[CONTRIBUTION FROM THE KANSAS AGRICULTURAL EXPERIMENT STATION.]

A PRELIMINARY STUDY ON THE CONDITIONS WHICH AFFECT THE ACTIVITY OF THE AMYLOLYTIC ENZYMES IN WHEAT FLOUR.

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The conditions for the production of sugars in the malting of barley have been carefully studied and are comparatively well known. That sugars are produced, in comparatively large amounts, when flour and water are mixed and allowed to digest at warm temperatures, is not so well known. Jago,[1] in determining the diastatic capacity of malt extract, found that flour alone when mixed with water and allowed to digest for four hours at 60° showed large diastatic activity.

Malt extract is used in bread baking for two reasons: (1) It contains large quantities of soluble carbohydrates, mostly in the form of maltose and glucose. (2) It also contains enzymes which produce soluble carbohydrates, probably mostly in the form of glucose and maltose. Both the soluble carbohydrates already present as well as those produced by amylolytic enzymes furnish food for the yeast in a very suitable form. If flour itself possesses such large diastatic activity that, by digesting it with water at a suitable temperature, more than one-fifth of its weight is transformed into soluble carbohydrates such as glucose and maltose, the conditions for such transformation deserve careful study.

The object of the present investigation was to determin some of the conditions which control the transformation of starch in wheat flour into reducing sugars. The conditions studied were: optimum temperature; duration of the digestion period; optimum proportion of water and flour; influence of chemicals acting as activators or paralysors.

In taking up this work a large number of preliminary trials were made in order to test the best methods of procedure. The results given in the following tables are repetitions of preliminary trials.

The following flour grades were used: patent, 80%; straight, 96 1/2%; clear, 16 1/2%; and low-grade, 3 1/2%. These were all made from the same lot of Kansas hard wheat.

The straight is simply a blend of the patent and the clear. The low-grade comes from the mill streams near the tail end of the mill. It contains a much higher percentage of ash and crude fiber than the other flours. The various charges used were based on the content of dry matter. In adding water, that present in the flour was figured in, so as to insure exact uniformity of conditions with respect to the proportion of dry substance and water.

The chemical analyses of these flours were as follows:

[1] "The Technology of Bread Making," by William Jago and William C. Jago.

Flour No.	Moisture.	Ash.	Protein.	Acidity. 0.05 N Cc. NaOH for 10-gram charge.
Patent	11.03	0.47	11.81	2.13
Straight	11.16	0.51	12.02	2.60
Clear	10.45	0.88	13.83	5.17
Low-grade	10.54	1.38	17.17	9.40

The digestion tank was a large galvanized iron tank, 75 x 75 x 38 cm. Inside this was placed a copper tank 65 x 35 x 18 cm. These were tanks which happened to be in the laboratory, and this double arrangement made it possible to keep a fairly constant temperature. The heating was done by means of gas burners.

The flour charges were weighed out in wide mouth, 300 cc. glass stoppered bottles and allowed to stand in the tank for 15 minutes before the water was added. That the flour acquired the desired temperature in this time was determined by inserting a thermometer. It was also determined in the preliminary trials that this digestion, dry, produced no appreciable amount of reducing sugars. Before adding the water it was brought to the same temperature as the flour. When chemicals were used they were dissolved in this water.

