyrosine	2.4	2.3	2.3	1.9	2.2	1.7	2.4
Valine	8.2	7.9	7.6	7.7	7.8	8.5	7.9
% Protein	12.0	11.3	16.3	...	11.7	5[d]	13.5[e]

[a]Given in residues of amino acid per 100 residues.
[b]Tryptophan is destroyed during acid hydrolysis. Some losses of cystine, methionine, and tyrosine occur during acid hydrolysis in the presence of carbohydrate.
[c]Unaccountably low, not included in mean value.
[d]Material extracted by water amounts to 0.7% by weight of the immersed cut rice grains.
[e]This material was extracted in 5-min treatment of cut grains with formic acid (no sucrose present) and amounted to 1.63% by weight of the whole rice grains.

had been immersed in water for three days was similar to that in dry grain (Bechtel and Pomeranz 1977). We observed, however, the occurrence of what appears to be a crystalloid inclusion (called protein-carbohydrate body in barley [Jacobsen et al 1971] and type II inclusion in wheat [Fulcher 1972, Morrison et al 1975]) inside the aleurone grains, which was not observed by Bechtel and Pomeranz (1977) in the two varieties they studied. The amount of starchy endosperm adhering to the aleurone cell preparation is shown in Fig. 1 to be very small.

The formic acid/sucrose treatment of cut rice grains, followed by peeling off the aleurone cells plus grain coat, causes breaks in the aleurone cell walls and disorganization of the cytoplasm, as shown by comparison of Figs. 4 and 5 with Figs. 1 and 3. The normal constituents of aleurone cells cannot be clearly identified in the formic acid-treated material. The broken cell walls in Figs. 4 and 5 have allowed some of the contents of the cell to escape, including the lipid bodies, which are likely to be soluble in formic acid. Nevertheless, the bulk of the protein was possibly still retained inside the cell, and this was monitored by amino acid analysis of the aleurone cells plus grain coat fractions produced by both methods.

Figure 6 is a light micrograph of fragments of grain coat layers produced by ultrasonication. Remnants of anticlinal aleurone cell walls adhering to grain coats indicate a fairly clean preparation of grain coats for analysis.

Comparison of Preparative Methods and Analyses of Aleurone Cells Plus Grain Coat

The amino acid analyses of the aleurone cells plus grain coat produced by the two methods, with and without subsequent washing, show no differences that are outside experimental error. The values have therefore been averaged in Table I to get a best value for the analysis of aleurone cells plus grain coat. The mean percent of protein in aleurone cells plus grain coat (11.7%) is the average value for the mild water immersion method, in which aleurone cell fine structure is retained. After three days' soaking of cut grain in water, 0.7% of the rice by weight is dissolved and this contains 5% protein. The protein contains more alanine and glycine and less arginine and cystine than do the aleurone cells plus grain coat. As would be expected, the composition of this soluble protein resembles that of albumin, the water-soluble protein fraction from brown rice (Juliano 1972). The total amount of protein extracted by the water immersion method (Table I) is negligible compared with the amount of protein present in the aleurone cells plus grain coat, and it is most likely of starchy endosperm origin (Jacobsen and Knox 1974).

The formic acid/sucrose treatment produces breaks in the aleurone cell walls, and the normal constituents of aleurone cells can no longer be clearly observed. The protein content of the aleurone cells plus grain coat prepared in this manner is 16.3%, which is much higher than that of the similar preparation produced by water soaking (11.7%). This indicates that the material removed from the cytoplasm of aleurone cells in the formic acid treatment is largely nonprotein. The treatment with formic acid dissolves 1.63% by weight of the rice, and the dissolved material contains 13.5% protein. The amino acid analysis of this protein is similar to that of glutelin, the protein component removed from rice by extraction with dilute acid (Juliano 1972). Because over 90% of the glutelin originates from the endosperm (Juliano 1972), most of the protein in the formic acid extract may come from the starchy endosperm rather than from the aleurone cells plus grain coat. This gives a reasonable explanation for the fact that the amino acid analysis of the aleurone cells plus grain coat produced by the two methods shows values that are about the same.

We concluded that the water immersion treatment is a satisfactory method of producing aleurone cells plus grain coat but that the formic acid/sucrose treatment cannot be recommended because it causes disruption of aleurone cell walls, disorganization of the cytoplasm, and removal of part of the contents of the cells.

Amino Acid Analyses of Histological Components of Rice

The amino acid analyses of rice and its major histological components are reported in Table II. The analyses of whole grain and of embryo have been recalculated on the basis of grams per 16.8 g of nitrogen and are similar to the values given by Juliano (1972).

Because the starchy endosperm makes up about 91% by weight of the rice grain (Juliano 1972), its amino acid analysis is very similar to that of the whole grain. As might be expected, the amino acid analyses of embryo, aleurone cells plus grain coat, and of grain coat are considerably different from those of the whole grain. Lysine and threonine are the most interesting amino acids from the nutritional point of view because for humans they are the first and second limiting amino acids in rice (Juliano 1972). Table II shows more lysine and threonine in the embryo and the aleurone cells plus grain coat than in the starchy endosperm.

