explanation for the present findings. The oral route of administration evidently results in relatively little systemic absorption of the unchanged herbicide. The ip studies indicate, however, that once absorbed, the compound may persist in fat for lengthy periods of time.

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LITERATURE CITED

Anbar, M., Aberth, W. H., *Anal. Chem.* **46**, 59A (1974).
Aue, W. A., Hastings, C. A., Kapila, S., *Anal. Chem.* **45**, 725 (1973).
Brodie, B. B., in "Absorption and Distribution of Drugs", Binns, T. B., Ed., Williams and Wilkins, Baltimore, Md., 1964, p 199.
Burchfield, H. P., Johnson, E. D., "Guide to the Analysis of Pesticide Residues", Vol. 1, U.S. Department of Health, Education, and Welfare, Bureau of State Services, Office of Pesticides, Washington, D.C., 1965.
Chen, C. N., Andrade, J. D., *J. Pharm. Sci.* **65**, 717 (1976).
Dagani, D., Archer, M. C., *J. Natl. Cancer Inst.* **57**, 955 (1976).
Djerassi, C., *Science* **169**, 941 (1970).
Downer, G. B., Hall, M., Mallen, D. N. B., *J. Agric. Food Chem.* **24**, 1223 (1976).
Elison, C., Elliott, H. W., Look, M., Rapoport, H., *J. Med. Chem.* **6**, 237 (1963).
Emmerson, J. L., Anderson, R. C., *Toxicol. Appl. Pharmacol.* **9**, 84 (1966).
Golab, T., Herberg, R. J., Day, E. W., Raun, A. P., Holzer, F. J., Probst, G. W., *J. Agric. Food Chem.* **17**, 576 (1969).
Hall, M., Mallen, D. N. B., *J. Chromatogr. Sci.* **14**, 451 (1976).
Hambleton, L. G., *J. Assoc. Off. Anal. Chem.* **54**, 125 (1971).
Horning, M. G., Haegele, K. D., Sommer, K. R., Nowlin, J., Stafford, M., Thenot, J.-P., *Proc. Int. Conf. Stable Isot. Chem. Biol. Med., 2nd*, **41** (1975).
McReynolds, J. H., Heck, H. d'A., Anbar, M., *Biomed. Mass Spectrom.* **2**, 299 (1975).
Payne, W. R., Jr., Pope, J. D., Jr., Benner, J. E., *J. Agric. Food Chem.* **22**, 79 (1974).
Pflugmacher, J., Ebing, W., *J. Chromatogr.* **93**, 457 (1974).
Plimmer, J. R., Klingebiel, U. I., in "Mass Spectrometry and NMR Spectroscopy in Pesticide Chemistry", Haque, R., Biros, F. J., Ed., Plenum Publishing Co., New York, N.Y., 1974, p 99.
Riegelman, S., Loo, J. C. K., Rowland, M., *J. Pharm. Sci.* **57**, 117 (1968).
Ruzicka, J. H., Thomson, J., Wheals, B. B., Wood, N. F., *J. Chromatogr.* **34**, 14 (1968).
Smith, A. E., *J. Chromatogr.* **97**, 103 (1974).
Soper, Q. F., U.S. Patent 3 257 190 (June 21, 1966).
Stalling, D. L., Tindle, R. C., Johnson, J. L., *J. Assoc. Off. Anal. Chem.* **55**, 32 (1972).
Tepe, J. B., Scroggs, R. E., *Anal. Methods Pestic. Plant Growth Regul. Food Addit.* **5**, 527 (1967).
Tong, S. C., Gutenmann, W. H., St. John, L. E., Jr., Lisk, D. J., *Anal. Chem.* **44**, 1069 (1972).
Yagupol'skii, L. M., Mospau, V. S., *Ukr. Khim. Zh. (Russ. Ed.)* **21**, 81 (1955).

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Extraction and Identification of the Major Metabolite of [*carbonyl*-^{14}C]Methabenzthiazuron after Degradation in the Soil

Werner Mittelstaedt,* Gerald G. Still, Hans Dürbeck, and Fritz Führ

After soil decomposition of [*carbonyl*-^{14}C]methabenzthiazuron, the metabolite fraction was extracted and separated from the unaltered methabenzthiazuron. Previous studies showed that due to small turnover rates, only limited concentrations of metabolites could be expected. Using HPLC, it was possible to isolate, concentrate, and purify the major metabolite. Its chromatographic properties were identical with 1-methyl-1-(2-benzthiazolyl)urea. The characterization was confirmed by mass spectroscopy fragmentation which yielded a parent ion at m/e 207. The manifold steps of separation and purification lead to relatively high losses. After separation from methabenzthiazuron, the metabolite fraction represented 2.4% of the applied radioactivity. After fractionation on μ-Porasil, only 0.4% was available for MS analysis as major metabolite. About 0.9% was separated and not identified, and 1.1% was used for TLC and LSC counting or lost during evaporation and redissolving, respectively.

