The Oxidation-Reduction Enzymes of Wheat. II. A Quantitative Investigation of the Dehydrogenases[1]

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ABSTRACT

Two hard red winter wheats, Triumph and Bison, and two hard red spring wheats, Lee and Selkirk, along with five milling fractions of each, were quantitatively assayed for dehydrogenases of glucose-6-phosphate, 6-phosphogluconate, malate, isocitrate, succinate, glutamate, lactate, and alcohol. Crude enzyme extracts were prepared in aqueous sucrose. Assays were conducted by conventional spectrophotometric methods and by densitometric tracings of reduced nitro-blue tetrazolium on polyacrylamide gels. Malate dehydrogenase showed no significant variation between winter and spring wheat and was 100 to 1,000 times more active than any other dehydrogenase studied. Glucose-6-phosphate, 6-phosphogluconate, isocitrate, and alcohol dehydrogenases were detected in significant amounts. Low levels of activity were detected for dehydrogenases of lactate, succinate, and glutamate. Generally, the spring wheats contained significantly higher dehydrogenase activity. High levels of activity were detected for many dehydrogenases in flour, particularly from spring wheat. Lee wheat contained more dehydrogenase activity than any of the other varieties.

A qualitative investigation of nine dehydrogenases in wheat seed and five milling fractions of four wheat varieties has been presented in a previous paper (1). A comparison was made of the isoenzyme patterns observed upon polyacrylamide disk electrophoresis of crude enzyme extracts. Wide variance was found among the various milling fractions, in both number and intensity of isoenzyme bands. In general, the flour contained fewer isoenzyme bands than the other fractions.

A literature review of dehydrogenases that have been detected in wheat was presented previously (1) and will not be repeated here.

It is the purpose of this paper to present the data of quantitative measurements of the activity of various dehydrogenases in extracts of whole wheat and five milling fractions of four wheat varieties previously described (1).

MATERIALS AND METHODS

Two quantitative methods of assay were used: 1) densitometric measurements of bands of enzyme activity on polyacrylamide disk electrophoresis gels and 2) conventional spectrophotometric measurements of enzyme activity in the crude extracts.

The methods of extraction and storage of crude enzyme extracts from the various wheat samples and of enzyme analysis on polyacrylamide gels was described earlier (1). The polyacrylamide gels were incubated with the substrate solution for a given dehydrogenase until readily observable bands

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were detected on the gels. The incubation time varied with the different substrates and milling fractions. Therefore, the values of activity levels as determined by the electrophoretic technique can be compared only between the same fractions of the four varieties and not among all fractions of a particular variety.

Quantitative data of the activity of the various dehydrogenases detected on polyacrylamide disk gels were obtained with a Joyce Chromoscan densitometer. The instrument is equipped with an automatic digital readout integrator which gives arbitrary numerical values that are directly proportional to the area under the peaks on the densitometer tracings of the polyacrylamide gels. Various interference filters may be used in the light path to obtain the desired wave length of light. For scanning the reduced nitro-blue tetrazolium bands of enzyme activity, a green filter with maximum transmittance at 532 mμ was used.

Conventional quantitative spectrophotometric data were obtained by recording changes in absorbance due to oxidation or reduction of pyridine nucleotide coenzymes at 340 mμ, with a Beckman Model B single-beam spectrophotometer with a 1-cm. light path. The 2.85-ml. reaction volume contained the components listed in Table I for each enzyme in addition to

TABLE I

SUBSTRATE SOLUTIONS FOR SPECTROPHOTOMETRIC ASSAY OF DEHYDROGENASES[a]

ENZYME	REFERENCE	SUBSTRATE AT pH 7.5	NAD, NADH, NADP, OR NADPH[b]
		μmoles	*μmoles*
Glucose-6-phosphate dehydrogenase	2	Sodium glucose-6-phosphate	5 NADP, 0.5
6-Phosphogluconate dehydrogenase	3	Sodium 6-phosphogluconate	5 NADP, 0.5
Malate dehydrogenase	4	Sodium oxalacetate	25 NADH, 0.5
Isocitrate dehydrogenase	5,6	Sodium isocitrate	20 NADP, 0.5
Glutamate dehydrogenase	7	Sodium glutamate	20 NAD, 0.5
Alcohol dehydrogenase	8	Ethanol	2500 NAD, 0.5
Lactate dehydrogenase	9	Pyruvic acid	45 NADH, 0.5

[a] Each solution contained 10 μmoles magnesium chloride and 170 μmoles tris-(hydroxymethyl) amino methane-hydrochloride in 2.85 ml. at pH 7.5 in addition to components listed.

