STUDIES ON WHEAT PLANTS USING CARBON-14 COMPOUNDS

XVII. The Pattern of Carbon-14 Incorporation into Some Fractions of Wheat Gliadin[1]

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ABSTRACT

Wheat proteins were labeled with carbon-14 by injection of acetate-1-C^{14} into the stems of different groups of wheat plants at each of five stages of maturity. The portion of gliadin soluble in 0.025*N* ammonium hydroxide was oxidized with performic acid and five major fractions were isolated therefrom by chromatography on DEAE-cellulose. The fractions differed from each other in chemical properties, amino acid composition, and carbon-14 content. Furthermore, the specific activity of glutamic acid isolated from acid hydrolysates of the gliadin fractions was found to depend markedly upon both the fraction from which it was obtained and the time at which tracer was administered. Although the relationships between the specific activity of glutamic acid and the time of tracer injection are complex, two possible explanations for the results are offered. First, the oxidation of intramolecular disulfide linkages facilitates separation of different gliadin proteins, in which the relative efficiencies of carbon-14 incorporation vary during kernel formation and maturation. Second, or probably as well, by rupturing interpeptide cross-links, the oxidation produces polypeptide chains each with its own chemical properties, amino acid composition, and biosynthetic history.

The portion of wheat kernel protein soluble in 70% ethanol (gliadin) can be separated by several techniques into a number of

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partially characterized components (2,5,11). Studies in this laboratory show that five "proteinlike" fractions can be obtained from a wheat gliadin fraction which has been oxidized with performic acid (2). The separations were done by chromatography on ion-exchange cellulose, and the results are readily reproduced with different samples of gliadin. The fractions differ from each other in amino acid composition, and a preliminary study with gliadin from plants which had been labeled with acetate-C^{14} show that the specific activity of several individual amino acids is characteristic of the fraction from which they are obtained. The results are analogous in many ways to those obtained by examining carbon-14 incorporation into kernel fractions designated as glutenin, gliadin, globulin, and albumin (1). The dependence of the specific activity of a particular amino acid upon the fraction from which it is obtained is evidence that the separation of the fractions is being effected, at least in part, on the basis of differences introduced at the time of protein formation. Thus the fractions are not merely the result of manipulations involved in the isolation and fractionation of the proteins.

The present report gives results of an investigation of the relation between the stage of wheat plant maturity when acetate-1-C^{14} was administered and the specific activity of several gliadin fractions. The effect of time of labeling on the specific activity of the glutamic acid from these fractions was also studied. The data indicate that the rate of formation of kernel proteins is reflected in their specific activity.

Experimental Methods

Production of Labeled Wheat. Thatcher wheat was sown in a field plot on May 26, 1961. The plants were irrigated as required for satisfactory growth and developed normally. When the plants were well headed and kernel formation was evident, a group of tillers which were judged to be of equal stages of maturity were selected and tagged. On July 28 sodium acetate-1-C^{14} in aqueous solution (0.15 ml., 0.168 mg., 10.0 μc.) was injected into the top internode of each of ten of the tagged stems. Similarly, ten plants were administered tracer on July 31, August 8, 11, and 17. The kernels were well formed and are described as being in the "late milk stage" at the time of the first injection. The kernels were quite mature and firm at the time of the last injection. The wheat was harvested at full maturity and air-dried at room temperature, and the kernels were collected. Additional nonradioactive wheat was harvested from the same plot and equal amounts of radioactive and nonradioactive wheat were mixed before milling. Kernels from each set of injections were pooled

and milled in an experimental mill. Approximately 19.0 g. of flour (50% extraction) were recovered from each run.

Isolation of Gliadin. Gluten-C^{14} (approximately 2.5 g.) was prepared from each flour sample by the method of Lusena (6), and the gliadin was then extracted from the gluten with 70% aqueous ethanol (1). Gliadin yields were from 50 to 60% based on the gluten.

Gliadin was separated into an aqueous ammonia-soluble fraction (50% of the gliadin nitrogen) and an aqueous alkali-soluble fraction (2). The ammonia-soluble material was oxidized by performic acid (3) and chromatographed on N,N-diethylaminoethylcellulose (DEAE-cellulose) (supplied by Brown and Co., Berlin, N. H.) (2). The elution curves are reproduced in Fig. 1. The appropriate fractions were pooled, dialyzed against distilled water, and freeze-dried.