Amino Acid Balance of Rice

To quantitate the amino acid analyses of the various components, we have calculated the amount of each amino acid

TABLE II
Amino Acid Analyses[a] of Rice and Its Components[b]

Amino Acid	Whole Grain IR-32	Starchy Endosperm	Embryo	Aleurone Cells Plus Grain Coat, Mean Value	Grain Coat
Alanine	8.6	8.9	9.8	10.2	11.5
Arginine	6.1	5.5	8.2	5.8	4.4
Aspartic acid	9.2	9.2	8.9	10.5	11.7
Cystine	0.47	0.44	0.43	0.44	0.25
Glutamic acid	16.4	17.1	13.8	13.2	8.5
Glycine	9.0	8.4	11.4	10.4	10.8
Histidine	2.5	2.3	3.0	2.7	1.9
Isoleucine	4.6	4.8	4.1	4.5	4.9
Leucine	8.7	9.0	6.8	8.3	9.2
Lysine	4.1	3.7	6.4	4.9	4.8
Methionine	1.6	1.4	1.3	0.85	0.70
Phenylalanine	4.5	4.5	3.8	4.5	5.1
Proline	6.3	6.9	4.9	6.1	7.8
Serine	4.5	4.7	3.6	3.9	4.5
Threonine	3.7	3.5	4.6	3.9	4.3
Tyrosine	2.3	2.3	1.7	2.2	1.9
Valine	7.4	7.4	7.3	7.8	7.6
% N recovered	97	94	91	94	96
% Protein	6.9	6.1	14.5	11.7	7.8

[a] Given in residues of amino acid per 100 residues.
[b] The amount of hydroxyproline in grain coats was 0.18 residues per 100 residues of amino acids; in all other components the hydroxyproline was below the level of detection.

TABLE III
Amounts of Protein and Amino Acids[a] in the Histological Components of Rice[b]

	Embryo (A)	Aleurone Cell Plus Grain Coat (B)	Starchy Endosperm (C)	Total (D = A + B + C)	Total from Whole Dry Grain (E)	Ratio (D/E × 100)
Protein	0.363	0.761	5.551	6.675	6.90	97
	(5.4)	(11.4)	(83.2)			
Alanine	0.0245	0.0494	0.3185	0.392	0.379	103
Arginine	0.0375	0.0657	0.4277	0.531	0.572	93
Aspartic acid	0.0373	0.0826	0.5460	0.666	0.662	101
Cystine	0.0040	0.0090	0.0463	0.059	0.061	97
Glutamic acid	0.0615	0.1112	1.1280	1.301	1.312	99
Glycine	0.0213	0.0390	0.2457	0.306	0.316	97
Histidine	0.0140	0.0254	0.1638	0.203	0.223	91
Isoleucine	0.0153	0.0371	0.273	0.325	0.338	96
Leucine	0.0248	0.0683	0.5187	0.612	0.624	98
Lysine	0.0263	0.0449	0.2366	0.308	0.334	92
	(8.5)	(14.6)	(76.9)			
Methionine	0.0060	0.0104	0.0910	0.107	0.110	97
Phenylalanine	0.0185	0.0507	0.3367	0.406	0.425	96
Proline	0.0147	0.0449	0.3367	0.396	0.363	109
Serine	0.0098	0.0228	0.2020	0.235	0.239	98
Threonine	0.0153	0.0267	0.1837	0.226	0.222	102
	(6.8)	(11.9)	(81.3)			
Tyrosine	0.0095	0.0267	0.1710	0.207	0.222	93
Valine	0.0235	0.0572	0.3731	0.454	0.470	97
Percent by weight of each component in dry rice[c]						
	2.5	6.5	91.0	100.0	...	...
% Protein in each component[d]						
	14.5	11.7	6.1	...	6.9	...

[a]Given in grams of amino acid residues or protein in 100 g of dry rice.
[b]Values in parentheses represent the percentage of the total amount of that material present in the particular component.
[c]Data from Juliano (1972).
[d]Data from Tables I and II.

residue in each histological component and compared this with the amount obtained by analyses of the whole dry grain. In Table III, the amino acid balance is seen to be satisfactory for all the amino acids. This also indicates that three days' soaking of cut grains in water did not induce any significant change in the amino acids of aleurone cells plus grain coat (containing living aleurone tissue) and of starchy endosperm (containing dead tissue).

The percentage of the protein and of the first two limiting amino acids (lysine and threonine) in each of the histological components is shown in parentheses in Table III. Although the aleurone cells plus grain coat and the embryo together amount to only an estimated 9% of the weight of the grain (Juliano 1972), they contain 17% of the protein, 23% of the lysine, and 19% of the threonine.

The bulk of the vitamins in rice are also located in these histological components, which are removed in the milling process as bran (Hinton 1948, Hinton and Shaw 1953, Juliano 1972). Thus the rice bran is a valuable source of protein and vitamins but is often used for stock fodder or even discarded. The bran protein, however, is not as digestible by man as is the protein from the starchy endosperm.[4] The nutritional advantage of brown rice compared with that of milled rice is its higher vitamin content and (probably) higher absorption of lysine. These findings indicate the desirability of promoting the consumption of brown rice and of improving the utilization of rice bran.

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[4]B.O. Juliano, personal communication.

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