[b] NAD = nicotinamide adenine dinucleotide; NADH = reduced nicotinamide adenine dinucleotide; NADP = nicotinamide adenine dinucleotide phosphate; NADPH = reduced nicotinamide adenine dinucleotide phosphate.

the crude extract (2–9). Enough crude extract was used to give an absorbance change of 0.002 to 0.008 O.D./min. The zero time of the reaction in each case was 15 sec. after addition of substrate, and the subsequent change in absorbance per 15-sec. interval was recorded. The enzymes were assayed as described below. For each of the enzymes investigated, the control consisted of the given reaction mixture with water in place of the particular substrate.

The data obtained from spectrophotometric assays were converted into units of enzyme per ml. of crude enzyme extract. One unit of enzyme was defined as that amount which catalyzes the conversion of 1 μmole of substrate to product per min. in a 1-cm. light path under the conditions of the assay. The protein content of all sample extracts was determined (10), and the specific activity of each enzyme was also calculated. The specific

activity was taken as the units of enzyme per mg. of protein in the crude enzyme extract.

Small differences would be expected in results obtained by the two quantitative methods of assay described above. In the case of the polyacrylamide disk technique, two factors are important: 1) there is substantial purification of the enzyme as gel electrophoresis separates it from loosely bound inhibitors and other components in the crude extract system that may antagonize the mode of action of the particular enzyme, and 2) there is an extremely high degree of concentration of enzyme in the polyacrylamide gel. The layer of sample applied to the gel was nearly 1 cm. thick and was concentrated to a few microns. These two factors are in contrast to the spectrophotometric method, in which all of the components of the crude system are present throughout the reaction mixture.

RESULTS AND DISCUSSION

In general, there was relatively good agreement between the units of enzyme per ml. of crude extract as determined by the spectrophotometric method and the numerical densitometer area of polyacrylamide gels as obtained in these investigations, with some differences in certain cases.

Dehydrogenases of the Pentose Shunt

Glucose-6-Phosphate Dehydrogenase. The level of activity of this enzyme was much higher in the spring wheats than in the winter wheats, as shown by the data presented in Table II. Only traces of activity were detected in the winter wheat flours, but significant levels of activity were measured in the spring wheat flours. The activities in the bran and shorts fractions of the winter wheats were low as compared to those of the spring wheat extracts. However, this was not so apparent in the red dog fraction.

An example of a densitometer tracing combined with a gel photograph of the activity in extracts of Lee whole wheat is shown in Fig. 1. Data of the same type for extracts of Lee flour are shown in Fig. 2. In Fig. 1 it is

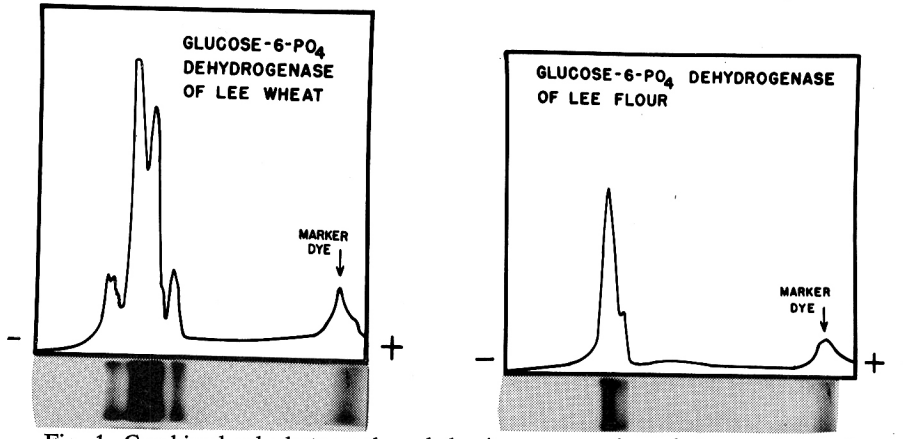

Fig. 1. Combined gel photograph and densitometer tracing of glucose-6-phosphate activity detected in an aqueous 12.5% sucrose extract of Lee whole wheat.

