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A Comparison of Methods for the Intrinsic Labeling of Wheat Protein with ^{35}S

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A hard red winter wheat variety (Brule), grown to maturity in a greenhouse, was intrinsically labeled with ^{35}S by either stem injection or addition of the isotope to the medium in which detached wheat heads were grown. Two levels of isotope were applied by each method at 5, 10, 15, or 20 days postanthesis. Significantly higher ($P < 0.05$) yield (weight/head) of grain was observed with injection compared to the incubation method. Of the isotope introduced by injection, 77% translocated into the wheat kernel, and of this amount at least 77% of the isotope in the kernel was associated with kernel protein and free sulfur amino acids. Data suggest that injection of wheat stems 15 days after anthesis, with doses up to 10 μCi of ^{35}S, would be a suitable method of obtaining intrinsically labeled wheat protein for bioavailability studies.

The use of intrinsically labeled nutrients offers investigators an effective means of conducting bioavailability studies. Nutrients intrinsically labeled with radioisotopes may be incorporated into diets fed to small animals and monitored throughout the organism.

Selected nutrients in wheat have been intrinsically labeled with radioisotopes with varying efficiency, depending in part on the maturity of the plant when the isotope is administered (McConnell and Ramachandran, 1956; Bilinski and McConnell, 1958; Lee and Wan, 1963; Lee and Reynolds, 1963). Effect of element concentration on uptake may also be a consideration. Starks and Johnson (1985) reported that the total zinc content of whole wheat flour was higher when stems were injected with $^{65}Zn + ZnSO_4$ than with ^{65}Zn alone, showing greater zinc uptake with increased injection load.

Mode of application may also influence results (Weaver, 1985). Two of the most commonly used procedures are injection of isotope solution into the hollow stem of the plant or incubation of detached wheat heads in liquid medium containing added isotope. Using the stem injection method, Lee and Reynolds (1963) obtained a maximum of 39% incorporation of ^{35}S in whole wheat flour.

Studies in which the isotope has been administered by incubation of detached heads have generally utilized short incubation periods, e.g., 12 h (Donovan et al., 1977) or 14 h (Graham and Morton, 1963). These studies were useful in defining the development of the plant at different stages of growth. However, for use in animal studies, a sufficient quantity of fully mature labeled grain similar in composition to grain used for human consumption is necessary. Donovan and Lee (1977) incubated wheat heads (detached at 8 or 20 days after flowering) in nutrient medium for 12 days, resulting in grain development similar to that of field-grown material.

The objective of this study was to optimize incorporation of ^{35}S into wheat kernel protein. Variables examined were concentration of dose, time after anthesis, method of isotope introduction (incubation vs injection), and extension of incubation period until wheat heads were mature.

MATERIALS AND METHODS

Hard red winter wheat (Brule) was grown in the greenhouse after it had been vernalized at 4 °C for 8 weeks. Potting mix contained 75% peat moss, 20% perlite, and 5% vermiculite (v/v/v). Greenhouse conditions: temperature, 16–27 °C; relative humidity, 50–65%; natural daylight conditions, August to January, 155 days. The first wheat head emerged after 48 days in the greenhouse, and anthesis was noted after an additional 19 days.

At 5, 10, 15, or 20 days after anthesis, carrier-free $^{35}SO_4$ ($Na_2{}^{35}SO_4$; New England Nuclear, Boston, MA) was introduced into the wheat heads by injection or incubation with 50 μL of one of two solutions (0.1 or 0.2 μCi/mL in sterilized incubation medium described below).

Stem Injection. An area 5 cm above the last node was cleaned with ethanol. A hole was pierced with an injection needle, and 50 μL isotope solution was slowly introduced with a Hamilton syringe. The hole was then covered with nitrocellulose glue to seal and to prevent infection.

Incubation. Wheat heads were cut above the last node. A slanted cut was then made 11–12 cm from the base of the head while the stem was kept in a 0.5% solution of sodium hypochlorite to prevent infection. Each excised wheat head was inserted into a 50-mL glass vial (25 × 100 mm) through a hole drilled in the cap lined with a 0.2-μm

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Table I. Yield of Brule Wheat Intrinsically Labeled with Carrier-Free ^{35}S

method of labeling[a]	wt/head,[b] g: days after anthesis			
	5	10	15	20
stem injection	0.68 ± 0.14[a]	0.59 ± 0.05[a]	0.74 ± 0.05[a]	0.72 ± 0.09[a]
cold incubn	0.27 ± 0.04[b]	0.26 ± 0.04[b]	0.27 ± 0.03[b]	0.55 ± 0.07[a]

[a] Isotope introduced 41–67 days before harvest. [b] Means ± SEM of six samples (three aliquots × two doses); data for 5- and 10-μCi doses were pooled, since yields were similar with either dose. Values with different superscripts within columns are significantly different ($P < 0.05$).

cellulose membrane filter. Vials contained 50 μL of the appropriate isotope dose in 2 mL of sterilized medium (0.05% nitrate nitrogen furnished by 20:20:20 N/P/K water-soluble fertilizer, with 2% sucrose added; Plantco Inc., Bramlea, Ontario, Canada). Vials were supported in racks placed in a cold bath (2 ± 1 °C). After most of the isotope solution was taken up by the wheat head (16–20 h), an additional 25 mL of sterilized medium was added.