The amount of reducing sugars, figured as glucose and maltose, which were produced under a given set of conditions was determined as follows: At the end of the digestion period 150 cc. of a dilute solution of sulfuric acid were added to the 300 cc. bottle. The concentration was such that when added to the water in the bottle the whole would make a 0.02 N solution of sulfuric acid. That this strength of acid stops further action was proved in several preliminary trials and is brought out by the figures in Table IV. The same figures also show that the hydrolytic action of the acid of this strength is so slight that the comparative results are not affected. After cooling to room temperature, filtering was done on a 24 cm. folded filter. A clear filtrate at this point is not secured nor is it necessary. 25 cc. of this filtrate, calculated to contain the extract from one gram of dry flour, were placed in a 100 cc. volume flask containing 5 cc. of a 10% solution of phosphotungstic acid; and then 25 cc. of 4% solution of sodium chloride were added and the whole made up to volume with distilled water. The use of phosphotungstic acid and sodium chloride at this point makes it possible to remove the proteins and suspended starch and a clear filtrate and distinct end point in the final titration is the result. Blank trials showed that these reagents do not interfere with the determination; and inasmuch as the same method was used throughout, the results are strictly comparable. As soon as the precipitate had settled, the supernatant liquid was filtered on 15 cm. folded filters and 50 cc. of the clear filtrate, containing the extract from half a gram dry flour, was added to 250 cc. volume flask which contained 50 cc. of the mixed Fehling solution, Soxhlet's modification. The whole was heated to boiling and boiled for exactly two minutes, after which it was cooled and made up to volume. After the cuprous oxide had settled filtering was done on double $12^1/_2$ cm. S & S blue ribbon filters. To 125 cc. of this filtrate 2 cc. of concentrated sulfuric acid were added, then 10 cc. of a solution of potassium iodide, made by dissolving 50 grams of the salt in 100 cc. of water. The liberated iodine was determined by titrating against a sodium thiosulfate solution of such strength that 1 cc. was equivalent to 0.005 gram of copper.

The determination of the residual copper, rather than that which is reduced as is usually done, results in a great saving of time and labor. The principles underlying this method are found in articles published in THIS JOURNAL by A. W. Peters.[1] The method as used here was tested in our laboratory by working with pure glucose.

The number of cc. of thiosulfate used in this titration is subtracted from the cc. used in a blank. The difference gives the equivalent of the copper reduced by one-half the charge. The readings in cc. are converted into the equivalent of copper by multiplying by the copper factor for the thiosulfate and this again by two to get the equivalent of the whole charge which was added to the Fehling solution. The percentages in glucose and anhydrous maltose are obtained from the tables, page 243, Bulletin 107, Revised, Bureau of Chemistry. In the following tables the weights

TABLE I.—OPTIMUM TEMPERATURE FOR PRODUCTION OF THE MAXIMUM AMOUNT OF REDUCING SUGAR.

	1 hr. at 20°.			1 hr. at 40°.		
		Sugar calculated as:			Sugar calculated as:	
Flour No.	Wt. of Cu reduced equivalent to 0.5 gm. flour. Gm.	Glucose. Per cent.	Maltose. Per cent.	Wt. of Cu reduced equivalent to 0.5 gm. flour. Gm.	Glucose. Per cent.	Maltose. Per cent.
Patent	0.0135	1.26	2.02	0.0280	2.66	4.58
Straight	0.0120	1.10	1.74	0.0272	2.59	4.46
Clear	0.0180	1.68	2.79	0.0332	3.15	5.52
Low-grade	0.0157	1.47	2.39	0.0307	2.92	5.07
	1 hr. at 60°.			1 hr. at 62.5°.		
Patent	0.1855	18.63	32.70	0.2250	22.87	39.74
Straight	0.1858	18.67	32.76	0.2165	21.96	38.22
Clear	0.1797	18.01	31.67	0.2305	23.46	40.69
Low-grade	0.1600	15.95	28.14	0.2022	20.42	35.66
	1 hr. at 65°.			1 hr. at 67.5°.		
Patent	0.2313	23.55	40.84	0.1852	18.60	32.64
Straight	0.2300	23.41	40.60	0.1705	17.06	30.02
Clear	0.2312	23.54	40.82	0.2141	21.68	37.78
Low-grade	0.2095	21.19	36.96	0.1693	16.92	29.78

[1] THIS JOURNAL, 34, 422–454, 928–954.

of the copper reduced are also given, as this weight remains the same whether the reducing substances are calculated as glucose, maltose or as some other sugar.

