QUANTITATIVE MICROSCOPIC EVALUATION OF ENDOSPERM BREAKDOWN IN CONDITIONED HARD RED WINTER WHEAT[1]

M. J. Wolf, W. F. Kwolek,[2] and J. R. McCarthy

ABSTRACT

The amount of free protein and free starch present in a flour was taken as an estimate of the degree of endosperm breakdown in wheats conditioned at various moistures and temperatures. Percentages of free protein, free starch, and endosperm were calculated (weight basis) from microscopic sizing data on flours. Free protein content of flours from tempered Wichita and Ponca wheats was ordinarily below 2% of flour weight. Free protein content was highest at low moisture levels (in dried wheat) regardless of holding temperature (3° to 70°C.). Tempered flour showed the same trend with respect to protein release as tempered wheat. Protein release in flour from immature wheat is significantly higher than at later stages. Only starch granules less than about 10 μ in diameter were free of protein. Free starch content of flours was independent of conditioning treatments. Highest free protein yields (about 4% of the flour) were noted under combined low-moisture and high-temperature treatment. A maximum of about 25% of the total protein, calculated on Kjeldahl nitrogen of the flour, was in the free form. Results suggest that highest free protein release may be expected by milling grain at around 16% moisture, where flour yield is optimal, then regrinding the flour after drying it to moisture levels below 10%.

Breakdown of endosperm after conditioning treatments of wheat is generally evaluated by air classification of the reground flours. The magnitude of the protein shift (1), estimated from Kjeldahl analyses of the air-classified fractions, is taken as an indication on the extent of protein release resulting from endosperm breakdown. This method is useful and convenient; however, it does not give a true picture of the amount of free protein present in flours, because the Kjeldahl nitrogen determination does not distinguish between particles of free

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[2]Biometrician, Biometrical Services, ARS, USDA, stationed at the Northern Laboratory.

protein and those still adhering to starch. Microscopic examination of high-protein and other air-classified fractions shows that much of the protein remains attached either to starch in small endosperm fragments or to individual starch granules (Figs. 1 and 2).

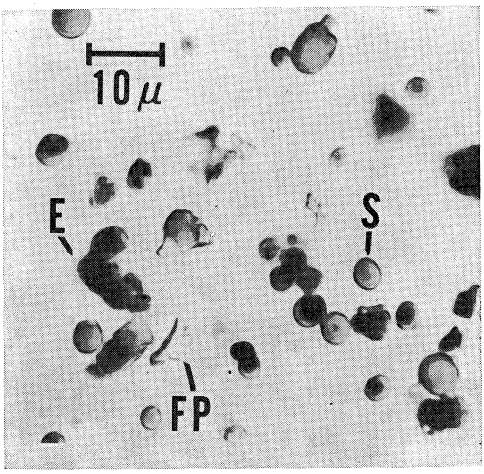

Fig. 1. Typical air-classified intermediate fraction of a HRW wheat showing free protein (FP), free starch (S), and endosperm particle (E). Stained with iodine vapor. ×1,000.

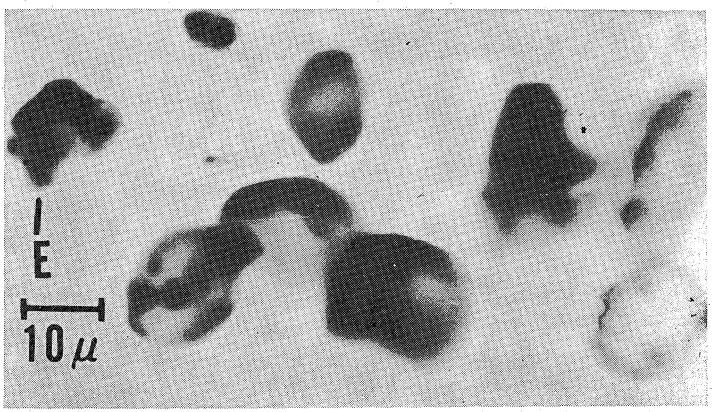

Fig. 2. Typical air-classified starch fraction of a HRW wheat, showing dark-staining interstitial protein adhering in patches to large starch granules. E, endosperm particle. Stained with iodine vapor. ×500.

A more dependable estimate of protein release can be obtained by repeated sedimentation from mixtures of carbon tetrachloride and benzene, a procedure similar to that of Hess (2). In our trials with

this sedimentation method, since more than eight successive sedimentations were required to approach quantitative recovery (unpublished data), the use of organic liquids did not appear suitable as a routine procedure for estimating protein release in flours.

Protein and starch are readily distinguishable microscopically in slightly hydrated flours stained with iodine vapor. Consequently, it appeared possible to estimate the amount of free protein and free starch in flours if each particle were not only sized but also identified as free protein, as free starch, or as an endosperm fragment made up of adhering starch and protein.

A relatively simple microscopic procedure was used to determine the amount of free starch and free protein in wheat flours. With this method, the effect of various conditioning treatments on endosperm breakdown was determined by estimating the amount of free starch and free protein released.

