STUDIES WITH RADIOACTIVE TRACERS
VII. Investigations with Flour Containing P^{32} [1]

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ABSTRACT

Phosphate-P^{32} was injected into the stems of maturing Thatcher wheat. The harvested kernels were milled and the P^{32} distribution in the milling products ascertained. Fractionation of the P^{32}-containing flour indicated assimilation of the injected activity into all fractions of the flour. Confirmatory data were obtained regarding earlier conclusions of other workers on the effectiveness of different solvents in extracting phospholipids and on the binding of lipids by the glutenin fraction of gluten.

The extensive literature on flour lipids and their role in baking has been reviewed recently by a number of authors (2,4,9). Of considerable interest is the binding of lipids to proteins and the involvement of phospholipids in the formation of the lipoprotein complex. Olcott and Mecham (11) have shown that making flour into dough or mere wetting caused the binding of a large portion of the originally free lipids, and particularly the phospholipids, of the flour. Fractionation studies further demonstrated that the bound lipids were associated with the glutenin rather than the gliadin fraction, thus suggesting that glutenin as it occurs in gluten may be a lipoprotein. More recently, X-ray evidence of Traub and co-workers (12) and X-ray and electron micrographic studies of Grosskreutz (5) have led to the suggestion that the lipoprotein complex of gluten may consist of protein fibers or platelets held together by layers of phospholipid molecules in the form of well-oriented bimolecular leaflets. Such interests in the phospholipids of flour have led us to undertake an investigation using flour into which radioactive phosphorus, P^{32}, has been incorporated.

Materials and Methods

Production of Flour Containing P^{32}. Wheat plants of the Thatcher variety were grown in the greenhouse. At various stages of growth, ranging from 10 to 40 days after the plants had headed out, a solution of carrier-free phosphoric acid-P^{32} (0.05 or 0.1 ml. initially containing 0.25 to 1.0 mc. of P^{32}) was injected into the stem of each plant. The technique of injection was the same as that described by McConnell and Ramachandran (6) in their work with C^{14}-labeled acetate. The plants were grown to maturity and harvested, and the kernels were

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milled with a mill designed by Geddes and Frisell (3) to provide active flour containing P^{32}.

Lipid Extraction. Dough samples were prepared each from 1.0 g. of active flour and 0.6 ml. of water containing 0, 20, or 40 p.p.m. potassium bromate, allowed to stand at 30°C. and 70% r.h. for 4 hr., and then freeze-dried and pulverized. Such dough samples as well as 1-g. samples of active flour were extracted in a Goldfisch extractor for 17 hr. with petroleum ether, diethyl ether, chloroform, or absolute ethanol. After removal of the solvent, the entire extract was used for P^{32} determination.

The extraction of flour with water-saturated 1-butanol was carried out by the procedure of Mecham and Mohammad (8). Either the entire extract or the chloroform-soluble portion was used for the P^{32} analysis.

Fractionation Methods. Two procedures for fractionation of the active flour were employed. Method I was essentially that described by McConnell and Ramachandran (6). The fractions assayed for P^{32} were lipid, albumin, water-soluble nonprotein (soluble in 5% potassium sulfate, not precipitated by T.C.A.), gluten, sodium hydroxide-soluble nonprotein (soluble in 0.02*N* NaOH, not precipitated by T.C.A.), and starch. Method II was the same as that used by Olcott and Mecham (11). The fractions of interest were glutenin, a middle fraction, and gliadin, as designated by these workers (11). Other fractions that were also assayed for P^{32} included starch and washing (after removal of the crude gluten), residual starch and insolubles (after dispersion of the crude gluten in 0.1*N* acetic acid), and solubles at pH 6.8 (after precipitation of the gliadin and middle fraction at pH 6.8).

Radioactivity Determination. The entire sample or an appropriate portion of the material to be assayed was covered with a solution of 10% calcium acetate and ashed in a muffle furnace at 600°C. overnight (1). The ash was dissolved in 2–3 ml. of 6*N* hydrochloric acid, quantitatively transferred to a plastic counting tube, made up with water to uniform volume of 5.0 ml., and then counted in a well-type scintillation counter designed for the counting of solutions of hard beta-emitting isotopes such as P^{32}. To correct for the decay of the P^{32} with time, a standard solution containing a fixed amount of P^{32} was counted before and after the counting of the sample. Since the standard decayed at the same rate as the sample, by setting the activity of the standard as 10,000 c.p.m. at an arbitrarily fixed zero time, if the activity of a given sample were y c.p.m. and the mean activity of the standard counted before and after the counting of the sample were,

say, 8,000 c.p.m., then the activity of the sample corrected to zero time would be (10,000/8,000)y c.p.m.

