THESIS

Subject THE DIASTASE AND INVERTASE CONTENT OF WHEAT FLOUR AND THEIR RELATION TO BAKING STRENGTH.

Name GEORGE P. KOCH.

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by

George P. Koch

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REPORT

of

COMMITTEE ON THESIS

THE undersigned, acting as a committee of the Graduate School. have read the accompanying thesis submitted by Mr. George Peter Koch for the degree of Master of Science. They approve it as a thesis meeting the requirements of the Graduate School of the University of Minnesota, and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science.

R. W. Thatcher
Chairman

F. J. Alcock

J. H. Dewey

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INTRODUCTION.

"Baking strength" is the property of a flour which enables it to produce a large well-piled and well-aerated loaf of bread. The term well-piled means the evenness of fine texture throughout. Many different interpretations of "baking strength" have been made because formerly "baking strength" had reference to the property of a flour of giving the greatest number of loaves per sack and the absorption of the greatest amount of water.

A review of the literature on flour strength will show clearly the contradictory opinions of investigators who base their conclusions upon research. Several investigators have laid much stress upon the quality and quantity of gluten in flour as an index of flour "strength." Jago[1] states that, "There must be sufficient gluten present to adequately retain the gas and confer elasticity. Too much may be injurious,

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inasmuch as it may offer too great a resistance to the action of the distending gas. The consequence of this is the production of small and what are sometimes called "gluten bound" loaves. It must be highly elastic, yielding readily to distention without breaking."

Willard and Swanson(2) are of the opinion that "The capacity to produce a good loaf depends upon the quality of the gluten that a given flour can yield. If the gluten is too weak the partitions between the globules are broken and the globules coalesce with the production of a coarse grained bread, while a weak gluten will yield under the weight of the loaf and instead of rising in a well rounded form will flatten out and run over the edge of the pan if possible."

Wood(3), in his investigations, comes to different conclusions, namely, "That a high content of gluten is frequently associated with "strength", but there are so many cases in which a flour high in gluten is not so strong as another which contains less of that substance that it can no longer be maintained that gluten content

can be considered as a measure of strength."

In their investigations on English wheats, Humphries and Biffin[4] concluded that the quality of gluten and the high nitrogen content of flour designate strength, summarizing their general opinion in the following words: "It appears rather the quality of the gluten than the amount which determines strength. On the whole, the estimation of total nitrogen percentage in either the grain or the flour gives results which are nearest to agreeing with the baking trials, those wheats with highest nitrogen content being strongest."

Shutt[5] as the result of a series of investigations on Canadian wheats concludes: "That there is a distinct relationship between the nitrogen compounds (protein, gliadin, and dry gluten) and "baking strength."

Guthrie[6] comes to the same conclusion from a series of experiments. In brief, his statement is as follows: "Strength clearly does not depend upon the amount of gluten, but on the difference in its constitution. And again, on the quantity of gluten in flour depends

the nutritiousness of the bread, and upon its quality depends the strength of the flour and its value for baking purposes."

Guthrie[7] who also worked with Australian wheats, concludes that there is no parallelism between gluten content and "strength" of flour. He states as follows: "At one time the opinion was generally held that gluten content and strength of flour were synonymous. This has been shown to be untenable as wheats giving highest gluten content namely, Durums, produced the weaker flours of any examined and in no case was there any regularity to be noted between the gluten contents and the strength of the flour."

Similar facts are reported by Armstrong[8] who believes that high gluten content is usually associated with strength but in many instances the flours low in gluten showed just as much "strength" as did flours of a much higher gluten content. In his conclusions he states: "Scientifically, gluten content cannot be considered as an absolute measure of "strength", although

obviously connected with it. Strength depends on the quality rather than on the quantity of the proteins of the flour. However, the protein content, when judging normal flours is undoubtedly the best single measure of strength."

Aside from the several authorities, whose conclusions that "baking strength" of flour depended upon the quality or quantity of gluten, have been quoted, there are many other investigators who attribute "baking strength" to the gliadin ratio, (the ratio of glutenin to gliadin).

Snyder[9] arrived at the conclusions that, "The size of the loaf is determined by the properties or character of the gluten, especially of the ratio of the gliadin to glutenin rather than the percentage amount of gluten in the flour. Snyder[10] also states (in Minnesota Bulletin No. 63) "When these two bodies (glutenin and gliadin) are present in about the same proportion, the flour is stronger than that which contains a larger proportion of glutenin. When glutenin predominates, strong tough

elastic non-adhesive gluten is the result, while when gliadin predominates, the gluten is weak, sticky and inelastic."