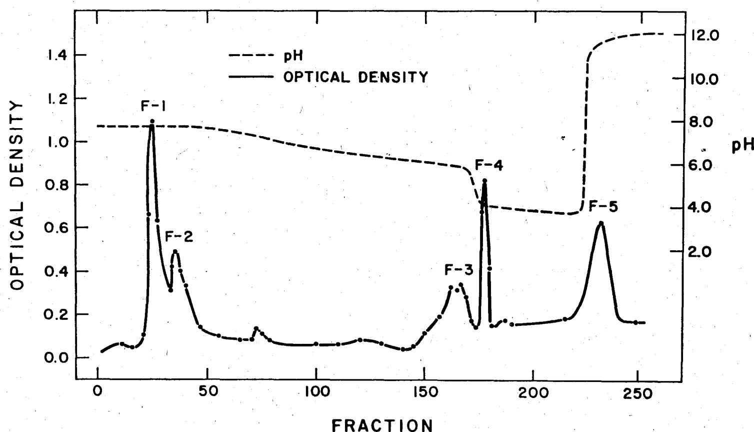

Fig. 1. Elution curve for the chromatography of the oxidized gliadin fraction on DEAE-cellulose.

Nitrogen was determined by the micro-Kjeldahl method.

Isolation of Glutamic Acid. A sample of the oxidized protein and each of the fractions obtained from another portion of the protein were hydrolyzed by refluxing in 6*N* hydrochloric acid for 20 hours. Glutamic acid was isolated from each hydrolysate by chromatography on Dowex-1 × 8 (acetate form) (4). The amounts of glutamic acid were estimated by the ninhydrin method of Moore and Stein (7) and their carbon-14 contents measured. The specific activities were confirmed after addition of a known amount of carrier L-glutamic acid and recrystallization.

Amino Acid Analyses. The amino acid compositions of the hydrolysates of each fraction from a nonradioactive gliadin preparation

were measured using a Beckman/Spinco automatic amino acid analyzer. A 92% recovery of amino acid nitrogen was realized from hydrolysates of F-1, 2, 3, 4, and CM-1. The values obtained for ammonia were not used.

Determination of Specific Activity. All samples, for measurement of specific activity, were converted to carbon dioxide by the wet combustion technique (9). The carbon-14 content of the gas was measured in a vibrating reed electrometer (Dynacon, Nuclear Chicago Corp.). Unless designated in the text, sufficient carbon-14 was given for assay, so that the probable error in the measurements was 2%. The specific activity is given in mμc./mg. N. This unit was used because one of the protein fractions contained nonprotein carbon and hence expressing specific activity on the basis of carbon would have given a low value.

Results and Discussion

The amino acid compositions of the fractions from a nonradioactive preparation of performic acid-oxidized protein are in Table I. The results show that the materials differ markedly from each other, the wide variation in the basic amino acid content being especially notable. The results agree in general with some partial analyses previously reported (2), except that in the earlier work arginine was not detected in F-1. CM-1 and CM-3 are fractions of F-1; CM-1 represents

TABLE I
AMINO ACID COMPOSITION OF THE OXIDIZED PROTEIN FRACTIONS
(Percent by weight of total amino acids recovered)

AMINO ACID	F–1	CM–1	CM–3	F–2	F–3	F–4	F–5	OXIDIZED PROTEIN
Glutamic acid	43.0	47.6	23.3	41.7	19.5	37.7	33.0	37.7
Proline	22.0	22.9	8.8	19.2	6.5	12.4	12.3	17.2
Serine	4.6	4.6	6.7	5.2	6.6	7.2	7.0	5.3
Leucine	5.2	5.6	8.9	5.8	8.9	6.4	6.0	7.0
Isoleucine	2.4	2.2	4.7	2.7	4.5	2.8	3.5	4.0
Valine	2.9	2.8	5.9	3.6	6.5	4.1	4.0	4.0
Glycine	3.4	2.4	10.2	6.5	16.6	5.9	9.6	4.7
Alanine	1.8	1.1	10.2	3.5	14.9	4.5	4.0	3.2
Phenylalanine	6.4	5.8	6.5	4.7	trace	3.0	4.2	4.5
Tyrosine	0	0	trace	0	0	0	0	trace
Threonine	1.9	1.6	5.0	2.7	5.5	3.5	3.4	2.3
Aspartic acid	2.0	1.4	8.2	2.9	7.5	4.1	5.0	3.0
Cysteic acid	0.5	0.7	0	1.0	trace	2.4	3.4	2.0
Methionine sulfone[a]	1.5	0.3	0	0.5	0	0.8	0.9	0.7
Lysine[a]	0.5	0	1.6	0	trace	0.9	1.3	1.2
Histidine[a]	0.90	0.10	0	0	0	1.3	2.0	1.2
Arginine[a]	1.1	0.4	0	0	0	2.0	3.3	2.4