Fig. 2. Combined gel photograph and densitometer tracing of glucose-6-phosphate activity detected in aqueous 12.5% sucrose extract of Lee flour.

TABLE II

ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN MILLING FRACTIONS OF FOUR WHEAT VARITIES[a]

FRACTION	TRIUMPH WHEAT			BISON WHEAT			LEE WHEAT			SELKIRK WHEAT		
	Gel Area	S.A.[c,d]		Gel Area	S.A.		Gel Area	S.A.		Gel Area	S.A.	
		units[b]*/ml.*[c]	*units/mg.*		*units/ml.*	*units/mg.*		*units/ml.*	*units/mg.*		*units/ml.*	*units/mg.*
Whole wheat	68	59	6.2	52	64	9.3	233	183	14.9	172	128	14.9
Flour	1	trace	trace	4	trace	trace	85	64	12.0	34	29	11.0
Break shorts	0	46	4.7	4	44	5.8	250	1,820	148	114	1,090	65
Reduction shorts	29	292	26.4	28	109	13.5	277	1,100	120	164	914	78
Bran	10	18	1.8	16	45	3.7	259	730	98	168	490	61
Red dog	109	361	51	53	438	92	339	1,280	143	260	460	43

[a] Two quantitative methods were used: 1) conventional spectrophotometric assay and 2) integration of the area under the peaks of enzyme activity on a densitometer tracing of a polyacrylamide disk electrophoresis gel. Densitometer values of the activity can be compared only horizontally, because different substrate incubation times were required to detect activity in various fractions.

[b] One unit of enzyme = that amount which catalyzes the conversion of 1 μmole of substrate to product per min. in a 1-cm. light path under the conditions of the assay.

[c] The units of enzyme as measured by NADP reduction at 340 mμ were multiplied by 10^6 to give the values in this column.

[d] S.A. (Specific Activity) = units of enzyme/mg. of protein in the extract.

TABLE III

ACTIVITY OF 6-PHOSPHOGLUCONATE DEHYDROGENASE IN MILLING FRACTIONS OF FOUR WHEAT VARIETIES[a]

FRACTION	TRIUMPH WHEAT			BISON WHEAT			LEE WHEAT			SELKIRK WHEAT		
	Gel Area	S.A.[b,c]		Gel Area	S.A.		Gel Area	S.A.		Gel Area	S.A.	
		units/ml.[b]			*units/ml.*			*units/ml.*			*units/ml.*	
Whole wheat	45	29	3.0	42	22	3.2	162	80	6.5	111	51	6.0
Flour	0	0	0	0	0	0	141	37	6.8	65	18	6.7
Break shorts	36	137	13.9	46	183	24.0	167	511	41.5	175	482	28.7
Reduction shorts	56	146	13.2	63	183	22.8	124	365	39.8	96	219	18.7
Bran	40	37	3.7	61	91	7.4	174	274	37.0	149	220	27.0
Red dog	174	128	18.2	141	110	23.0	309	292	32.6	251	220	20.5

[a] Densitometer band area values of the activity can be compared only horizontally, because different substrate incubation times were required to detect activity in various fractions.

[b] The units of enzyme as measured by NADP reduction at 340 mμ were multiplied by 10^6 to give the values in this column.

[c] S.A. = Specific activity.

apparent that in sensitivity and reproduction, densitometer tracings are superior to the photographic process. The densitometer tracing indicates the level of activity not only in the whole sample but also in individual isoenzyme bands observed on the gel. As shown in the figures, most of the glucose-6-phosphate dehydrogenase activity was expressed by two isoenzyme forms.

6-Phosphogluconate Dehydrogenase. The activity of this enzyme in crude extracts of whole wheat was much higher in the spring wheats, as shown in Table III. No activity was detected in winter wheat flour, but high levels were detected in spring wheat flour. A comparison of specific activities shows that the specific activity in spring wheat was twice that in winter wheat. A striking observation is that the specific activity of spring wheat flour extracts was higher than that of the corresponding whole-wheat extracts.

As with glucose-6-phosphate dehydrogenase, the activity in the bran fraction was quite low in relation to the shorts and red dog fractions. High levels of activity were detected in the break shorts, reduction shorts, and red dog fractions, and no large seasonal variations were demonstrated by the data.