The urea derivative methabenzthiazuron [1,3-dimethyl-3-(2-benzthiazolyl)urea] is a very effective herbicide in grain and certain vegetable crops (Hack, 1969; Kolbe and Zimmer, 1970). In lysimeter studies, under field conditions, using [*phenyl*-U-^{14}C]methabenzthiazuron, 83% of the applied radioactivity was recovered in soil 111 days after spray application to spring wheat (Führ, 1975; Führ and Mittelstaedt, 1976). The rate of methabenzthiazuron metabolism was very slow. At harvest, 90% of the chloroform extractable radiocarbon was shown to be unchanged herbicide. In addition to methabenzthiazuron, thin-layer chromatography resolved four other radiolabeled components from this soil extract (Table I). Even after 16 months, the metabolite fraction did not exceed 10% of

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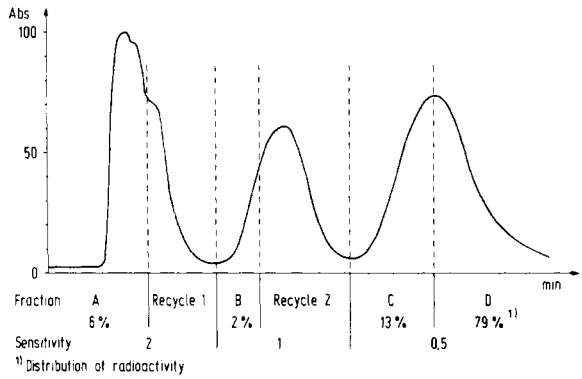


Figure 1. GPC separation of soil metabolites of [*carbonyl*-^{14}C]methabenzthiazuron on μ Styragel (4 × 100 Å). Solvent: chloroform 2.0 mL/min; chart 10 mm/min.

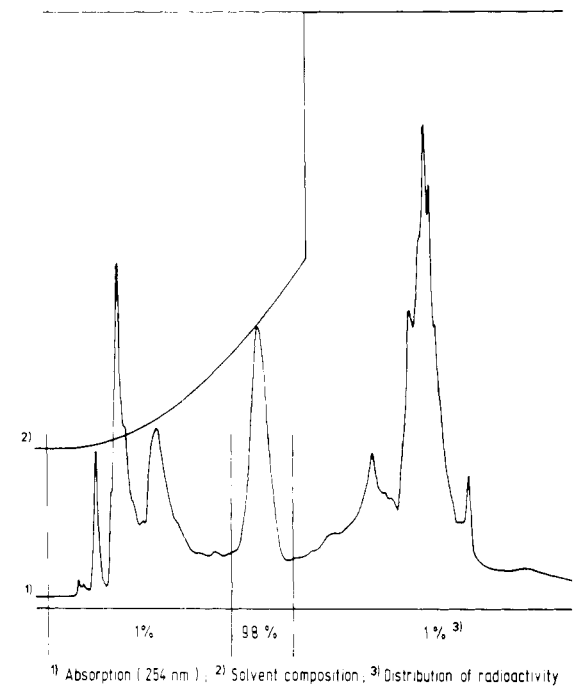


Figure 2. Reverse phase separation of soil metabolites of [*carbonyl*-^{14}C]methabenzthiazuron on μ Bondapak C/18. Solvent program no. 7, 15 min, 15–100% acetonitrile, flow rate 1.5 mL/min, chart 10 mm/min.

the extracted radioactivity. In degradation studies under constant conditions (Cheng et al., unpublished), even lower mineralization rates were observed. Therefore, only small quantities of metabolites were available for further identification. The object of this study was to use new chromatographic methods to purify the major methabenzthiazuron metabolite (3.1% of total chloroform extractable radiocarbon).