Methods and Materials

Two hard red winter (HRW) wheats were studied: Wichita, grown in Kansas in 1960, and Ponca, grown in Illinois in 1963. Analytical data on the two wheats are summarized in Table I on dry-weight basis. Only immature samples of Ponca were studied miscroscopically.

TABLE I
COMPOSITION OF WICHITA AND PONCA HRW WHEATS

VARIETY	DAYS AFTER FLOWERING	PROTEIN (N × 5.7)	ASH	STARCH
		%	%	%
Wichita	(Mature)	17.8	1.7	...
Ponca	15	11.2	2.8	48.4
	20	10.7	2.2	64.1
	27	11.8	2.1	64.9
	(Mature)	12.3	2.2	65.3

Wheat was conditioned in a glass cylinder 7.5 cm. i.d. and 14 cm. long. Approximately 100 g. of wheat was placed in the conditioner; the chamber was closed and the calculated amount of water was added through a valve. The chamber was slowly rotated for 22 hr. in a water bath at a constant temperature in the range 30° to 70°C. (±1°). For the run at 3°C., the wheat was tempered in a cryostat, and aliquots for moisture determination were ground directly in the cryostat with a micro Wiley mill. Moisture content of the conditioned wheat was determined on a small aliquot ground to pass 40-mesh. The remainder of the conditioned wheat was removed from the chamber and immediately milled in a Brabender Quadruplex mill. A small portion (2–3 g.)

of the flour from each sample was reground with a Pitchford selective particle size grinder equipped with a tungsten carbide chamber, balls, and a 400-mesh sieve. The percent moisture, Kjeldahl nitrogen, and ash were determined on all whole flours. Microscopic analyses were made on both the whole and reground flour samples.

Two-gram samples of whole flour (from Wichita wheat) were tempered by absorption or desorption of water vapor at 25°C. in air at various relative humidities. The flours were exposed to laboratory air or held over different saturated salt solutions in desiccators to yield a series of flours varying in equilibrium moisture content. Equilibrium moisture levels were approached in a few days, but all samples were held for 14 days under the respective conditions. Before microscopic analyses, whole flours were reground after treatment.

Whole wheat and flour were assayed for moisture by drying at 130°C. for 1 hr. (3). Protein-nitrogen and ash were determined as described in *Cereal Laboratory Methods* (4) (crude protein, micro-Kjeldahl method 46–13; ash, basic method, 08–03). Polarimetric starch was determined by the method of Earle and Milner (5).

Microscopic Analyses of Flours. Mounting media for flour should be inert, nonaqueous fluids that do not swell the protein. Dow-Corning silicones, available in a wide range of viscosities and refractive indices, meet requirements satisfactorily. Silicone No. 200 with a viscosity of 50 centistokes at 25°C. and a refractive index, $n_{546}^{25°C.}$ 1.4038, was used most frequently.

A small sample of flour was dispersed on a slide in the silicone with a spear-shaped dissecting needle. Loose clumps were broken up under the dissecting microscope. The sample on the slide can be stirred vigorously in the silicone oil with little possibility of breaking down endosperm fragments. Uniform particle distribution is not required; rather, the main concern is to separate loose aggregates. Tapping the cover glass lightly while observing particles under the microscope is also useful in detecting or separating loose clumps of flour.

The sample in silicone was then stained with iodine vapor by inverting it over an aqueous solution of iodine containing an excess of iodine crystals.

The microscope was equipped with a 4-mm. 0.95 N.A. apochromatic objective and 10× compensating eyepieces. An ocular scale, 5 mm. long, divided into 50 units, was calibrated with a stage micrometer; each unit measured 2.84 μ. Lighting was adjusted for Kohler illumination with an outside 100-watt light source.

A band the full width of the ocular scale (0.142 mm.) was scanned at random with the aid of the mechanical stage. Parallel sweeps were

made, care being taken to avoid overlapping. All individual particles in the path of the sweep within the width of the ocular scale were counted, identified, and measured.

Particles subtending less than 0.5 of an ocular unit were omitted. Less than 0.03% of the weight of the sample ordinarily falls in the size range, 0–0.5 unit; consequently, any error from neglecting these particles is small.

Each particle was identified from its iodine-staining and morphological characteristics as free starch, as free protein, or as an endosperm fragment made up of both starch and protein (Figs. 1 and 2).

The class interval in all frequency distributions was taken as the ocular scale unit, equivalent to 2.84 μ.

The largest particle in whole flours ranged as high as 180 μ in diameter; however, the top particle size was generally much smaller. Particle size in reground flours rarely exceeded 40 μ in diameter.

Particles were sized to the nearest unit midpoint by using the horizontal dimension of the particle as the diameter. With this procedure, a microscopist can size and identify 400 particles in about 20 min. with the aid of an assistant to tally the data. Additional time is required for computations.