Dehydrogenases of the Citric Acid Cycle

Malate Dehydrogenase. An interesting feature of studies on this enzyme is that the levels of activity were extremely high as compared to other enzymes studied. This is shown by the data in Table IV. The level of activity was from 100 to 1,000 times higher than any other dehydrogenase that was investigated. The specific activity was higher in Bison than in Lee for all samples that were investigated, except bran, where a slightly higher activity was detected in Lee. As determined by the spectrophotometric assay, the specific activity was higher in the flour than in the whole wheat, except for Triumph, where the level of activity was extremely low. The remaining milling fractions of all varieties contained high levels of activity with no large differences.

Figure 3 shows an example of gel quantitative data of malate dehydrogenase in Bison flour extract. Most of the activity was located in just four

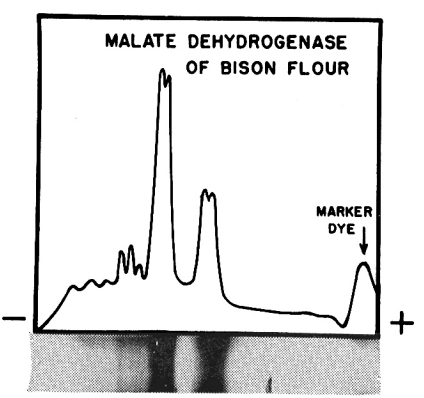

Fig. 3. Combined gel photograph and densitometer tracing of malate dehydrogenase activity in aqueous 12.5% sucrose extract of Bison flour.

TABLE IV

ACTIVITY OF MALATE DEHYDROGENASE IN MILLING FRACTIONS OF FOUR WHEAT VARIETIES[a]

FRACTION	TRIUMPH WHEAT			BISON WHEAT			LEE WHEAT			SELKIRK WHEAT		
	Gel Area		S.A.[b, c]	Gel Area		S.A.	Gel Area		S.A.	Gel Area		S.A.
		units/ml.[b]			*units/ml.*			*units/ml.*			*units/ml.*	
Whole wheat	418	20	2.1	418	37	5.3	407	60	4.8	391	55	6.4
Flour	157	6	0.6	525	18	5.8	260	29	5.5	300	29	10.8
Break shorts	225	92	9.3	363	113	14.8	425	128	10.4	269	122	7.3
Reduction shorts	417	113	10.2	400	111	13.7	395	109	11.9	310	119	10.0
Bran	828	92	9.3	865	100	8.2	605	82	11.0	525	82	10.2
Red dog	593	146	20.7	660	146	30.5	888	128	14.3	910	128	11.9

[a] Densitometer band area values of the activity can be compared only horizontally, because different substrate incubation times were required to detect activity in various fractions.

[b] The units of enzyme as measured by NADH oxidation at 340 mμ were multiplied by 10^3 to give the values in this column.

[c] S.A. = Specific activity.

TABLE V

ACTIVITY OF ISOCITRATE DEHYDROGENASE IN MILLING FRACTIONS OF FOUR WHEAT VARIETIES[a]

FRACTION	TRIUMPH WHEAT			BISON WHEAT			LEE WHEAT			SELKIRK WHEAT		
	Gel Area		S.A.[b, c]	Gel Area		S.A.	Gel Area		S.A.	Gel Area		S.A.
		units/ml.[b]			*units/ml.*			*units/ml.*			*units/ml.*	
Whole wheat	32	37	3.9	22	46	6.6	67	75	6.1	43	56	6.5
Flour	32	19	1.9	22	37	11.3	135	19	34.8	52	11	41.5
Break shorts	0	75	7.6	0	149	19.5	79	292	23.8	40	261	15.6
Reduction shorts	121	200	18.1	100	91	11.4	275	186	20.2	135	109	9.3
Bran	0	109	11.1	0	82	6.7	36	146	19.6	28	183	22.8
Red dog	38	183	26.0	0	73	15.3	248	328	36.5	28	146	13.6

[a] Densitometer band area values of the activity can be compared only horizontally, because different substrate incubation times were required to detect activity in various fractions.

[b] The units of enzyme as measured by NADP reduction at 340 mμ were multiplied by 10^6 to give the values in this column.

[c] S.A. = Specific activity.

molecular forms of the enzyme, even though a total of ten isoenzyme bands were detected on the polyacrylamide disk gels.

Isocitrate Dehydrogenase. The activity of isocitrate dehydrogenase per ml. of crude extract was roughly twice as high in extracts of whole spring wheat than winter wheat (Table V). However, when these values are expressed as specific activities it is observed that Bison, Lee, and Selkirk contained roughly the same specific activity values, about one and one half times as much as Triumph.