In their investigation on "baking strength" Saunders and Shutt[11] arrived at the conclusion, that there is a distinct relationship between the proteins, gliadin and wet and dry gluten, but add that it is impossible to state that there is any absolute ratio and "Gliadin number datum is not to be considered as definitely related to the nitrogen compounds or the "Baking Strength."

Guess[12] in his investigations agrees with Snyder's results that the ratio of gliadin to glutenin was an index of quality or "baking strength."

The same statement is confirmed by Fleurent[13] in which he states, "Ignoring the actual percentage of gluten, the proportion of glutenin and gliadin which are found in flours giving the best bread-making results are gliadin 25% and glutenin 75% or the ratio of (1:3). Flours with the ratio of 1:4 develop well during fermentation but collapse and become compact during baking.

When the ratio is (1:2) the flour is almost unworkable." While Shutt[5], on the other hand, working with Canadian wheats, concludes that the gliadin ratio is erratic and of little value either from a scientific or a practical standpoint.

Guthrie[6] summarizes his results by stating that, "Gluten consists of two distinct proteid substances, gliadin and glutenin. These occur in different proportions in the gluten from different flours, and according as one or the other predominates the flour is strong or weak.

Shutt[14] summarizes another investigation with wheats by saying that "Canadian flours do not conform to any gliadin ratio as the index of flour strength."

And again, Shutt[15] in research data published in 1909 expressed his opinion that "The amount of gluten is largely a product of the season and that the character of the gluten is fixed by the variety."

While the gliadin ratio is held by some investigators to be the index of baking strength, others think

that such proof is entirely erroneous, and real index of baking qualities is rather in the physical properties than the chemical makeup. The following conclusion by Wood[3] represents the results of his research: "It has been shown that the chemical composition of gliadin and glutenin of strong and weak flours are identical in all flours examined." He based "strength" upon the amounts of acid, bases, salts and sugars which are present in the flour. In brief, his conclusions are as follows: "Strength was generally found to be associated with a high ratio of proteid to salts and weakness to low ratio." He states in the same article "Size and volume is determined by the amount of sugar contained in flour, together with that formed in dough by diastatic action."

Armstrong[8], previously cited, agrees with Wood's conclusions that it is the physical properties of the gluten rather than its amount which determines the behavior of flour when it is made into bread.

Hardy[16], as the result of flour investigations,

lays much stress upon the electrolytes which may be added in making bread. He is quoted as follows: "Electrolytes do more than confer on the gluten its mechanical properties, they determine its power of holding water and determine the water-holding power of any other colloidal matter present in the dough."

Baker and Hulton[17] are of the same opinion that the mineral constituents of flour play an important part in determining flour "strength."

Wood and Hardy[18] are still of the opinion, as stated in a very recent publication, that the physical state of gluten is conditioned by the electrolytes which are present. They conclude by saying "Gluten is peculiarly sensible to low concentration of acid or alkali. Very dilute acids cause dispersion while strong acids above a certain concentration maintain the cohesion. The power of dispersing of gluten varies with the concentration of the acid. Measured by the concentration of salt needed to prevent dispersion, the dispersive power of an acid increases with increasing con-

centration, and then falls until the critical concentration is reached when dispersive action is nil. In the case of alkalis the action is similar to that of acids but the electric sign is negative."

Jessen-Hansen, H.[19] advance still another theory relative to the baking "strength" of flour; namely, that there should be a certain amount of acid present in the flour in order to get the best results. Their statement reads as follows: "There exists an optimum concentration of hydrogen ions for general "baking quality."

Saunders and Shutt[11] arrived at the conclusion, "That if the size of the loaf produced is controlled by the volume of the gas evolved in bread making, then this volume is dependent on the degree of enzyme action which may affect the proteid as well as the carbohydrates rather than the amount of sugar that is present in the flours."

Shutt[5], in his recent investigations, could find no relation between the percentage of sugar present in flour and the volume of the loaf, but states his experience, "That some relationship existed between maturity

and gliadin content, the more fully ripened wheat containing the larger proportion of gliadin."