Statistical Computations and Analyses

Statistical studies of microscopic data of flour particles were based on three sets of analyses. The first set (Table II) consisted of a sample from each of 25 flours subjected to various temperature and humidity conditions before milling. In the second set (Table III), three different whole and reground flours were used. Four samples were taken from each of the six flours shown in Table III. In the third set (Table IV), two examiners each sized two samples of particles from each of four prepared microscope slides. The flour in the third analysis was reground. Data resulting from the three sets served as the basis for statistical tests of significance and examination of sources of variation in microscopic analyses.

Estimation of Percent Free Protein. Percent free protein is estimated as the ratio of 100 times the protein volume divided by the total volume of the protein, starch, and endosperm particles. Since total volume of the pure starch particles was less than 5% for all samples, these particles were omitted from the calculations. Omission of the starch volume leads to a very slight increase in the estimate of free protein; assuming free starch at 5%, the increase is less than 0.3% when the percent free protein is 5%, and less than 0.05% when the free protein is 1%.

TABLE II

PERCENT FREE PROTEIN FROM PARTICLE VOLUME DETERMINATIONS OF WHOLE FLOURS FROM WICHITA WHEAT SUBJECTED TO VARIOUS TEMPERATURE AND MOISTURE CONDITIONS BEFORE MILLING

KERNEL TREATMENT		TYPE OF PARTICLE					FREE PROTEIN		
		Endosperm			Free Protein				
Temp.	Moist.	V_1[a]	V_2[b]	V_3[c]	$*V_1$[a]	V_4[d]	P_1[e]	P_2[f]	P_3[g]
°C.	%	vol. ($\times 10^{-4}$), μ^3			vol. ($\times 10^{-2}$), μ^3		%	%	%
3	16.2	442	258	267	258	285	0.58	1.09	1.06
	17.0	651	324	294	407	398	0.62	1.21	1.34
30	9.7	849	462	373	426	362	0.50	0.78	0.96
	11.7	154	146	169	333	188	2.12	1.27	1.10
	12.6	360	244	239	360	359	0.99	1.45	1.48
	13.1	717	391	366	336	232	0.47	0.59	0.63
	17.0	1,809	965	717	50	43	0.03	0.04	0.06
40	9.6	492	159	135	188	190	0.38	1.18	1.39
	12.9	51	48	51	102	146	1.96	2.95	2.78
	16.9	1,306	1,408	391	157	293	0.12	0.21	0.74
	20.4	2,598	2,069	1,173	584	218	0.22	0.11	0.19
50	9.0	1,180	2,333	216	665	666	0.56	2.85	2.99
	11.7	935	412	378	215	224	0.23	0.54	0.59
	13.6	2,278	1,970	1,201	307	466	0.13	0.24	0.39
	15.2	922	306	269	169	150	0.13	0.49	0.55
	17.1	1,348	880	674	136	116	0.10	0.13	0.17
60	9.0	421	185	192	464	531	1.09	2.79	2.69
	12.0	551	614	491	499	433	0.90	0.70	0.87
	13.6	1,991	804	426	133	141	0.07	0.18	0.33
	19.0	3,569	1,889	1,283	91	63	0.03	0.03	0.05
	20.7	4,641	4,309	2,825	76	54	0.02	0.01	0.02
70	8.0	98	101	121	633	428	6.07	4.07	3.42
	9.8	239	189	197	469	480	1.93	2.48	2.38
	11.4	193	205	201	163	142	0.84	0.69	0.70
	13.2	203	232	201	262	234	1.27	1.00	1.15

[a] See equation 1.
[b] See equation 2.
[c] See equation 3.
[d] See equation 4.
[e] $P_1 = (*V_1)/(V_1 + *V_1) \times 100$.
[f] $P_2 = (V_4)/(V_2 + V_4) \times 100$.
[g] $P_3 = (V_4)/(V_3 + V_4) \times 100$.

The volume of the endosperm particles or the protein particles can be obtained by computing

$$V = S \sum_{i=1}^{k} n_i d_i^3$$

where n_i is the frequency of occurrence of particles of diameter d_i, the midpoint of the class interval; k is the number of class intervals; and S is the shape and density factor.

When the frequencies of the particle diameters can be described by a statistical distribution, the estimated volume can be computed on the basis of this knowledge. If the log normal distribution is appro-

TABLE III

PERCENT FREE PROTEIN FROM PARTICLE VOLUME DETERMINATIONS BASED ON MICROSCOPIC MEASUREMENTS OF FLOURS; COMPARISON OF WHOLE AND REGROUND FLOURS FROM IMMATURE PONCA WHEATS TEMPERED AT 30°C.