The figures in Table I show the effect of temperature on the amount of reducing sugars produced. The four flours were digested for one hour at the temperatures given. The greatest increase occurs between 40° and 60°. The increase is much greater between 60° and 62.5° than between 62.5° and 65°. As at 67.5° there is a decided drop in the amount, 65° is apparently very near the optimum temperature for the maximum activity of the amylolytic enzymes in wheat flour. The amount of transformation is remarkable. Digesting flour with water in the proportion of 1 to 10 for one hour results in transforming nearly one-fourth of the weight of flour, figured as glucose, or over two-fifths figured as maltose, into such soluble carbohydrates as reduce Fehling's solution. The method of determination used takes no account of non-reducing carbohydrates present such as sucrose or soluble starch.

TABLE II.—EFFECT OF THE DURATION OF THE DIGESTION PERIOD ON THE AMOUNT OF REDUCING SUGARS.

	1 hr. at 62.5°.			2 hrs. at 62.5°.		
		Sugar calculated as:			Sugar calculated as:	
Flour No.	Wt. of Cu reduced equivalent to 0.5 gm. flour. Gm.	Glucose. Per cent.	Maltose. Per cent.	Wt. of Cu reduced equivalent to 0.5 gm. flour. Gm.	Glucose. Per cent.	Maltose. Per cent.
Patent 80%	0.2250	22.87	39.74	0.2437	24.91	43.91
Straight 96½%	0.2165	21.96	38.22	0.2417	24.69	42.70
Clear 16⅓%	0.2305	23.46	40.69	0.2440	24.95	43.10
Low-grade 3½%	0.2022	20.42	35.66	0.2140	21.67	37.76
	4 hrs. at 62.5°.			6 hrs. at 62.5°.		
Patent 80%	0.2582	26.51	45.62	0.2552	26.19	45.12
Straight 96½%	0.2540	26.06	44.90	0.2565	26.34	45.34
Clear 16⅓%	0.2500	25.60	44.18	0.2535	25.99	44.80
Low-grade 3½%	0.2190	22.22	38.65	0.2175	22.07	38.40

For this trial the temperature 62.5° was used. The figures in Table I show that near this point a small fluctuation in temperature will effect the results but little.

By far the greatest amount of reducing sugar is produced during the first hour. The increase during the second hour was much greater than during the two hours following the second. After four hours there is a small decrease in two flours and a small increase in two. These small changes are within the limits of the analytical error. That no further transformation appears after four hours is due either to the fact that the action stops entirely after this time or, what is more probable, the action is reversible to such an extent that the results are neutralized. In four hours' time the maximum amount of reducing sugars is produced. But in the three hours beyond the first, there is a comparatively small increase as compared with the first hour and this increase gradually diminishes as the time of digestion increases.

TABLE III.—THE OPTIMUM PROPORTION OF FLOUR AND WATER.

1 hr. at 65°.

Flour No.	1:1. Wt. of Cu reduced equivalent to 0.5 gm. flour. Gm.	1:1. Sugar calculated as: Glucose. Per cent.	1:1. Sugar calculated as: Maltose. Per cent.	1:3. Wt. of Cu reduced equivalent to 0.5 gm. flour. Gm.	1:3. Sugar calculated as: Glucose. Per cent.	1:3. Sugar calculated as: Maltose. Per cent.
Patent	0.0860	8.38	14.94	..	...	...
Straight	0.0782	7.60	13.56	0.2210	22.44	39.00
Clear	0.0957	9.36	16.67	..	...	...
Low-grade	0.0832	8.09	14.44	0.1937	19.51	34.16
	1:4.			1:5.		
Patent	..	...	...	0.2295	23.35	40.52
Straight	0.2382	24.31	42.08	0.2360	24.07	41.67
Clear	..	...	...	0.2325	23.70	41.06
Low-grade	0.2027	20.46	35.75	0.2015	20.35	35.54
	1:10.			1:15.		
Patent	0.2313	23.55	40.84	..	...	...
Straight	0.2300	23.41	40.60	0.2015	20.35	35.54
Clear	0.2312	23.54	40.82	..	...	...
Low-grade	0.2095	21.19	36.96	0.1795	17.99	31.64
	1:50.			1:100.		
Patent	0.1190	11.72	20.84	0.0280	5.32	9.16
Straight	0.1097	10.76	19.17	0.0267	5.06	8.76
Clear	0.1377	13.64	24.16	0.0312	5.94	10.32
Low-grade	0.1092	10.71	19.08	0.0230	4.34	7.40