EXPERIMENTAL SECTION

Material. [*carbonyl*-^{14}C]Methabenzthiazuron (4.4 μCi, 10 ppm) was applied to a degraded loess soil (195 g, 1.1% C, 12.4% clay, 27.4% silt, 58.1% sand, pH 6.3; three replicates).

Extraction and Fractionation. After decomposition for 12 weeks under constant aeration with about 3 L of CO_2-free air/h at 65% water holding capacity (WHC) and at a simulated standardized temperature program (20 °C with daily or weekly increase up to 25 or 30 °C, respectively), all three replicates were immediately extracted using the Cheng–Führ procedure (1976). All extracts were partitioned into organic and aqueous phases. The organic

Table I. Distribution of ^{14}C in the Chloroform Fraction (Two-Dimensional TLC) of Soils Extracts 111 Days after Application of [*phenyl*-U-^{14}C]Methabenzthiazuron to Spring Wheat under Field Conditions (Time of Application: May 2, 1973)

	Rel distribution of chloroform extractable radioactivity				
	Substance no.				
Soil layer, cm	1[a]	2	3	4	5
0–5	94.1	1.5	3.1	0.8	0.5

[a] (Methabenzthiazuron).

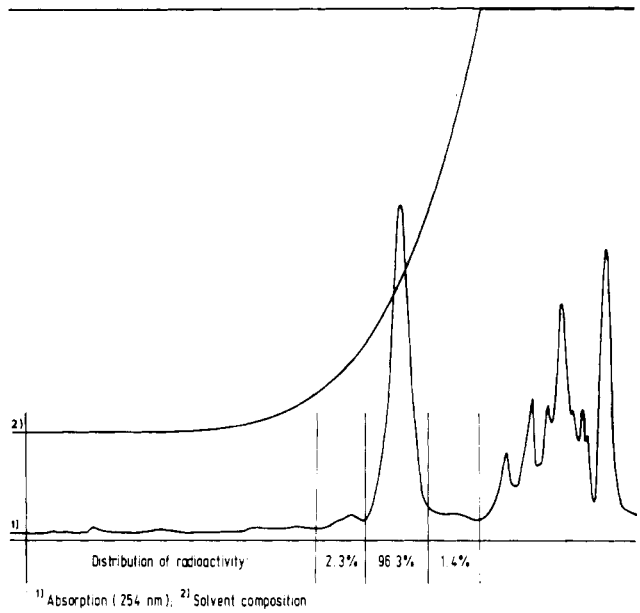


Figure 3. Reverse phase separation of soil metabolites of [*carbonyl*-^{14}C]methabenzthiazuron on μ Bondapak C/18. Solvent program no. 10, 20 min, 20–100% acetonitrile, flow rate 1.5 mL/min, chart 10 mm/min.

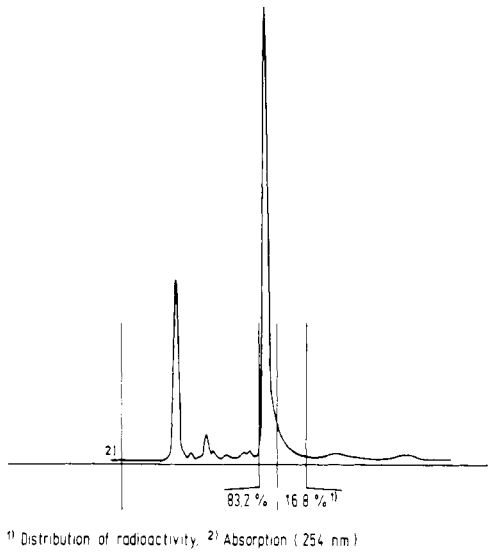


Figure 4. Normal phase separation of soil metabolites of [*carbonyl*-^{14}C]methabenzthiazuron on μ Porasil. Solvent: chloroform, 1.5 mL/min; chart 10 mm/min.

fraction was concentrated (evaporated) to 130 mL and dried over 10 g of anhydrous Na_2SO_4 and filtered through