WHEAT		TYPE OF PARTICLE					FREE PROTEIN[b]		
		Endosperm[b]			Free Protein[b]				
Age	Moist.[a]	V_1	V_2	V_3	$*V_1$	V_4	P_1	P_2	P_3
days[c]	%	*vol.* ($\times 10^{-4}$), μ^3			*vol.* ($\times 10^{-2}$), μ^3		%	%	%
					Whole flour				
15	9.6	2,673	2,644	3,692	1,126	965	0.42	0.36	0.26
		6,405	2,850	2,945	988	790	0.15	0.28	0.27
		9,041	2,998	2,658	1,064	970	0.12	0.32	0.36
		2,919	2,062	1,638	1,208	1,233	0.41	0.59	0.75
					Reground flour				
		442	479	521	2,265	1,278	4.87	2.60	2.39
		308	419	589	3,200	1,915	9.41	4.37	3.15
		411	505	549	967	880	2.30	1.71	1.58
		399	528	648	963	856	2.36	1.60	1.30
					Whole flour				
20	10.0	1,523	1,419	2.780	1,447	1,566	0.94	1.09	0.56
		7,341	2,781	2,523	526	535	0.07	0.19	0.21
		9,345	2,368	2,185	569	548	0.06	0.23	0.25
		3,803	2,791	3,084	963	979	0.25	0.35	0.32
					Reground flour				
		309	409	727	1,131	802	3.53	1.92	1.09
		302	394	432	872	788	2.81	1.96	1.79
		328	412	519	600	600	1.80	1.44	1.14
		650	973	1,535	717	747	1.09	0.76	0.48
					Whole flour				
27	11.3	8,772	3,430	3,802	1,626	1,776	0.18	0.52	0.46
		13,867	6,787	6,083	879	803	0.06	0.12	0.13
		19,457	9,110	7,971	892	912	0.05	0.10	0.11
		12,165	3,954	4,550	588	644	0.05	0.16	0.14
					Reground flour				
		686	720	1,084	480	452	0.69	0.62	0.42
		315	230	352	725	584	2.25	2.48	1.63
		330	387	548	625	576	1.86	1.47	1.04
		303	285	501	631	628	2.04	2.16	1.24

[a] Milled at moisture shown.
[b] See footnotes, Table II.
[c] Days after flowering.

priate, then volume can be computed as

$$V = SN\left[\text{antilog}\ (3m_g + 10.362\ s_g^2)\right]$$

where $N = \Sigma n_i$, the total number of particles,

$$m_g = \frac{\Sigma n_i \log d_i}{N}$$

TABLE IV

PERCENT FREE PROTEIN FROM PARTICLE VOLUME DETERMINATIONS BASED ON MICROSCOPIC MEASUREMENTS OF PONCA WHEAT FLOUR[a]

OPERATOR	TYPE OF PARTICLE					FREE PROTEIN[b]		
	Endosperm[b]			Free Protein[b]				
	V_1	V_2	V_3	*V_1	V_4	P_1	P_2	P_3
	vol. ($\times 10^{-4}$), μ^3			*vol.* ($\times 10^{-2}$), μ^3		%	%	%
1	216	166	155	383	336	1.74	1.98	2.12
	336	303	358	452	467	1.33	1.52	1.29
	186	167	179	482	501	2.53	2.91	2.72
	214	212	284	612	704	2.78	3.21	2.42
	144	128	187	592	581	3.95	4.34	3.01
	258	220	201	529	511	2.01	2.27	2.48
	394	213	432	426	387	1.07	1.78	0.89
	288	232	619	473	523	1.62	2.20	0.84
2	232	204	230	943	875	3.91	4.11	3.66
	961	1,371	2,335	498	487	0.52	0.35	0.21
	586	955	1,157	692	726	1.17	0.75	0.62
	482	595	752	497	428	1.02	0.71	0.57
	488	683	852	667	478	1.35	0.69	0.56
	278	282	508	273	267	0.97	0.94	0.52
	410	559	588	579	536	1.39	0.95	0.90
	180	177	241	300	272	1.64	1.51	1.12

[a] Tempered at 12.3% moisture, 30°C.; reground. Sample size, 350 particles.
[b] See footnotes, Table II.

and

$$s_g^2 = \sqrt{\frac{\Sigma n_i (\log d_i - m_g)^2}{N-1}}$$

Numerical values for m_g and s_g can be obtained either by computing, or graphically by plotting, the cumulative percent particles *vs.* diameter on log probability paper. The log of the particle diameter corresponding to the cumulative 50% point gives m_g; s_g is given by the log of the diameter at the 84% point minus m_g. The parameters of the log normal distribution provide a basis for comparing different flour types. The use of an underlying distribution to describe the observed count helps to smooth irregularities associated with sampling variation.

The volume for the endosperm particles is given in Tables II–IV according to three different estimating equations, namely:

$$V_1 = \Sigma n d_i^3 \quad (1)$$

$$V_2 = N \text{ antilog } (3m_g + 10.362\, s_g) \text{ (computed)} \quad (2)$$

$$V_3 = [\text{antilog } (3m_g + 10.362\, s_g^2)] \text{ (2-point graphical)} \quad (3)$$

For equation 3, the data were treated as though the particle diameters were classed as falling into three groups:

$$d_i \leq d_1$$
$$d_1 < d_i \leq d_2$$
$$d_i > d_2$$

The values selected for d_1 and d_2 were 2.5 and 10.5 or 1.5 and 7.5 units, respectively. The percent particles less than d_1 and less than d_2 were plotted on log probability, and graphical estimates of m_g and s_g were obtained. For estimating protein volume, use of the log normal distribution was unsatisfactory. In many instances the distribution was actually J-shaped; however, this factor may reflect the particular equal-sized class intervals used.