To determin what proportion of flour and water will produce the maximum amount of reducing sugar the following proportions were digested for one hour: 1 : 1, 1 : 3, 1 : 4, 1 : 5, 1 : 10, 1 : 15, 1 : 50, 1 : 100. The results are given in Table III. The optimum proportion as measured by the amount of reducing sugars produced is 1 : 4. From this point the decrease is in both directions. The amount of reducing sugars obtained when the proportion 1 : 10 was used was very little less than from the proportion 1 : 4. Apparently between these two limits the proportion in which flour and water are mixed has very little influence on the amount produced, but above and below these proportions the change is very marked.

The Effect of Chemicals on the Production of Reducing Sugars.

The following chemicals were tried: sulfuric acid, sodium hydroxide, sodium chloride and "Merck's" dibasic potassium phosphate. Because but little difference had appeared in the previous trials between the patent, straight, clear, and low-grade flours only the straight and the low-grade were used in the subsequent work. The chemicals were made up to such concentrations that one solution was progressively twice the strength of the next weaker. In each table the results obtained by digesting with water in the proportion of 1 : 10 are inserted for comparison.

TABLE IV.—EFFECT OF DIGESTING WITH SULFURIC ACID AT DIFFERENT CONCENTRATIONS FOR ONE HOUR AT 65° C.

	100 cc. 0.00125 N H_2SO_4.			100 cc. 0.0025 N H_2SO_4.		
		Sugar calculated as:			Sugar calculated as:	
Flour No.	Wt. of Cu reduced equivalent to 0.5 gm. flour. Gm.	Glucose. Per cent.	Maltose. Per cent.	Wt. of Cu reduced equivalent to 0.5 gm. flour. Gm.	Glucose. Per cent.	Maltose. Per cent.
Straight	0.2245	22.82	39.64	0.1632	16.28	28.72
Low-grade	0.2110	21.36	37.22	0.2065	20.86	36.44
	100 cc. 0.005 N H_2SO_4.			100 cc. 0.01 N H_2SO_4.		
Straight	0.0052	0.45	0.54	0.0045	0.39	0.43
Low-grade	0.1885	18.94	33.24	0.0125	1.15	1.84
	100 cc. 0.02 N H_2SO_4.			100 cc. distilled H_2O.		
Straight	0.0107	0.98	1.50	0.2300	23.41	40.60
Low-grade	0.0067	0.59	0.83	0.2095	21.19	36.96

The sulfuric acid used had the following normalities: 0.02, 0.01, 0.005, 0.0025 and 0.00125. Sulfuric acid of normality 0.00125 apparently has a small inhibiting effect on the production of reducing sugar in the straight flour, but a small activating effect on the low-grade. The next concentration, 0.0025 N, has a very marked inhibiting effect on the straight flour, but apparently none on the low-grade. The greatest difference between the two flours appears when the normality 0.005 was used. This concentration of sulfuric acid produces complete inhibition in the straight flour, but only a small retarding effect on the low-grade. Complete inhibition in the low-grade did not take place until the concentration was 0.02 N, but this strength of acid seems to produce a small hydrolyzing effect on the starch in the straight flour.

The low-grade flour has a much higher ash and protein content than the straight flour. Whether this retardation was due to the higher ash content, or to the higher and the different nature of the protein, or both, will remain for further investigation to show. The large differences between these two flours indicate a fruitful field of investigation.

TABLE V.—EFFECT OF DIGESTING WITH SODIUM HYDROXIDE OF DIFFERENT CONCENTRATIONS.

1 hr. at 65° C.