Spring wheat flours contained much more activity than winter wheat flours. Triumph flour was particularly low in activity of this enzyme as compared to Bison flour, even though they are both winter wheats. This is similar to the data obtained for malate dehydrogenase in these same two flours.

As shown in Table V, no activity could be detected in certain fractions of the winter wheats by the polyacrylamide gel method. This may have been because the activity in these same fractions from spring wheat was much higher and the reaction was quenched when the gels containing spring wheat samples had developed intense bands of activity. The reaction time was then the same for a given fraction of all four varieties. The level of activity in bran of spring wheat was about twice as high as in winter wheat. Such variation was not observed in break shorts, reduction shorts, and red dog fractions.

Succinate Dehydrogenase. No attempt was made to conduct spectrophotometric assays of succinate dehydrogenase, since many factors can affect the measurement of activity of this enzyme in a crude enzyme extract system (11,12). Data were obtained from polyacrylamide gel assays and these have been presented in Table VI. A relatively long substrate incubation

TABLE VI

RELATIVE TOTAL ACTIVITY OF SUCCINATE DEHYDROGENASE IN MILLING FRACTIONS OF FOUR WHEAT VARIETIES[a]

VARIETY	WHOLE WHEAT	FLOUR	BREAK SHORTS	REDUCTION SHORTS	BRAN	RED DOG
Triumph	8	0	28	42	40	23
Bison	trace	0	17	93	15	8
Lee	62	trace	108	239	118	165
Selkirk	22	0	60	263	76	80

[a]The activity is expressed as the integrated area under the peaks on densitometer tracings of the polyacrylamide disk gels. The activities can be compared only vertically for the four varieties, because different substrate incubation times were required to detect activity in various fractions.

time was required for the whole-wheat and flour fractions. Lee flour was the only flour fraction in which activity was detected, and then it was only an unmeasurable trace. In all other fractions, the activity was much higher in the spring wheat fractions. Generally, the spring wheats contained three to five times as much activity.

Other Dehydrogenases

Glutamate Dehydrogenase. The data of glutamate dehydrogenase assays have been presented in Table VII. Extracts of Bison and Lee whole wheats

TABLE VII

Activity of Glutamate Dehydrogenase in Milling Fractions of Four Wheat Varieties[a]

Fraction	Triumph Wheat			Bison Wheat			Lee Wheat			Selkirk Wheat		
	Gel Area		S.A.[b]	Gel Area		S.A.	Gel Area		S.A.	Gel Area		S.A.
		units/ml.[c]			*units/ml.*			*units/ml.*			*units/ml.*	
Whole wheat	31	90	9.3	52	179	25.9	41	358	29.1	38	90	10.4
Flour	70	0	0	51	0	0	33	179	33.5	49	0	0
Break shorts	40	90	9.1	81	121	15.8	77	448	36.4	35	358	21.3
Reduction shorts	89	90	8.1	56	300	37.3	16	628	68.3	22	448	38.1
Bran	46	179	18.2	32	224	18.2	15	538	71.9	30	448	55.6
Red dog	63	0	0	33	179	37.5	50	448	49.9	71	269	25.1

[a] Densitometer band area values of the activity can be compared only horizontally, because different substrate incubation times were required to detect activity in various fractions.

[b] S.A. = Specific activity.

[c] The units of enzyme as measured by NAD reduction at 340 mμ were multiplied by 10^7 to give the values in this column.

TABLE VIII

Activity of Alcohol Dehydrogenase in Mlling Fractions of Four Wheat Varieties[a]

Fraction	Triumph Wheat			Bison Wheat			Lee Wheat			Selkirk Wheat		
	Gel Area		S.A.[b]	Gel Area		S.A.	Gel Area		S.A.	Gel Area		S.A.
		units/ml.[c]			*units/ml.*			*units/ml.*			*units/ml.*	
Whole wheat	31	66	6.8	30	58	8.5	96	117	9.5	86	131	15.2
Flour	0	44	4.3	0	29	9.3	136	168	31.4	trace	95	35.2
Break shorts	0	7	0.8	0	29	3.8	135	777	62.3	0	146	8.7
Reduction shorts	0	15	1.3	0	7	0.9	141	904	98.2	94	548	46.6
Bran	0	66	6.7	0	22	1.8	27	73	9.8	11	51	6.4
Red dog	85	365	51.8	trace	128	26.7	237	16,050	1,789	23	212	19.8

[a] Densitometer band area values of the activity can be compared only horizontally, because different substrate incubation times were required to detect activity in various fractions.