The gamma distribution,

$$f(x) = \frac{1}{\alpha!\beta^{\alpha+1}} x^{\alpha} e^{-x/\beta}$$

was used to fit the distribution of protein particles. The fitting was accomplished by selecting values for α at increments of 1/2 and by using $\alpha/(m+1)$ (the maximum likelihood estimate) for β, where m is the arithmetic mean particle diameter. Useful tables for the computations are given in Pearson and Hartley (6). A chi-square test of goodness-of-fit was computed for each α (7), and at least three chi-square values were determined for each sample. A second-degree polynomial was fitted to the chi-square values as a function of α, and the α associated with a minimum chi-square value was obtained. An example of this procedure is given by Kendall (8).

The minimum chi-square value was less than 3.84 for 22 of the 25 samples, 16 of the 16 samples for comparing operators, and 21 of the 24 samples of three flour types. The probability of a chi-square greater than 3.84 with 1 degree of freedom is 0.05. Although the gamma distribution gave excellent fits to the data in several instances, a larger number of class intervals would have been better.

The total volume of the protein particles was computed for each sample as the cubed diameters (V_1) and also, on the basis of the gamma distribution, as

$$V_4 = \frac{S(\alpha+3)(\alpha+2)Nm^3}{(\alpha+1)^2} \quad (4)$$

The percent free protein was obtained by combining volume V_1 to V_4 in three different ways; namely, using only the cubed diameters (V_1), or using the log normal (V_2) or graphical log normal (V_3) volume with the gamma distribution (V_4) volume. Estimates for these three experiments are given in Tables II–IV.

Tests of Sampling Consistency. There were eight distributions each

of endosperm and of protein particles for both microscopists (Table IV). The distributions for each microscopist were tested for homogeneity by chi-square tests. For the less-experienced microscopist the chi-square values were 115.9 and 38.7 with 42 and 14 degrees of freedom for the eight endosperm and eight protein distributions. The probability of larger chi-square values in each case is less than 0.01. For the experienced microscopist the respective chi-square values were 33.2 and 8.19 with 28 and 14 degrees of freedom. The probability of more extreme chi-square values is greater than 0.10 in both instances. The chi-square values indicate that the distributions for the less-experienced microscopist were heterogeneous. The particle distributions for the experienced microscopist were consistent with random sampling variation. This shows that despite the relative simplicity of the microscopic procedure, some skill in measurement and identification of flour particles must be acquired before the microscopist can obtain consistent results.

When these data are used to estimate percent free protein, sampling variation in the estimates can be expected to be considerably greater for the less-experienced microscopist.

Distributions from the study of whole and reground samples of the flours were also tested for heterogeneity. Four distributions were obtained for each of the endosperm and free protein particles from each of the six combinations of flour and whole or reground condition.

Distributions of endosperm particles from whole and reground flours were homogeneous, as were those of the protein particles from reground flours. The whole-flour protein particles showed statistically significant sampling variation (0.01 probability level) for two of the three flours.

Estimation of Precision. Analyses of the variation in estimates of the percent free protein were computed by using the logarithm of the percent free protein for each of the three experiments. Separate analyses were computed for percentages based on cubed diameters and on both the fitted and graphical log normal and gamma distributions. An estimate of the variance (variance is the standard deviation squared) was obtained for each of the three experiments after variation associated with imposed conditions was removed. Since these variances were essentially the same for the three experiments, they were pooled. The resulting standard deviations were 0.311, 0.264, and 0.255, each with 47 degrees of freedom for the respective log percent free protein based on cubed diameters, log normal and gamma, and the log normal graphical and gamma volume estimates.

A measure of the precision with which a mean is estimated is given

by $s/\sqrt{n}$ where s is the standard deviation and n is the number of samples of 400 particles used in estimating a mean percent free protein. If this measure of precision is designated P_m and the standard deviation s, as 0.301, then

$$\log P_m = s/\sqrt{n} = 0.301/\sqrt{n}$$

since s is in log units and

$$P_m = \text{antilog } 0.301/\sqrt{n}$$
$$= 2^{1/\sqrt{n}}$$

The factor P_m when multiplied by and divided into the mean percent free protein, because logarithms are used, determines upper and lower 68% limits for the mean (analogous to the mean plus and minus the standard error of the mean). If s is first multiplied by the proper t value before the antilog is taken, factors giving 95 or 99% confidence limits may be obtained.

If 4, 9, or 16 samples are taken of 400 particles each, the precision with which the mean is estimated would be given by factors of 1.41, 1.26, and 1.19, respectively, corresponding to precisions of approximately ±41, ±26, and ±19%.