Wheat heads were harvested when all of the treated heads had dried. A total of 160 wheat heads were labeled in a 2 × 2 × 4 × 10 factorial design: two methods of isotope introduction (stem injection or incubation of excised heads), two dose levels (5 or 10 μCi/head of carrier-free $^{35}SO_4$), four time points (5, 10, 15, or 20 days after anthesis), and 10 replicate heads per treatment. Radioactivity was determined in the wheat kernel and protein as soon as possible to minimize further loss of activity.

Determination of ^{35}S Activity. Wheat kernels from two to seven heads were separated, and total weight was recorded. Ground wheat samples were solubilized in 0.5 M Protosol tissue solubilizer (New England Nuclear), and activity was counted in scintillation fluid with a Packard Tri-Carb scintillation counter (Packard Instrument Co., Downers Grove, IL). Samples were analyzed in triplicate along with unlabeled controls.

Protein and free sulfur amino acids were solubilized in 0.062 M Tris solution (containing 2% SDS and 5% 2-mercaptoethanol), pH 6.8, by the procedure described by Fullington et al. (1983). Activity in the supernatant was counted as described for wheat kernels.

Data were statistically analyzed by Duncan's new multiple-range test.

RESULTS AND DISCUSSION

Yield. Since yield (weight/head of air dried kernels) was not affected by dose levels, data for both levels were pooled. Significantly higher ($P < 0.05$) average yield was observed when the isotope was introduced by injection, compared to incubation of detached heads, starting at 5, 10, or 15 days postanthesis (Table I). However, at 20 days postanthesis there were no significant differences in yield between injected and incubated wheat heads. Donovan and Lee (1977), comparing field-grown wheat to incubated wheat heads (cv. Penjamo), found no significant negative effect of incubation on grain dry weight when heads were excised from field-grown plants at 8 or 20 days after anthesis and incubated for 12 days in growth medium. In the present study, wheat heads excised at 5, 10, or 15 days postanthesis and incubated for 41–62 days yielded kernels weighing 51–64% less than all other treatments. Mold contamination may have contributed to the reduced yield with incubation, since heads excised at 5, 10, or 15 days postanthesis were much less developed than those at 20 days, and mold growth occurred during the lengthy incubation period in spite of the precautions taken. Cold temperature (2 °C) and/or lack of growth factors and micronutrients in the incubation medium may also have contributed to reduced uptake of nutrients in the incubated detached heads, relative to intact plants.

Table II. Incorporation of ^{35}S into Brule Wheat Kernel

	days after anthesis	% of dose[a] at	
		5 μCi/head	10 μCi/head
injection	5	66.50 ± 1.02[a,b]	61.28 ± 0.84[a,b]
	10	61.84 ± 1.42[a–c]	63.54 ± 0.39[a,b]
	15	77.21 ± 0.19[a]	78.26 ± 1.54[a]
	20	36.37 ± 0.61[d]	21.79 ± 0.08[c]
cold incubn	5	46.23 ± 0.19[c,d]	57.45 ± 2.02[b]
	10	57.72 ± 1.68[b,c]	48.92 ± 0.70[b]
	15	44.12 ± 1.01[c,d]	46.64 ± 0.58[b]
	20	64.78 ± 0.72[a,b]	57.49 ± 0.48[b]

[a] Means ± SEM of three aliquots from composites of two to seven heads. Values with different superscripts within columns are significantly different ($P < 0.05$). SEM determined on aliquots of composite samples represents variability in aliquot sampling and counting technique and not variability of isotope uptake head to head.

^{35}S Incorporation into Wheat Kernel. Efficiency of incorporation of ^{35}S is best expressed in terms of percent of administered dose (corrected for isotope decay), since radioactivity decays with time and absolute amounts will vary, depending on the age of the isotope at the time activity is measured. The percent incorporation of ^{35}S after injection or incubation of wheat heads and influence of dose and time of application after anthesis are presented in Table II. The largest proportion (78%) of dose incorporated was achieved with injection of 10 μCi at 15 days postanthesis. Injection 5 days later (20 days postanthesis) resulted in a significantly lower proportion of isotope incorporation with either dose. This suggests that the rate of protein synthesis in intact plants reached a maximum by 15 days postanthesis and declined thereafter. All incubation treatments had significantly lower ($P < 0.05$) incorporation than injection at 15 days with the exception of incubation at 20 days with 5 μCi.