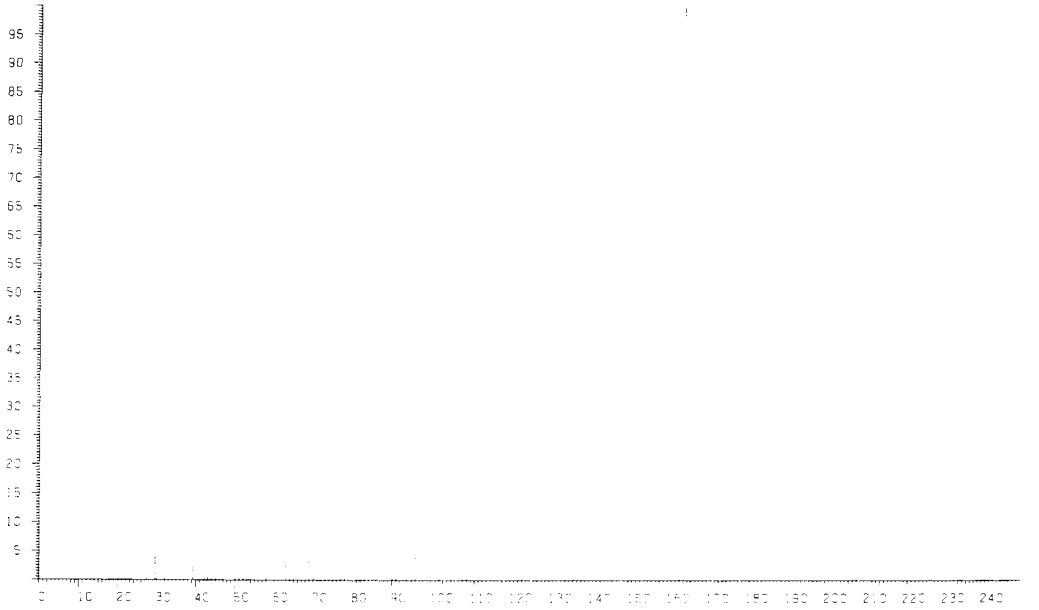


Figure 5. Mass spectrum of 1-methyl-1-(2-benzthiazolyl)urea.

a 100-μm Teflon filter. After evaporation to dryness, the sample was dissolved in 5 mL of chloroform which was placed on a silica gel column for separation of methabenzthiazuron from its soil metabolites using the method of Jarczyk (1972). The column (40 × 2 cm diameter) was filled with 58 g of silica gel 60 (0.063–0.2 mm, Merck). The first eluting solvent was a mixture of carbon tetrachloride–acetonitrile (100:3). The first 650 mL of eluate was discarded, while the second 640 mL containing the methabenzthiazuron fraction was collected.

The column was then eluted with 885 mL of acetonitrile. The first 420 mL contained no radioactivity; however, the following 460 mL yielded almost quantitative elution of the metabolite fraction. The metabolite and the methabenzthiazuron fractions were each concentrated to 100 mL, filtered (Teflon filter 5 μm), further evaporated to dryness and dissolved in 2 mL of chloroform.

Purification. The metabolite fraction was then partially purified, according to molecular size, using four 100-Å μ-Styragel columns in series (Waters Associates prepacked columns, 4 mm i.d., 300 mm long). A Waters Associates high-performance liquid chromatograph (HPLC) with a Model 440 detector (UV detector with a fixed wavelength of 254 nm) used for this separation. The HPLC conditions were: chloroform solvent; flow rate, 2 mL/min; chart speed, 10 mm/min. Best separation was achieved with the use of the recycling procedure which is graphically represented in Figure 1. Using the UV absorption curve to follow the elution patterns, fractions A, B, C, and D were collected and recycle 1 and 2 were recycled through the μ-Styragel columns. Radioassay and TLC showed that fraction D contained the radiolabeled methabenzthiazuron metabolite.

The metabolite, fraction D, was further purified using HPLC on the reverse phase column (Waters Associates) μ Bondapak C/18 (Still and Mansager, 1975). For this separation a solvent elution gradient was delivered to the column by two M-6000 A pumps that were controlled by a Model 660 solvent programmer. Pump A used 5% acetonitrile in water while pump B used 100% acetonitrile. The flow rate through the column was 1.5 mL/min. The metabolite was first separated from its contaminants using program no. 7 (15–100% acetonitrile, over 15 min, Figure 2) The radiolabeled fraction from this separation was concentrated and rechromatographed using the same