To compare the percent free protein associated with two different methods or treatments, the ratio of the two means can be used, as it has the advantage that shape factors cancel (assuming that the method does not affect the shape factor). The ratio would be a measure of relative efficiency of two methods for yielding free protein and the precision of the estimate would be given by

$$P_r = \text{antilog } s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

when n_1 and n_2 are the number of samples from each treatment. If $n_1 = n_2 = n$ and $s = 0.301$

$$P_r = 2.67^{1/\sqrt{n}}$$

The respective 95% confidence limit factors for the mean and for the ratio of two mean percent free proteins with these data are given by

$$P_m = 4^{1/\sqrt{n}}$$

and

$$P_r = 7.13^{1/\sqrt{n}}$$

For example, if the ratio of two mean percent free proteins, each associated with a different treatment and each based on nine observations, was 3.5, the 95% limits (assuming s has more than 15 degrees of freedom) would be 3.5/1.92 and 3.5×1.92 or 1.82 to 6.72. Thus the prob-

ability would be 0.95 that the limits 1.8 to 6.7 include the true relative efficiency of the two treatments. If the confidence interval includes 1.00, the methods would not be significantly different at the 95% significance level.

Figure 3 shows a plot by experiment of the percent free protein by the cubing and log normal plus gamma procedures. The correlation coefficient for the two methods is 0.902. The cubing procedure tends to give slightly higher estimates, but the points are generally evenly scattered about the line for equal values by either procedure.

Figure 4 shows agreement of the graphic estimate of combined

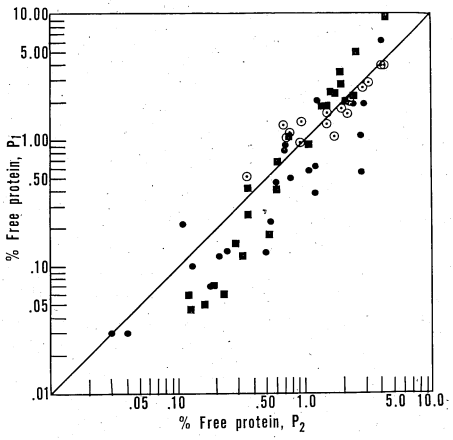

Fig. 3. Comparison of two methods for computing percent free protein. Symbols: solid circle, set 1; solid square, set 2; dot in circle, set 3.

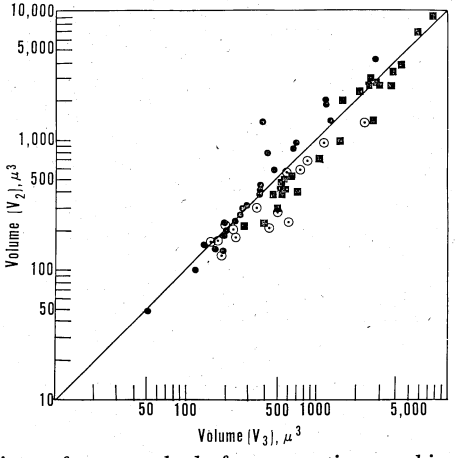

Fig. 4. Comparison of two methods for computing combined particle volume. Symbols, same as for Fig. 3.

particle volume as compared to an estimate based on a computed mean and variance. The agreement is good and the correlation between the two is 0.963.

The three equations for estimating the percent free protein all gave practically the same results. The estimate designated P_2 (see footnote, Table II is preferred, since it provides the best approximation to the underlying particle distributions. However, if adequate computing facilities are not available, estimate P_1 may be chosen, because the calculations are relatively simple.

Results and Discussion

Effect of Temperature and Moisture. In conditioning over the temperature range of 50° to 70°C., the highest free protein content occurred at low moisture levels, and the amount decreased sharply as the moisture level of the wheat increased (Tables II and V). Differences in protein release between high and low moisture levels were statistically significant.

In the range 3° to 40°C., the relation of protein release *vs.* moisture appears to be different from that in the higher range. At 30° and 40°C., there is a peak in protein release around 12% kernel moisture; the position of the peak shifts to lower tempering moistures as the temperature increases above 40°C. Tentatively, until further work is completed, it is assumed that the general pattern of protein release with change in moisture over the entire temperature range is the same as that described for the 50° to 70°C. range.

Tempering moisture levels influenced protein release. The slopes of moisture *vs.* log-free protein lines at the different temperatures were significantly (95% level) different from zero and were parallel, suggesting that the effect of moisture on endosperm breakdown was independent of temperature (Fig. 5). Since the lines were parallel, all data were adjusted to the same moisture level, and variation in percent free protein associated with the effects of temperature was found not significant. Although relatively high levels of protein release were noted when wheat was conditioned at 70°C. (Tables II and V), the values were not significant at the 95% level in comparison with free protein released at lower temperatures. The effect of relatively high conditioning temperature on endosperm breakdown, particularly in combination with low grain moisture, needs to be investigated more fully.