[a] These amino acids were present in very small quantities in some fractions, hence these figures of percent composition may be less accurate than those for the other amino acids. A subfraction of F–1 (CM–2, 3% of F–1) was not analyzed.

about 82% of F-1 and CM-3 about 6% (2).

The nomenclature used is the same as that used in a previous report (2) and is explained in Fig. 1.

The weights of the fractions isolated here are proportional to those isolated in other nonradioactive runs where the weights of starting material were greater. The amino acid analyses of radioactive and nonradioactive runs are in excellent agreement. We have assumed, therefore, that the amount of carbon-14 incorporation is proportional to specific activities of the fractions. The radioactive data are from single experiments and we have found that the fractionation of the oxidized protein is quite reproducible.

The specific activities of the individual fractions are clearly dependent upon the time of tracer injection (Table II), and the amount of carbon-14 incorporated into them is a maximum when the tracer was given about 73 days after seeding.

TABLE II

SPECIFIC ACTIVITIES

EXPERIMENT	INTERVAL AFTER SEEDING	OXIDIZED PROTEIN	F–1	F–3	F–4	F–5 [a]
	days		*mμc./mg.N*	*mμc./mg.N*	*mμc./mg.N*	*mμc./mg.N*
			Protein Fractions			
1. July 28	63	17.1	15.5	1.40	2.90	
2. July 31	66	19.8	17.5	1.40	4.80	
3. August 8	73	22.8	23.4	11.4	16.1	
4. August 11	77	11.6	13.6	1.12	4.20	
5. August 17	81	10.5	11.0	1.0	2.10	
			Glutamic Acid			
1. July 28	63	49.7	49.2	7.8	38.8	40.0
2. July 31	66	59.5	63.4	28.3	20.6	49.5
3. August 8	73	85.5	74.8	45.5	78.3	67.7
4. August 11	77	47.7	44.5	14.7	53.4	58.8
5. August 17	81	31.3	52.5	10.7	20.4	20.0

[a] The specific activities of F–5 are not known. F–5 was eluted from the DEAE-cellulose with 0.25*M* alkali, and its specific activity could not be accurately determined because of losses of carbon-14 during dialyses.

The F-1 fractions have specific activities similar to those of the corresponding oxidized proteins. Furthermore, the glutamic acid samples isolated from them have specific activities similar to those of the glutamic acid samples from the corresponding oxidized proteins. An exception occurs in the last experiment. The specific activity of glutamic acid from F-1 shows a marked increase between the 76th and 81st days (Table II), indicating a preferential increase of carbon-14 incorporation as glutamic acid-C^{14} in the late stages of the plant's

development. Since F-1 is a mixture of proteins or polypeptides (2), it is possible that one of its fragments shows a rapid increase in carbon-14 incorporation just as the plant is reaching maturity.

Fraction-2 (F-2) is a minor component of the oxidized protein (2), and in these experiments sufficient quantities of it could not be isolated for degradative work.

The amount of carbon-14 incorporated into F-3 when tracer was given on the 63rd day after seeding is low compared with the other fractions on the same date. In addition, the glutamic acid from this run was weakly labeled and contained so little carbon-14 that the radioactivity determinations are of doubtful accuracy. Since the glutamic acid from the other fractions has a much higher specific activity (Table II) in experiments for tracer given on the 63rd day, it appears that the synthesis of F-3 may be relatively slow at this time in the plant's development. The results also suggest that the glutamic acid-C^{14} is used up fairly rapidly for incorporation into other fractions, so that it has been extensively diluted with nonradioactive glutamic acid by the time that incorporation into F-3 takes place. The amount of carbon-14 incorporated into F-3, as measured by the glutamic acid specific activity, increases to a maximum much more rapidly than with the other fractions, but this amount is always less than the average value (Fig. 2).