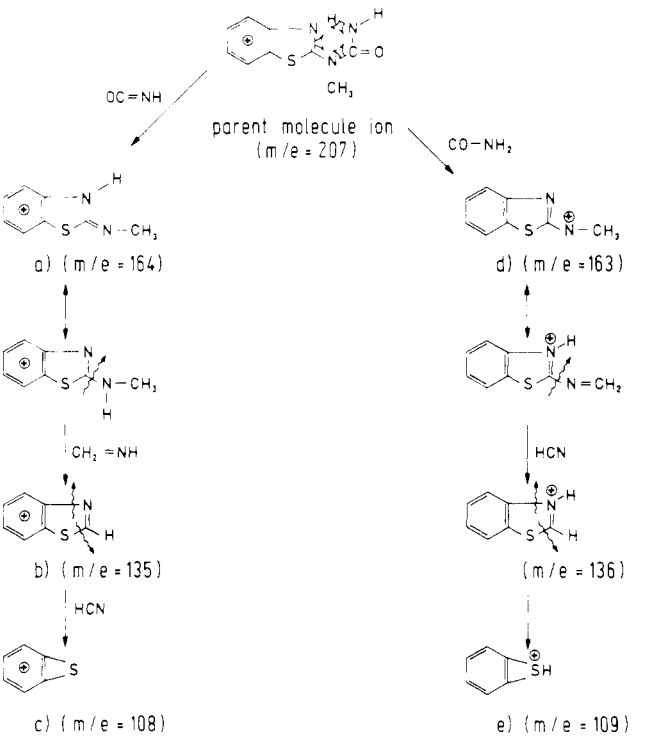


Figure 6. Fragmentation of 1-methyl-1-(2-benzthiazolyl)urea.

system but program no. 10 (20–100% acetonitrile, over 20 min). A single sharp elution peak was collected, concentrated, and prepared for further HPLC purification (Figure 3).

Reference substances of several hypothetical metabolites were chromatographed using the same conditions. The elution volume of the different materials were compared with the unknown methabenzthiazuron metabolite (Table II) which showed the same elution volume as 1-methyl-1-(2 benzthiazolyl)urea.

The final purification used normal phase HPLC on a μ-Porasil column (Waters Associates). The elution solvent, chloroform, was pumped at a flow rate of 1.5 mL/min. This chromatographic system yielded nearly baseline separation of the metabolite from its contaminants (Figure 4). For comparison, reference substances were also

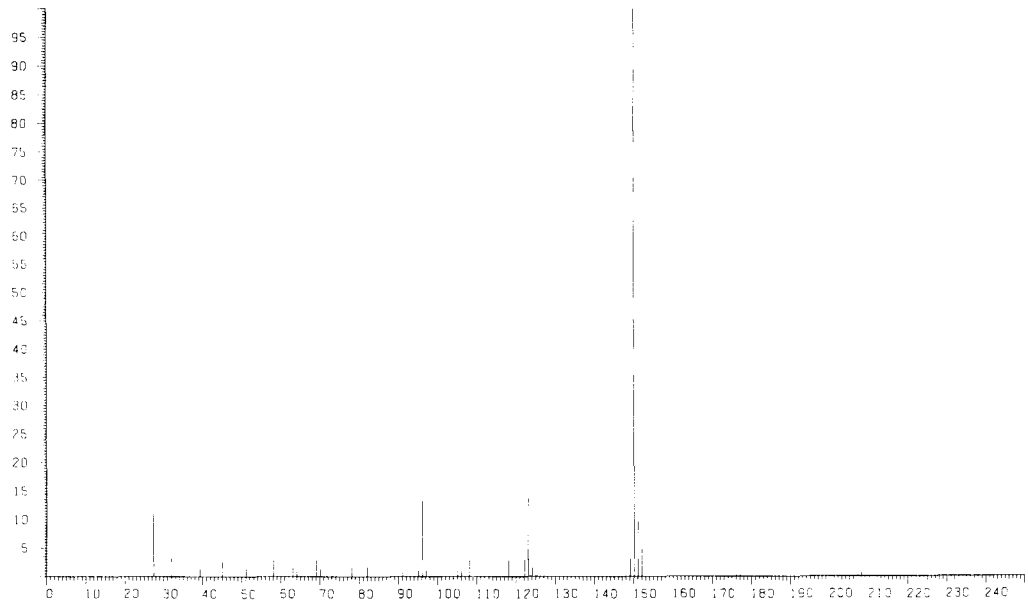
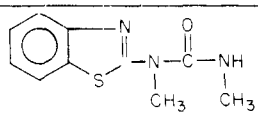
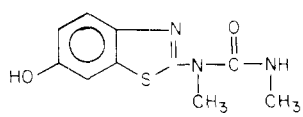
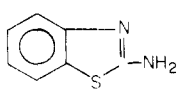
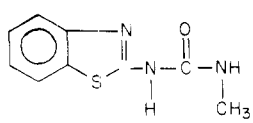
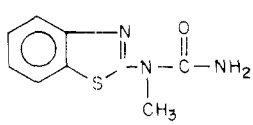


Figure 7. Mass spectrum of 3-methyl-1-(2-benzthiazolyl)urea.