A special point is the observation that optimum flour yields occur at much higher tempering moistures than does high free-protein release. This result suggests that the highest free-protein recoveries can be

TABLE V

PERCENT ASH, MEAN PARTICLE DIAMETER, AND FREE PROTEIN IN WHOLE FLOURS; WICHITA, HRW

CONDITIONING TEMPERATURE	MOISTURE	ASH (d.b.)	MEAN PARTICLE DIAMETER[a]	FREE PROTEIN[b] P_2
°C.	%	%	μ	%
3	13.2	0.59	10.0	. .
	16.2	0.60	11.4	6.4
	17.0	0.58	16.0	7.0
30	9.7	0.77	11.4	4.7
	11.7	0.64	9.1	7.6
	12.6	0.66	11.4	8.5
	13.1	0.63	13.0	3.6
	17.0	0.68	15.8	0.24
40	9.6	0.74	10.1	7.5
	12.9	0.69	4.8	18.0
	16.9	0.80	14.1	1.3
	20.4	0.98	17.3	0.67
50	9.0	0.76	11.1	16.0
	11.7	0.67	13.5	3.1
	13.6	0.57	15.9	1.5
	15.2	0.65	10.0	3.2
	17.1	0.65	14.8	0.83
60	9.0	0.87	9.5	16.9
	12.0	0.64	12.9	4.1
	13.6	0.61	11.8	1.1
	19.0	0.67	18.3	0.21
	20.7	0.79	23.9	0.07
70	8.0	0.82	9.0	25.2
	9.8	0.71	. . .	14.3
	11.4	0.67	9.4	4.4
	13.2	0.66	9.4	6.1

[a] Number-average diameter, all particles.
[b] Percent free protein = $(P_2 \times 100)/(\%$ Kjeldahl protein).

expected when wheat is milled at relatively high tempering moistures (12–16%) followed by regrinding of the flour at moistures below 10%. Flours obtained by milling grain at low moisture levels are generally characterized by a relatively high ash content (Table V).

The mean flour particle diameter (all flour particle types) rises as the tempering moisture is increased (Table V). At the same time, the free-protein content drops. At 30° and 40°C. there is a dip in mean particle size which coincides with the peaks in free protein discussed above. At 70°C., however, the mean particle diameter remains essentially unchanged around 9 μ regardless of conditioning moisture level. Despite the relatively small difference in particle diameter at this temperature, there is a greater tendency for the endosperm to break along starch-protein interfaces at 8% moisture than at higher moisture levels.

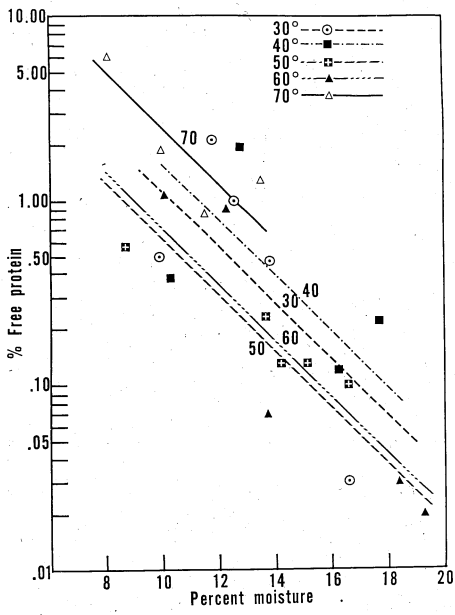

Fig. 5. Percent free protein (Σ d^3) *vs.* percent flour moisture and temperature. Symbols: dot in circle, 30°; solid square, 40°; cross in square, 50°; solid triangle, 60°; open triangle, 70°.

Relation of Kernel Maturity and Regrinding to Endosperm Breakdown. The effect of flour regrinding on protein release in mature wheats is well known. Tables III and VI show a significant increase in protein release at all kernel maturity levels in reground flours *vs.* the corresponding whole flours. Protein release in reground flours at 15 days after flowering is significantly higher than at 20 or 27 days; however, there is no further change beyond 20 days after flowering.

TABLE VI

FREE PROTEIN AS PERCENT OF KJELDAHL PROTEIN IN IMMATURE PONCA WHEAT

KERNEL		FLOUR		FREE PROTEIN [b]
Age [a]	Moist.	Type	Protein (N × 5.7)	
days	%		%	%
15	9.6	Whole	10.3	3.8
		Reground	...	25.0
20	10.0	Whole	10.0	4.6
		Reground	...	15.2
27	11.3	Whole	11.3	2.0
		Reground	...	14.9

[a] Time after flowering.
[b] See footnote *b*, Table V.

Similarly, there is only a minor change in starch and protein content with increasing maturity beyond 20 days after flowering (Table I).

Free-protein levels in whole flours are comparable to those in mature Wichita flours (Tables II and V) and show no significant change with maturity. Differences in composition of immature endosperm protein or in the physical state of starch and protein at early stages of kernel development may account for the greater ease of breakdown of immature endosperm.

Relation between Starch Granule Size and Endosperm Breakdown. Sullivan *et al.* (9) noted that a sharp drop in percent ash in hard wheat flour having a particle size or around 15 μ diameter was associated with an increase in free starch content. Their observation is in general agreement with the results noted here.