Determination of Total and Inorganic Phosphorus. Total phosphorus was measured by a colorimetric procedure given in *Cereal Laboratory Methods* (1). The same solution of a given ashed sample was used for P^{32} counting as well as for total phosphorus determination. The portion of the P^{32} present as inorganic phosphate in a given fraction of flour was estimated with the aid of added inactive phosphate as carrier. If the fraction studied was solid, it was thoroughly stirred for 20 min. with 10 ml. of water containing 200 mg. of sodium dihydrogen phosphate as carrier. The supernatant, separated by centrifugation, was then treated with 2.5% ammonium molybdate in 3*N* nitric acid to precipitate the phosphate as ammonium phosphomolybdate. The collected ammonium phosphomolybdate was dissolved in ammonium hydroxide and the solution counted to give the inorganic P^{32} content. For a liquid sample, such as the water-soluble nonprotein, 2.0 ml. of solution containing 200 mg. of sodium dihydrogen phosphate carrier were added to an appropriate aliquot of the sample and mixed thoroughly, and then inorganic phosphate was precipitated, redissolved, and counted as described above.

Results and Discussion

The results are discussed below under various subheadings. All tabulated data, with the exception of Table I, are mean values of duplicate experiments.

Incorporation of P^{32} into Maturing Wheat. Three crops of wheat were grown, harvested, and milled. The results are shown in Table I. Crops I and II indicated that of the radioactivity recovered in the milling products, bran contained the largest, and flour the least, proportions. The P^{32} injected into the plants grown for Crop II amounted

TABLE I

P^{32} CONTENTS IN MILLING PRODUCTS

Crop No. and Duration of P^{32} in Plants		Wt. of Kernels Milled	Wt. of Milling Product			Total Activity Recovered			Distribution of Recovered Activity		
			Bran	Shorts	Flour	Bran	Shorts	Flour	Bran	Shorts	Flour
	days	*g.*	*g.*	*g.*	*g.*	10^3 *cpm*	10^3 *cpm*	10^3 *cpm*	%	%	%
I	20	15.0	3.5	2.4	8.9	1,810	695	596	58.4	22.4	19.2
II	20	16.5	4.5	2.5	8.5	5,290	2,920	2,270	50.5	27.8	21.7
III-1[a]	20	40.0			24.0			1,200			
III-2[a]	15	48.0			27.0			1,060			
III-3[a]	10	32.4			20.0			948			
III-4[a]	5	38.0			23.5			112			

[a] The same amount of P^{32} was injected to each of these four groups of plants.

TABLE II

PERCENTAGE OF P^{32} IN EXTRACTS OF FLOUR FROM VARIOUS CROPS[a]

SOLVENT	CROP					
	I	II	III-1	III-2	III-3	III-4
Diethyl ether	1.5	1.3	1.5	1.4	1.3	1.3
Petroleum ether	2.3	1.9				
Chloroform	4.5	4.1				
Ethanol	5.7	6.2	5.6	5.5	5.6	5.5
Water-saturated 1-butanol	11.8	11.0 (7.9[b])				

[a] P^{32} contents in extracts expressed as % of the total P^{32} in the flour.
[b] Chloroform-soluble portion.

to 60.5×10^6 c.p.m. From the activities of the milling products derived from this crop (Table I), it can be seen that the recoveries in the bran, shorts, and flour were respectively, 8.7, 4.8, and 3.8% of the total injected. Crop III tested the effect of time of P^{32} administration on the degree of P^{32} incorporation. When the activity was injected between 5 and 20 days before the harvest, the results showed that the longer the duration of the P^{32} in the plants, the greater its incorporation into the resulting flour.

Studies on Lipids. It is well known that different "fat solvents" can extract different amounts of "lipids" from flour. The results given in Table II also show that different solvents will extract different amounts of P^{32} or phospholipids. It may also be noted that water-saturated 1-butanol is the most effective of the solvents studied for the extraction of phospholipids. The P^{32} content of the chloroform-soluble portion of the water-saturated 1-butanol extract, which should exclude any nonlipid phosphorus in this extract, is greater than the P^{32} content of the entire extract of any one of the other solvents. This is in agreement with the finding of Mason and Johnston (7), who had concluded that water-saturated 1-butanol is more effective than ethanol in removing lipids, particularly phospholipids.