The conclusions of Shutt are confirmed by Saunders and Shutt (20) in a later investigation, in which the results of the research pointed to the conclusion that immaturity directly affected the protein content, the immature grain having a poorer quality of gluten and thus a poorer "baking strength."

Purpose of the Investigation.

One of the projects in the Agricultural Chemistry Division of the University of Minnesota is "The Strength of Wheat Flour." This project is divided into several sub-projects, or problems of investigation, concerning several of the important phases of flour chemistry which may possibly have some direct influence upon the "baking strength" of flour.

The problem selected by the writer was "The Enzymes of the Normal Wheat Kernel, Their Isolation, Identification, and Effect Upon Baking Strength." In this investigation it was proposed to study the kind and nature of

enzyme action in the normal wheat kernel and to ascertain if there was any direct relation between the character and amount of enzyme action and "baking strength" of flours.

Inasmuch as there has been comparatively little material published upon the relation of enzyme action to "baking strength", the writer was considerably handicapped at the outset of his research because there were no available methods by means of which the enzyme activity of the flours could be determined, consequently, the time permitted no further investigation than the study of the carbohydrate-splitting enzyme. It is, however, the intention to continue this study of the protein-splitting enzymes of the wheat flour and to further investigate the carbohydrate-splitting enzymes, particularly the "diastase" enzyme.

Review of Literature Concerning Enzymes of Wheat.

The chemical composition, the relation of quantity and quality of gluten in flour, its physical property and the effect due to electrolytes, the ratio of gliadin to glutenin, and the percentage content of organic and inorganic salts in flour to "baking strength", have been a matter of greatest public interest and scientific investigation within the last half century. Very few investigators have considered the enzyme activity and its effect upon bread making. Probably the most extensive work upon this phase of research has been carried out by Baker and Hulton[21] who is 1909 published an article as the result of research on the "Conditions Affecting the Strength of Wheat Flours." They conclude that there is no one single test or analysis of wheat flour which is capable of giving a conclusive index of baking quality of flour. The most certain method, and the one of last resort, is the "baking test." These authors are of the opinion that the diastase of the

flour plays an important part during the latter part of fermentation and the early part of the baking period, because some of the carbon dioxide which is formed during this period is the result of the fermentation of the maltose which has been produced by the action of diastase upon the starch granules of the flour.

In their investigation they have noted that the carbon dioxide given off was not concordant with the diastase which they were able to extract and they also found that a weak flour has as much or more diastatic power than did a strong one. Their statements relative to the kind of diastases in the flour are as follows: "The diastase of some flours contain a liquefying enzyme, while in others, it is either absent or unable to exert its influence." These investigators are of the opinion that during doughing this insoluble matter may be rendered soluble and "That there is a connection between the gas volume and the additional matter rendered soluble during the process of doughing." In brief, they conclude that "It seems probable that we might establish

a connection between strength and the relative amount of starch-liquefying enzyme in flour."

Reycler[22] found, as the result of some of his research studies that an opalescent liquid which gave Lintner's diastase reaction could be obtained from gluten of wheat when it had been acted upon by dilute acid and he also stated that "A slight increase in acidity increased the activity."

This latter statement was confirmed by Ford[23], who states that "The activity of diastase may be increased even ten times by the addition of mere traces of certain substances, as lactic acid. The restrictive influence of acids depends upon their dissociation, the greater the amount of free hydrogen ions the greater the restriction."

In studying amyolytic and proteolytic ferments of wheaten flours, Ford and Guthrie[24] found that the amyolytic activity was destroyed with very great rapidity, consequently the enzyme activity of flours, which were being tested, could not be measured with very great ac-

curacy. Ford and Guthrie[25] further found in some investigations with barley that "with pāpain(a protein-splitting enzyme) increasing the amylolytic activity consists in liberating latent amylase present in the barley in an occulated insoluble form."

Duggar[26] was also of the opinion that it was impossible to entirely separate the diastase from other substances.

Securing Samples for the Investigations.

The unsatisfactory condition of the present knowledge concerning the enzymes of flour and methods for their isolation and determination, as shown in the preceding review of the literature concerning them, made it necessary to plan these investigations so as to eliminate, as far as possible, the possibility of drawing conclusions from a few unusual samples.