In both Ponca and Wichita varieties, the only starch granules free of adhering protein were those below 10 μ in diameter (Fig. 6). This condition in wheat is apparently related to the bimodal starch granule size frequency distribution (not shown in Fig. 6). The protein-free granules correspond in size to the small-granule fraction defined by the first peak of the distribution. The small-granule fraction of wheat starch arises in later stages of kernel development, after the large, lenticular granules have already been deposited (10). Possibly because of the later origin of the small starch granules, the bond with the surrounding protein is only partially formed or more easily broken, resulting in release of the small granules.

Although most wheat starch granules fall into the small size class,

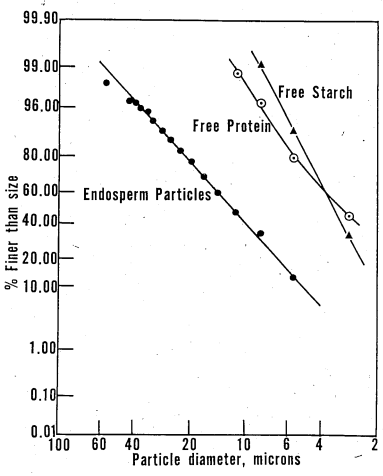

Fig. 6. Typical cumulative size distribution of the three basic types of particles; fractions plotted as percent of respective particle type; Wichita, HRW.

on a weight basis this fraction constitutes less than 3 or 4% of the total starch. Consequently, even if all of these granules were free of adhering protein, the total amount of small free-starch granules available would be low. The mean size of these granules (approx. 4 μ) is such that many of them find their way into the fine fraction on air classification of flours.

In general, the amount of free starch present in a flour is fairly constant and undergoes little change with variations in protein release resulting from different conditioning treatments. This situation suggests that the additional protein fragments are derived primarily from the protein attached to the larger starch granules. However, few of the latter granules are completely freed of protein, in the varieties studied, by any of the treatments used. Apparently only minor quantities of free protein are liberated along with the freeing of small starch granules; it appears, therefore, that most protein liberation is associated with breakdown of endosperm fragments involving large starch granules. Because the surface area of wheat starch granules above 10 μ diameter is greater than that below 10 μ (the ratio of the number of large to small granules in wheat endosperm is about 1:20), this fact further supports the idea that production of free starch and free protein is primarily a matter of breaking down the association of protein with large starch granules.

Tempered Flour. In tempered flours, the same relation was noted between flour moisture level and free-protein release as was observed in tempering the intact wheat kernel. Over the moisture range 10.7 to 17.9%, free protein dropped from 7.7 to 2.7%. Because of the extensive variability in the data, this difference was not statistically significant at the 95% level with a single analysis.

Free protein in tempered flour was relatively high compared with that in flours from tempered wheat; however, the highest value, 7.7%, was not significantly greater than the highest levels obtained when wheat grain was tempered at 30°, 40°, or 70°C.

A more extensive study of flour tempering is needed. Accessibility of moisture to flour particles, and possibly the better control of conditions, makes flour tempering attractive experimentally.

General Discussion

Data comparable to those reported here on the effect of various conditioning treatments on endosperm breakdown are not available for comparison. The range of conditions reported in the literature was generally too narrow for us to evaluate trends from protein-shift data.

Particles classified microscopically as protein in the course of our work are not equivalent to Kjeldahl protein. However, the error in treating the iodine-stained, yellow-brown particles as pure protein is not great. Starch-free wedge protein isolated by sedimentation contained about 92% protein (unpublished data). This value for the protein content of wedge protein is identical with that of Röhrlich and Niederauer (11) if their data are recalculated with a factor of 5.7 instead of 6.25.

Quantitative determination of starch granule breakage was not attempted in the course of our study; however, relatively few damaged starch granules were observed. Consequently, subcellular endosperm breakdown proceeds primarily along starch-protein interfaces and through interstitial protein. From microscopic appearance of flour particles it may be inferred that both paths are followed in varying degree, probably depending on the nature of the endosperm and on previous treatment.

Expressing free protein, determined microscopically, as a percentage of the total protein (by Kjeldahl N) of the flour, about 0.1 to 25% (Tables V and VI) of the protein occurred in the free state. Usually free protein constituted less than 8 to 10% of the total protein. Under ordinary conditions of pretreatment and milling, fracture of the endosperm appears to proceed almost always through the protein rather than along the existing starch-protein interfaces. Again, because increased production of free protein on drying or regrinding is not accompanied by a corresponding increase in free starch, further support is provided for the idea that subcellular reduction of endosperm occurs to a large extent by facture of the protein. Measuring the free starch surface area before and after regrinding, or other treatment, would be useful in studying the mode of endosperm breakage.

Interstitial protein appears to be the most labile component of the starch-protein complex of the endosperm. Its physical state is readily changed over a wide range by moisture. Absorption of small amounts of water increases the plasticity of the protein and renders it less subject to fragmentation; conversely, withdrawal of moisture makes the protein friable and increasingly susceptible to fragmentation.

Quantitative microscopic analyses provide an estimate of the amount of free protein available for separation. Actual recovery of free starch and free protein from treated flours, however, is a separate problem outside the scope of the present study.

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