Measurement of the percent by weight and percent P^{32} in the ether- and ethanol-extracted lipids of flour and doughs (Table III) confirmed the earlier observations of Olcott and Mecham (11) that doughing greatly reduced the amount of ether-extractable lipids. Ether-extractable radiophosphorus was also greatly reduced by doughing. No great effects were noticeable when bromate was included in the dough, again in agreement with a previous finding of Mecham and Weinstein (10). Ethanol apparently can break lipoprotein bonds, since making the flour into dough neither changes the total weight nor the P^{32} content of the ethanolic extract of flour or dough.

TABLE III

COMPARISON OF PERCENT BY WEIGHT AND PERCENT OF P^{32} IN ETHER AND ETHANOL EXTRACTS OF FLOUR AND DOUGHS

Solvent	Weight Experiments (Ordinary Flour)				P^{32} Experiments (Active Flour)			
		Dough (p.p.m. $KBrO_3$)				Dough (p.p.m. $KBrO_3$)		
	Flour	0	20	40	Flour	0	20	40
	%	%	%	%	%	%	%	%
Ether	1.39	0.53	0.49	0.46	1.3	0.4	0.4	0.3
Ethanol	3.00	3.33	3.22	3.02	6.2	6.2	6.1	6.0

TABLE IV

DISTRIBUTION OF P^{32} IN FLOUR FROM CROP I. FRACTIONS OBTAINED BY METHOD I

Lipid Solvent	P^{32} in Fraction [a]						
	Lipid	Albumin	Water-Soluble Nonprotein	Gluten	NaOH-Soluble Nonprotein	Starch	Total Recovery
	%	%	%	%	%	%	%
Ether	1.5	4.0	40.7	15.0	6.4	18.6	86.2
Petroleum ether	2.3	5.2	48.0	13.6	6.5	16.8	92.4
Chloroform	4.5	5.5	42.3	15.7	6.0	14.6	88.6
Ethanol	5.7	4.1	41.6	15.8	7.2	16.3	90.7

[a] Expressed as a percentage of the total P^{32} in the flour.

Distribution of P^{32} in Flour Fractions. Radiophosphorus was found in all fractions of the flour obtained by either of the two fractionation methods used in the present studies. Table IV shows the distribution of P^{32} in various fractions obtained by method I. Although different fat solvents extracted different amounts of P^{32}, the P^{32} in the other fractions from various defatted flours all showed a similar type of distribution, with the water-soluble nonprotein containing the largest amount of activity. With the exception of the gross differentiation between inorganic and organic P^{32}, which is to be discussed, no attempts were made to elucidate the chemical natures of the phosphorus compounds in the various fractions.

Table V shows the P^{32} distribution from a similar fractionation together with the total phosphorus of the fractions. The fact that the ratios of P^{32} to total phosphorus obtained in this experiment were of the same order of magnitude for all fractions indicated that the administered phosphate-P^{32} was incorporated more or less uniformly into all fractions of the flour.

From another set of fractionation studies, each fraction was separated into organic and inorganic P^{32}. The results are also included in Table V. It was expected that the water-soluble nonprotein would

TABLE V

COMPARISON OF P^{32} RECOVERY AND TOTAL PHOSPHORUS AND OF INORGANIC AND ORGANIC P^{32} IN FRACTIONS OF ETHER-EXTRACTED FLOUR FROM CROP II

FRACTION	P^{32} RECOVERY	TOTAL P	P^{32}/MG. P	INORGANIC P^{32} [a]	ORGANIC P^{32} [b]	INORGANIC P^{32}/ ORGANIC P^{32}
	%	*mg.*	%	%	%	
Albumin	4.2	0.07	60	0.8	3.2	0.25
Water-soluble nonprotein	38.6	0.52	73	9.8	34.0	0.29
Gluten	17.4	0.29	60	4.6	10.2	0.45
NaOH-soluble nonprotein	8.3	0.12	69	0.5	6.1	0.08
Starch	19.0	0.34	56	0.9	19.4	0.04

[a] Portion precipitated with added phosphate carrier as ammonium phosphomolybdate.
[b] Including both solid residue and supernatant after removal of phosphomolybdate.

contain most of the inorganic P^{32}, and this was actually observed. The ratio of inorganic P^{32} to organic P^{32} for this fraction, however, was not extraordinarily high. If the phosphate-P^{32} administered to the plants were simply transported to the kernels as phosphate, one would expect a very high proportion of inorganic P^{32} in the water-soluble nonprotein, which would result in a very high ratio of inorganic P^{32} to organic P^{32} in that fraction. Since this was not the case, much of the P^{32} incorporated into the kernel must have been converted from inorganic to organic forms during the process of assimilation by the plant.