Flour No.	Wt. of Cu reduced equivalent to 0.5 gm. flour. Gm.	Sugar calculated as: Glucose Per cent.	Sugar calculated as: Maltose. Per cent.	Wt. of Cu reduced equivalent to 0.5 gm. flour. Gm.	Sugar calculated as: Glucose. Per cent.	Sugar calculated as: Maltose. Per cent.
	100 cc. 0.00125 N NaOH.			100 cc. 0.0025 N NaOH.		
Straight	0.1500	14.99	26.48	0.0070	0.63	0.88
Low-grade	0.1610	16.04	28.32	0.0880	8.60	15.32
	100 cc. 0.005 N NaOH.			100 cc 0.01 N NaOH.		
Straight	0.0050	0.43	0.51	0.0035	0.29	....
Low-grade	0.0120	1.10	1.74	0.0067	0.59	0.83
	100 cc. 0.02 N NaOH.			100 cc. distilled H_2O.		
Straight	0.0022	0.17	....	0.2300	23.41	40.60
Low-grade	0.0075	0.67	0.98	0.2095	21.19	36.96

Sodium hydroxide was used in the same normalities as the sulfuric acid. The paralyzing effect of sodium hydroxide is more marked than that of sulfuric acid. Sodium hydroxide, 0.00125 N, shows marked inhibiting effect. A concentration of 0.0025 N produces practically complete inhibition in the straight flour and the reducing sugar formed in the low-grade is less than half the amount when pure water was used.

A comparison of the results in Tables IV and V shows that the amylolytic enzymes present in the flour tolerate a greater concentration of the hydrogen ions than of the hydroxyl ions and that with the low-grade flour with its larger ash and protein content the toleration is greater toward the concentration of both these ions than in the straight flour.

A comparison between the amount of sodium hydroxide used in determining the acidity value of flour according to the method usually employed, and the amount of sodium hydroxide which here produces a paralyzing effect on the amylolytic enzymes is interesting. The extract from 10 grams of low-grade flour requires 9.4 cc. of a 0.05 N solution of sodium hydroxide to produce a pink color with phenolphthalein. The same amount of extract from 10 grams of straight flour requires 2.6 cc. This is equivalent to 0.0188 gram sodium hydroxide for the low-grade flour and 0.0052 gram for the straight. Turning to the figures in Table V, the 100 cc. of 0.0025 N solution of sodium hydroxide contain 0.01 gram of sodium hydroxide and the 100 cc. of the 0.005 N, 0.02 grams. The 0.01 gram of sodium hydroxide or a little more than the amount required to produce a neutral reaction in the water extracts used in the acidity determinations is sufficient to produce complete inhibition in the action of the amylolytic enzymes of the straight flour. Again the 0.02 gram sodium hydroxide or only a little more than the amount used in the acidity determination produces complete inhibition in the low-grade flour. This discussion shows a possible reason why the low-grade flour tolerates a larger amount of the hydroxyl ion than the straight flour. The same line of reasoning will probably explain the difference between the two flours in regard to the toleration of the hydrogen ion. The larger ash and protein content of the low-grade flour will neutralize the effect of a greater concentration of the hydrogen ion.

TABLE VI.—EFFECT OF DIGESTING WITH DIFFERENT AMOUNTS OF "MERCK'S" DIBASIC POTASSIUM PHOSPHATE.

1 hr. at 65° C.

	100 cc. M/800 K_2HPO_4.			100 cc. M/400 K_2HPO_4.		
		Sugar calculated as:			Sugar calculated as:	
Flour No.	Wt. of Cu reduced equivalent to 0.5 gm. flour. Gm.	Glucose. Per cent.	Maltose. Per cent.	Wt. of Cu reduced equivalent to 0.5 gm. flour. Gm.	Glucose. Per cent.	Maltose. Per cent.
Straight	0.1937	19.51	34.16	0.1065	10.45	18.60
Low-grade	0.1592	15.86	28.00	0.1177	11.58	20.60
	100 cc. M/200 K_2HPO_4.			100 cc. M/100 K_2HPO_4.		
Straight	0.0245	2.32	3.96	0.0094	0.86	1.26
Low-grade	0.0517	4.98	8.84	0.0169	1.58	2.60
	100 cc. M/50 K_2HPO_4.			100 cc. distilled H_2O.		
Straight	0.0027	0.21	....	0.2300	23.41	40.60
Low-grade	0.0057	0.50	0.64	0.2095	21.19	36.96

The amounts of this salt used are given in terms of molar concentration. The weakest concentration used, M/800, shows an inhibiting action in both flours. As with the other chemicals used, the low-grade tolerates a larger amount than the straight.