Table II. Elution Volume of Methabenzthiazuron and of Different Metabolites in Comparison to the Major Metabolite

No.	Compound structure	Elution volume [mL] μ-C 18[a]	Elution volume [mL] μ-Porasil[b]
1		30.98	4.95
2[c]		13.65	
3		15.60	
4		21.15	
5		24.83	8.40
Major metabolite		24.75-24.83	8.36

[a] Solvent program no. 10, 20-100% acetonitrile, 20 min; flow rate 1.5 mL/min; chart 10 mm/min. [b] Chloroform, flow rate 1.5 mL/min, chart 10 mm/min. [c] Found as degradation product (Wallnofer et al., 1976).

chromatographed (Table II). The metabolite was concentrated for mass spectral analysis.

MS Confirmation. The 70-eV mass spectral data were obtained on a MAT CH 7 spectrometer equipped with the "Spectrosystem 100" computing system. The metabolite was introduced by the solid sample probe at a temperature of about 70 °C to keep source pressure at 10^{-6} mmHg. The normalized and total ion current corrected (peak intensites are corrected for changes in the total ion current) spectrum (Figure 5) had a base peak at m/e 164, while the parent molecular ion was observed at m/e 207 with a relative intensity of 15.5%.

The major fragmentation process (Figure 6) involves initial loss of OC=NH to give methyl-2-benzthiazolylamine (m/e 164, fragment a) which then decomposes to fragment b (m/e 135) by loss of a CH_2=NH group. Elimination of HCN finally yields compound c (m/e 108), a fragmentation which is typical for benzthiazol (Clark et al., 1966).

In a less favorable fragmentation route, elimination of the $CONH_2$ radical from the parent molecular ion leads to the fragment d (m/e 163), which by two succesive losses of HCN yields fragment e (m/e 109).

These data and the related conclusions fit very well with the corresponding chromatographic results. Therefore the major soil metabolite of methabenzthiazuron has been identified as 1-methyl-1-(2-benzthiazolyl)urea (compound 5, Table II), with structural confirmation verified by the mass spectrum of an authentic sample.

As additional evidence, 3-methyl-1-(2-benzthiazolyl)urea (compound 4, Table II), another hypothetical metabolite, was subjected to electron impact mass spectrometry. The spectrum (Figure 7) is quite different from that of the major soil metabolite and has a base peak at m/e 150 and only three peaks with abundances 10% at m/e 207 [parent molecular ion (RI = 12.1%), m/e 123 (RI = 19.7%), and m/e 96 (RI = 13.2%)]. The initial fragmentation showed a loss of an OC=N-CH_3 molecule (m/e 57) to give m/e 150 which further decomposes by two successive losses of HCN, yielding the thiopyrylium cation (m/e 96), a known rearrangement process (Kinney and Cook, 1952).

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LITERATURE CITED

Cheng, H. H., Führ, F., *J. Agric. Food Chem.* **24**, 421 (1976).
Cheng, H. H., Führ, F., Jarczyk, H. J., Mittelstaedt, W., to be submitted to *J. Agric. Food Chem.*
Clarke, G. M., Grigg, R., Williams, D. H., *J. Chem. Soc. B*, 339 (1966).
Führ, F., Jül-Bericht Jül-1234, Habil-Schrift University of Bonn, 1975.
Führ, F., Mittelstaedt, W., *Landwirtscha. Forsch.* **32**, 286 (1976).

Hack, H., *Pflanzenschutz-Nachr. (Am. Ed.)* **22**, 341 (1969).
Jarczyk, H. J., Bayer AG, Pflanzenschutz, unpublished data, 1972.
Kinney, I. W., Jr., Cook, G. L., *Anal. Chem.* **24**, 1391 (1952).
Kolbe, W., Zimmer, K., *Pflanzenschutz-Nachr. (Am. Ed.)* **23**, 292 (1970) (283–354, English edition).
Still, G. G., Mansager, E. R., *Chromatographia* 8(3), 129 (1975).
Wallnöfer, P., Tillmanns, G., Thomas, R., Wünsche, C., Kurz, J., Jarczyk, H. J., *Chemosphere* **5**, 377 (1976).