Somewhat surprisingly, the ratio of inorganic P^{32} to organic P^{32} was found to be highest for the gluten fraction. Since the inactive phosphate carrier was added in the aqueous extract of the gluten and then precipitated as ammonium phosphomolybdate, it is possible that the aqueous extract of the gluten may have contained some active dispersed protein particles which were carried down with the phosphomolybdate precipitate, thus giving an erroneously high level of inorganic P^{32}.

The distribution of P^{32} in various fractions of the active flour obtained by method II is given in Table VI. Of the P^{32} found in the three protein fractions, by far the largest amount, over 90%, was present in the glutenin portion, although the amounts of glutenin and gliadin recovered were nearly equal (11). This is in agreement with the suggestion of Olcott and Mecham (11) that glutenin as it occurs in gluten may be a lipoprotein in which phospholipids play an important role in the binding between lipids and protein.

The Binding between Lipids and Proteins of Gluten. To demonstrate that lipid phosphorus can be bound by the glutenin fraction of gluten, the ether extract of 1.0 g. of active flour was added to 10.0 g.

TABLE VI
DISTRIBUTION OF P^{32} IN FRACTIONS OBTAINED BY METHOD II

Fraction	Active Flour from Crop III-1		Ordinary Flour Treated with Ether Extract of Crop III-1 Flour	
	P^{32} Recovery	P^{32} in Protein Fractions [a]	P^{32} Recovery	P^{32} in Protein Fractions [a]
	%	%	%	%
Starch and washing	63.6		60.8	
Residual starch and insolubles	4.7		12.4	
Solubles at pH 6.8	2.2		3.5	
Glutenin	16.6	91.7	18.8	95.0
Middle fraction	0.7	3.9	0.6	3.0
Gliadin	0.8	4.4	0.4	2.0
Total recovery	88.6		96.5	

[a] Sum of P^{32} contents in glutenin, middle fraction, and gliadin taken as 100%.

of ordinary flour, dried, and then fractionated by method II. The results are also given in Table VI. Although only about 20% of the added lipid-P^{32} was recoverable in the three protein fractions (glutenin, middle fraction, and gliadin), of the P^{32} recovered in these fractions, 95% was associated with the glutenin, indicating that this fraction of gluten is involved in the binding of phospholipids.

Saltlike Linkages between Lipids and Proteins. Mecham and Weinstein (10) have noted that the presence of salt decreased the binding of both total lipid and phospholipid when flour is made into dough. It was stated that these effects of salt suggest that the binding of protein and lipids may be partly ionic in nature. Glass (4) further pointed out that ionic or saltlike linkages would readily exist between phospholipids and protein, for example, between the trimethylammonium group of lecithin and a negatively charged group of a protein molecule. To investigate the possibility of ionic bindings between lipid phosphorus and flour protein, the glutenin fraction was prepared from 10.0 g. of active flour or 10.0 g. of ordinary flour that had been treated with the ether extract of 1.0 g. of active flour. If the lipid-P^{32} bound in the glutenin involved saltlike linkage, the lipid-P^{32} may be extractable from the glutenin by polar solvents such as water or a salt solution. The glutenin thus was extracted first by stirring for 10 min. with 10 ml. of water and then with 10 ml. of 10% sodium chloride solution. The P^{32} activities and total phosphorus in the two extracts and in the residue were measured. The results are tabulated in Table VII.

It can be seen that extractions with water and with sodium chloride solution together removed about 30% of the P^{32} activity from the glutenin of the active flour. These two extractions also removed

TABLE VII

P^{32} CONTENT AND TOTAL PHOSPHORUS IN AQUEOUS AND SALT EXTRACTS OF GLUTENIN

	Glutenin from Active Flour of Crop II			Glutenin from Ordinary Flour Treated with Ether Extract of Crop II Flour		
	P^{32} Content	Total P	Percent P^{32} per mg. Total P	P^{32} Content	Total P	Percent P^{32} per mg. Total P
	%	*mg.*	%	%	*mg.*	%
Aqueous extract	25.2	0.05	504	50.6	0.08	633
Sodium chloride extract	4.4	0.04	110	25.7	0.06	429
Residue	70.4	0.18	390	23.9	0.19	121

about 76% of the P^{32} activity in the glutenin of ordinary flour that had been treated with an ether extractive of active flour. In the latter experiment, the high ratio of percent P^{32} per mg. total P for the aqueous and salt extracts indicate that much of the P^{32}, originally added to the flour as lipid phosphorus and then bound to the glutenin fraction, can be removed preferentially by aqueous and salt extractions. These results suggest that at least a portion of the lipid phosphorus is loosely bound to glutenin and the binding may be saltlike or ionic in nature.

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