TABLE VII.—EFFECT OF DIGESTING WITH SODIUM CHLORIDE OF DIFFERENT CONCENTRATIONS.

1 hr. at 65° C.

	100 cc. 0.00625*N* NaCl.			100 cc. 0.0125 *N* NaCl.		
		Sugar calculated as:			Sugar calculated as:	
Flour No.	Wt. of Cu reduced equivalent to 0.5 gm. flour. Gm.	Glucose. Per cent.	Maltose. Per cent.	Wt. of Cu reduced equivalent to 0.5 gm. flour. Gm.	Glucose. Per cent.	Maltose. Per cent.
Straight	0.2357	24.03	41.63	0.2294	23.34	40.50
Low-grade	0.1837	18.44	32.36	0.1762	17.65	31.03
	100 cc. 0.025 *N* NaCl.			100 cc 0.05 *N* NaCl.		
Straight	0.2119	21.45	37.38	0.1914	19.26	33.73
Low-grade	0.1607	16.01	28.26	0.1404	13.91	24.66
	100 cc. 0.10 *N* NaCl.			100 cc. distilled H_2O.		
Straight	0.1319	13.04	23.14	0.2300	23.41	40.60
Low-grade	0.1059	10.38	18.50	0.2095	21.19	36.96

Although sodium chloride was used in relatively stronger concentration, its effect was less than that of any of the other chemicals tried. There is a progressive inhibition proportionate to the concentration. The most marked difference between the effect of sodium chloride and the other chemicals tried is that it shows as large an effect, if not larger, on the low-grade as on the straight flour.

The different behavior of these two flours toward these various chemicals indicates a very profitable line of investigation. The chemical study of flour has been confined too much to the constituents which occur in relatively large quantities. If measured quantitatively the amount of amylolytic enzymes present in the flour would be very small as compared with other constituents, yet their power is so great that digestion with water at a suitable temperature results in transforming more than two-fifths of the weight of the flour into soluble substances calculated as maltose. Yet the sensitiveness of these enzymes is such that a very small amount of acid and still smaller amount of alkali inhibits action.

Summary.

As the line of investigation presented in this paper is comparatively new with respect to flour, the methods used have been fully described. We have shown that the optimum temperature for the production of the maximum amount of reducing sugars is very near 65°; that the best proportion of water and flour lies between 1 : 4 and 1 : 10, and that there is little difference between these two limits. It has also been shown that the largest transformation takes place during the first hour; approximately 88% of the total change occurs during the first hour. The inhibiting effect of various chemicals has been shown. The inhibiting action is greater toward straight flour than toward low-grade.

ENZYMES. ASYMMETRIC SYNTHESES THROUGH THE ACTION OF OXYNITRILASES. PART I.

By Vernon K. Krieble.

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In a previous communication to This Journal,[1] it was pointed out that certain samples of emulsin when acting on amygdalin leave a residue of *l*-mandelonitrile. As the active nitrile in amygdalin is the dextro form, the nitrile found must be a synthetical product. The same sample of emulsin, however, when allowed to act on benzaldehyde and hydrocyanic acid, gave *d*-mandelonitrile, a result which had been previously noted by Rosenthaler. Rosenthaler,[2] however, found that his emulsin left *d*-mandelonitrile as a residue when acting on amygdalin. A plausible explanation for these divergent results is that there are varying quantities of two

[1] Krieble, This Journal, **34**, 716 (1912).

[2] Rosenthaler, *Arch. Pharm.*, **246**, 365 (1908).