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Studies on the Proteins of the Mutants of Barley Grain. 2. Fractionation and Characterization of the Alcohol-Soluble Proteins

U. Singh[1] and L. V. S. Sastry*

Fractionation and characterization of the alcohol-soluble proteins of a barley variety, its mutants, and Hiproly, a high-protein and high-nutritive barley isolated from the world barley collection, indicate quantitative and qualitative changes in the mutants. The 35% ethanol-soluble subfraction is higher in proportion in the mutants and Hiproly, and this could have resulted from the higher proportions of the polar amino acids in these proteins among which are also some limiting amino acids like lysine and threonine. The digestibility, as observed by the in vitro procedure, of this subfraction in all the varieties also is higher and, presumably as a result, the hordein fraction of these mutants also shows better digestibility. It appears that, in view of the presence of more polar amino acids which include lysine in this subfraction and better digestibility, its enrichment could be a nutritionally favorable index of better grain quality.

Earlier studies on the proteins of a barley variety, NP-113, its mutants (Notch-1 and Notch-2), and Hiproly indicated considerable changes in the classical protein fractions of the mutants, and these changes appeared to be nutritionally favorable (Singh and Sastry, 1977). Such changes might be attributed to either the enrichment of the protein species already existing as indicated in the case of wheat gluten or due to the synthesis of the new species of the proteins as in triticale or both (Yong and Unrau, 1964). In the present investigation, attempts have been made to identify these changes in the mutants and their relationship to nutritional quality. Consequently, the alcohol-soluble proteins have been fractionated, amino acid composition of the subfraction determined, and nutritional quality evaluated by preliminary in vitro evaluation procedures.

MATERIALS AND METHODS

The grain samples, namely, NP-113, Notch-1, Notch-2, and Hiproly, used in this investigation, and the protein fractions therefrom, were obtained as described earlier (Singh and Sastry, 1977). The determination of the protein content and electrophoresis of the proteins were also described earlier. The gel system (Singh and Sastry, 1977) with an operating pH 4.0 was employed for characterization of the protein subfractions.

Fractionation of Hordein. After extraction of hordein with 70% ethanol, distilled water was added to the extract until a final concentration of 35% ethanol was obtained. After allowing the protein to precipitate for 30 min, the suspension was centrifuged. The residue was washed with 35% ethanol and ethanol and dried under vacuum. This formed the 35% ethanol-insoluble fraction. The supernatant was "flash-evaporated" at 37 °C and the residue dried under vacuum. This fraction was designated as 35% ethanol-soluble fraction.

Preparation of α- and β-Hordeins. The procedure of Turner et al. (1965) was followed. Two hundred milligrams of the hordein was suspended in 5 mL of 95% ethanol. The suspension was shaken continuously for 2 h and centrifuged. The residue was washed twice, and the washings were pooled with the first supernatant and flash-evaporated at 37 °C. This fraction is referred to as the α-hordein. The pellet which was dried in vacuum was designated as β-hordein.

Gel Filtration of Hordein. The protein sample was dissolved in a sample containing 4 M dimethylformamide (DMF) and 0.1 M acetic acid. The solution was dialyzed against the same solvent for 24 h to remove contaminating pigments. After adjusting the protein concentration to 20 mg/4 mL of the solution, the sample was applied at the top of the Sephadex G-100 column (2 × 50 cm) previously equilibrated with the same solvent after measuring the total absorption at 280 nm. Elution was effected with the same solvent. Three-milliliter fractions were collected with the aid of a LKB-Radi Rac Siphon control automatic fraction collector. Fifty fractions were collected at the rate of 12 mL/h. The optical density of each fraction was also monitored at 280 nm. The tubes containing the protein under each peak of the elution profile were pooled, dialyzed against distilled water, and lyophilized to dryness.

Determination of Amino Acid Composition of Protein. About 10 mg of protein (N × 6.25) was hydrolyzed with 10 mL of distilled hydrochloric acid (6 N) for 24 h at 110 ± 1 °C in evacuated sealed tubes. Excess acid was removed by repeated washing with distilled water and it was subsequently evaporated under reduced pressure at 50 °C in a rotary flash-evaporator. The residue was taken up in citrate buffer (pH 2.0), and an aliquot was analyzed on a Technicon Sequential Multisample amino

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