[b] S.A. = Specific activity.

[c] The units of enzyme as measured by NAD reduction at 340 mμ were multiplied by 10^6 to give the values in this column.

had nearly equal specific activities, and their values were roughly three times as high as values of Triumph and Selkirk varieties.

An interesting point was observed when the flour fractions were assayed. Activity was detected in all flours by the polyacrylamide gel assay, but only in Lee flour by the spectrophotometric assay. These data would suggest that an inhibitor of this enzyme may be present in the extracts of flour from Triumph, Bison, and Selkirk that was separated from the enzyme upon electrophoresis. The same explanation may be offered in the case of Triumph red dog. Substantial activity was detected on polyacrylamide gels but none by the spectrophotometric method.

There were some differences in the data of the two quantitative methods for this enzyme. The polyacrylamide gel assays indicate a generally higher level of activity in winter-wheat fractions, but the spectrophotometric assays tend to show the opposite in a few cases. These can readily be observed in Table VII.

Alcohol Dehydrogenase. In extracts of whole wheat the activity of alcohol dehydrogenase was nearly twice as high in spring as in winter wheat (Table VIII). More pronounced were the differences in levels of activity in flour fractions where the spring wheats had from four to eight times more activity. The break shorts and reduction shorts fractions of the spring wheats contained extremely high levels as compared to the winter wheats. The differences with winter and spring varieties were not so pronounced in the case of bran and red dog, except Lee red dog, where the activity was almost 100 times that of the other red dog fractions.

On polyacrylamide gels no activity was detected under the conditions of the assay in extracts of flour, break shorts, reduction shorts, and bran of the winter wheats. If these gels were incubated with substrate for a long period, light bands appeared, but by then the bands representing activity in spring wheats had become too intense for quantitative data to be obtained.

It is somewhat surprising that such a high level of activity was detected for this enzyme in the Lee red dog fraction.

Lactate Dehydrogenase. In all samples, no activity was detected by spectrophotometric assays for this enzyme. Quantitative data were obtained by polyacrylamide gel assays. The difference in level of activity was greater between the two varieties of each seasonal group than between the two seasonal groups. Densitometer areas for whole wheat were Triumph, 22; Bison, 49; Lee, 67; and Selkirk, 32. No activity was detected in flour extracts of any varieties. Other milling fractions contained levels of activity that were roughly equal, except that the break shorts and red dog fractions of spring wheats were a little higher than extracts of winter wheat for these particular fractions.

GENERAL DISCUSSION

A few general statements can be made about the quantitative data presented. In general, levels of activity of dehydrogenases were higher in spring wheats than in winter wheats. This was especially pronounced in whole wheat and flour fractions and existed to a lesser degree in other milling fractions. The Lee variety contained significantly higher activities of most

enzymes that were investigated than any of the other varieties. A comparison of the specific activities of the enzymes studied indicated that glucose-6-phosphate, 6-phosphogluconate, isocitrate, and alcohol dehydrogenase were at about the same intermediate level of activity. Malate dehydrogenase was 1,000 times more active; glutamate dehydrogenase was about 10 times less active. No appreciable activity of lactate dehydrogenase was detected by spectrophotometric assay.

Significant enzyme activity was detected in the nonaleurone portion of wheat, namely, the endosperm and corresponding milling fraction, flour. Depending on the wheat milling process, various amounts of wheat germ may be present in the flour after milling. The extent to which this occurs can affect the enzyme content of the flour extensively. If these enzymes are important in doughs, doughmaking properties of a given flour could be altered because of the way in which it was milled.

It was observed that many enzymes for which spectrophotometric data were obtained have a higher specific activity in the flour fraction than in whole wheat. Examples are glutamate dehydrogenase, isocitrate dehydrogenase, malate dehydrogenase, 6-phosphogluconate dehydrogenase, and alcohol dehydrogenase. The high level of activity of these enzymes in the flour fraction of the spring wheats, in particular, suggests that such enzymes should not be ignored, because they may affect the properties of doughs from these flours. More information is required about these enzymes and the role that they may play in doughs during mixing.

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