In fraction-4 (F-4) the specific activity of the glutamic acid passes through a minimum with plants labeled on about the 66th day. This low specific activity is not reflected in the protein specific activity (Table II). No ready explanation can be found for this observation,

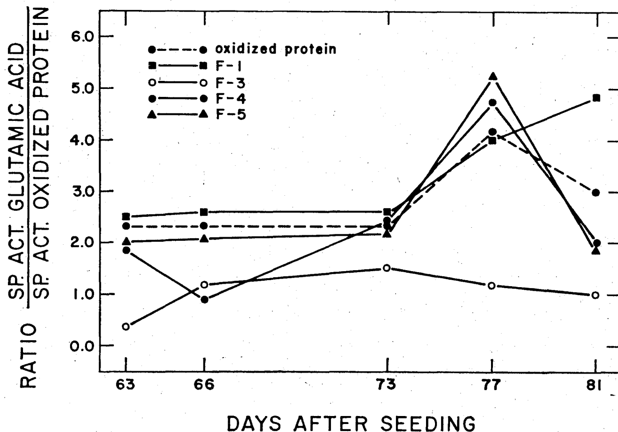

Fig. 2. Specific activity of glutamic acid/specific activity of oxidized protein plotted against days after seeding of the plants.

unless it shows that some other amino acid of very high specific activity is being incorporated into F-4 during this period. The rate of carbon-14 incorporation rapidly increases to a maximum when tracer was given on the 73rd day (Table II), and the relative rate of increase is greater with F-4 than with F-1. The specific activities of F-4 fractions and the glutamic acid isolated from them decline to a very low value by the 81st day, suggesting that by this time the incorporation of glutamic acid into F-4 is almost completed.

Fraction-5 (F-5), eluted from the DEAE-cellulose by 0.25*N* sodium hydroxide, is probably a mixture of polypeptides. The amount of incorporation of carbon-14 into F-5 as glutamic acid is greater at a time late in the plant's development relative to other labeled amino acids (Fig. 2).

The composition data (Table I) and the specific activities (Table II) show that the specific activities of the various protein fractions do not depend solely on the amounts of glutamic acid-C^{14} in them. For instance, fraction-4 (F-4) has the average amount of glutamic acid present in it and its specific activity in experiment 4 is one-quarter that in experiment 3 (73rd day). However, the specific activity of glutamic acid from No. 3 is less than twice that from No. 4. Hence, the other amino acids in F-4 must account for more of the carbon-14 in experiment 3 than they do in No. 4. Figure 2 shows the ratio of the specific activities of glutamic acid to the specific activities of the oxidized protein plotted against the time of injection of acetate-1-C^{14}. Although the time of maximum specific activity of both glutamic acid and proteins occurs for tracer injected on the 73rd day, the graph shows the above ratio to be a maximum for the 76th-day injection. Hence, the percent contribution of glutamic acid-C^{14} to the over-all specific activity of the protein is greater on the 76th-day experiments than it is on the 73rd day.

Fraction-1 (F-1) is known to be a mixture of polypeptides (2), and it must be considered probable that all fractions may be mixtures of similar proteins or peptides. Nevertheless, certain conclusions may be drawn from the results.

It appears that the relative rates of incorporation of carbon-14 into these protein fragments change during maturation of the plant. These changes may be shown by a comparison of the amounts of carbon-14 incorporation into the proteins. For instance, the ratio of the specific activities of the oxidized protein samples labeled by tracer given on the 63rd and 73rd days is $17.1/22.8 = 0.75$, while that for F-3 is 0.12 and that for F-4 is 0.18. There is much more rapid incorporation of

carbon-14 into F-3 and F-4 during this period than there is on the average or into F-1.

Each fraction, except for CM-3, contains cysteic acid (Table I). Hence, if the performic acid has oxidized intramolecular disulfide bonds in the gliadin complex of proteins, then the change in solubilities produced by the oxidation has permitted a chromatographic separation of some gliadin proteins, each having its own relative rate of formation during kernel formation and maturation. On the other hand, or possibly as well, the oxidation may have broken interpeptide disulfide bonds and produced polypeptide chains each with its own chemical properties and biosynthetic history. If this second suggestion is true, then one may conclude that the disulfide bond formation is one of the later steps in the formation of the kernel proteins since the carbon-14 results certainly suggest that the different parts of gliadin are synthesized at different rates, assuming that the disulfide bonds are stable to the isolation procedures (8), and there is no disulfide bond interchange (10).

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