Lee and Reynolds (1963) achieved 39% incorporation of ^{35}S-labeled sulfate by injection of 10–40 μCi/head into Thatcher wheat 20 days before harvest (plants harvested 91 days after seeding). Also, by injecting plants from 8 to 28 days before harvest, they found that longer periods of exposure to the isotope resulted in greater incorporation of the isotope into flour. Although a different variety of wheat (cv. Brule) was used in the present study, the isotope was introduced at presumably an earlier stage of development (from 41 to 67 days before harvest). This may partly explain the 2-fold increase in percent incorporation in this study compared to that reported by Lee and Reynolds (78 vs 39%). These authors did not include the time of anthesis relative to when injections were made in their study, which does not allow for a more precise comparison between stage of development and injection time. They did state that more than half of the injected radioactivity remained in the stem at maturity, possibly bound in the scar tissue or in the collodion seal. It appears that less isotope was bound to the stem in the present study.

^{35}S Incorporation into Wheat Kernel Protein and Free Sulfur Amino Acids. The proportion of isotope incorporation into wheat kernel protein and free sulfur amino acids (saa) (Table III) followed a trend similar to that of the whole kernel. The highest percent of isotope dose incorporated into wheat kernel protein components resulted with injection at 15 days postanthesis (60–63% of dose). Distribution of ^{35}S among wheat proteins was not investigated.

Table III. Incorporation of ^{35}S into Brule Wheat Kernel Protein and Free Sulfur Amino Acids

	days after anthesis	% of dose[a] at 5 μCi/head	% of dose[a] at 10 μCi/head
injection	5	50.60 ± 0.50^{b}	45.99 ± 0.05^{b,c}
	10	50.51 ± 0.60^{b,c}	47.63 ± 1.05^{b}
	15	63.09 ± 0.41^{a}	60.35 ± 0.46^{a}
	20	27.54 ± 0.59^{f}	16.88 ± 0.14^{e}
cold incubn	5	32.45 ± 0.36^{f}	36.43 ± 0.27^{c,d}
	10	41.47 ± 0.88^{b-e}	33.65 ± 0.34^{d}
	15	31.01 ± 0.26^{f}	32.91 ± 0.35^{d}
	20	42.61 ± 0.48^{b-d}	38.85 ± 0.87^{b-d}

[a] Values are means of three subsamples from a composite of two to seven heads. Values with different superscripts within columns are significantly different ($P < 0.05$). SEM determined on aliquots of composite samples represents variability in aliquot sampling and counting technique and not variability of isotope uptake head to head.

Table IV. Proportion of ^{35}S Incorporated into Brule Wheat Kernel Associated with Protein and Free Sulfur Amino Acids

	days after anthesis	% of incorporated dose[a] at 5 μCi/head	% of incorporated dose[a] at 10 μCi/head
injection	5	76.15 ± 1.91^{a,b}	75.08 ± 1.10^{a,b}
	10	81.75 ± 1.63^{a}	74.98 ± 2.06^{a,b}
	15	81.31 ± 0.62^{a}	77.15 ± 1.11^{a,b}
	20	75.77 ± 2.26^{a,b}	77.46 ± 0.96^{a}
cold incubn	5	70.19 ± 0.98^{b,c}	63.53 ± 1.79^{c}
	10	71.54 ± 4.62^{b,c}	68.80 ± 0.48^{a-c}
	15	70.38 ± 2.20^{b-d}	70.58 ± 1.06^{a-c}
	20	65.79 ± 0.97^{c}	67.56 ± 1.02^{b,c}

[a] Means ± SEM of three aliquots from composites of two to seven heads. Values with different superscripts within columns are significantly different ($P < 0.05$). SEM determined on aliquots of composite samples represents variability in aliquot sampling and counting technique and not variability of isotope uptake head to head.

A minimum of 64% of the incorporated isotope was associated with kernel protein and free saa, irrespective of dose, time, or means of introduction of isotope (Table IV). The lower values resulted from the incubation procedure, suggesting a lower rate of synthesis of sulfur-containing protein components.

Our observation of maximum protein synthesis at approximately 15 days postanthesis is entirely consistent with other reports, since in field-grown (cv. Gabo) wheat storage protein synthesis was found to begin at about 12 days and increase dramatically by 19 days postanthesis (Jennings and Morton, 1963). Future studies should address ^{35}S distribution among wheat proteins as well as compare deposition and distribution of sulfur in field grown and greenhouse wheat.

CONCLUSIONS

Data from this study indicate that the injection procedure produces the optimum conditions for label incorporation and is superior to the incubation procedure for that purpose. Total grain yield and amount of isotope incorporated were higher, and proportion of ^{35}S incorporated into protein was greater with injection compared to incubation. Control of mold contamination becomes increasingly difficult with the lengthy incubation period required for heads to reach maturity. Concentration of dose did not affect the yield or percent uptake of isotope, thereby presenting the possibility of increased enrichment of nutrient with isotope by using solutions of higher activity.

Time of introduction of isotope was critical, and most likely related to the stage of protein synthesis in the plant. Under the conditions of the current study, maximum incorporation of ^{35}S into protein in intact Brule wheat plants resulted from injection of isotope 15 days postanthesis. Investigators using other varieties of wheat with different maturation cycle lengths would need to adjust the time of isotope injection to correspond to the equivalent stage of growth.

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