In order to eliminate variations due to climatic conditions and further complications which might be introduced if the different varieties were used in such investigations, samples of wheat of a single variety, Turkey Red, and of the flour made from it, were secured from the most important wheat districts of greatest climatic variations. In so doing we have a standard common to all districts, but the "strength" varying with the climatic conditions under which the wheat was produced. To further reduce complications and to standardize this investigation to such an extent that variation due to

quality might be ascertained, different grades of flour which had been milled from the same wheat were secured from the several different wheat districts.

Samples were secured from the Experiment Station or some reliable milling firm, from the following states, Nebraska, Washington, Illinois, Kansas, Montana, North Dakota, and Minnesota. In some instances it was impossible to secure the different grades of flour from the persons to whom letters were sent because of the fact that there were no mills in their immediate vicinity where the samples could be milled. In those cases a sample of wheat was sent and Mr. Bailey, Cereal Technologist of this Station, milled the sample, recovering only a patent flour.

The letter which was sent to the different firms read as follows: "I should be very glad if you would ship me a five-pound sample each of Turkey Red wheat, the patent flour and first and second clear flours made from it.

The samples of the Nebraska flours and wheat were

obtained from the Gibbon Roller Mills, Gibbon, Nebraska. The following letter accompanied the samples: "Complying with your request, am forwarding samples of wheat, patent, clear and straight flours. We do not make a second clear."

Samples of Washington wheat and flour were solicited from two sources, namely, from the Prosser Flouring Mills, Prosser, Washington, and from the Ritzville Flouring Mills, Ritzville, Washington. The comments on the samples sent by the Prosser Flouring Mills read as follows: "As we do not make a high patent flour from this wheat, we are sending you our Baker's patent which is practically a straight grade, with about 6 per cent cut off. We are also sending you a sample of the cut off, with about 2 per cent taken out and put into the feed. So you will understand that the two samples of flour represent a purely straight grade if they were mixed together, 96 per cent of the Baker's patent and 4 per cent of the cut off." It was impossible to secure the different grades of flour milled from Turkey

Red wheat from the Ritzville Flouring Mills, due to the fact that that variety of wheat was not milled by that Company as the remarks indicate: "We have sent you a sample of Turkey Red wheat and trust that the same will reach you in due time. We do not mill this wheat so we are unable to give you any flour milled from this."

A sample of Turkey Red wheat was all that was possible to obtain from the Illinois Agricultural Experiment Station. Comments from that Station with reference to flour samples are as follows: "It will be impossible for me to get different grades of flour from Turkey Red wheat, since our millers get so little wheat in the state and I doubt that if flour were obtained, that we could be sure that it was from wheat grown locally."

Samples of Kansas wheat and flour were secured from the Lyons Milling Company, Lyons, Kansas. The comments on the samples sent by that Company read as follows: "This wheat is our milling blend after having left the milling separator. The wheat has not been scoured nor tempered. The patent is about 75 per cent, the first

clear 50 per cent, the second clear about 25 per cent. In other words, the patent and second clears together would make our full straight run of flour, and the first clear is the lower half of a full straight run from which a top 50 per cent patent has been taken off."

Samples of North Dakota wheat, and patent and first clear flour made from the same wheat, were solicited from the North Dakota Experiment Station. The comments upon the samples received from M. L. M. Thomas, of that Station, are as follows: "I take pleasure in forwarding to you five pounds of Hard Winter wheat grown at the North Dakota Experiment Station, Fargo, North Dakota, and five pounds each of Patent and First Clear flour made therefrom. Our miller apparently misunderstood my directions in regard to this sample and did not save the second clear flour, consequently we are unable to supply you with this."

A sample of Turkey Red wheat grown in Minnesota was secured from Jos. Meyer, LeSueur. The comments on the sample of wheat are as follows: "Am sending you a five pound sample of choice Turkey Red wheat." This

wheat was milled by Mr. Bailey, as mentioned above.

The Montana samples were furnished by the Experiment Station of that State, but were secured from a commercial mill at Bozeman. No comments on the character of the samples, except the labels on the sacks, were received.

Analyses and Baking Tests of Samples.

Since the samples of wheat which were secured were grown under very widely different conditions and were also milled by very varied processes, it was apparent that fairly complete chemical analyses and baking tests of the samples must be made. From the summary of these analyses and tests the flours of best quality and "baking strength" could easily be selected.

The chemical analyses of the samples were carried out by the official method in every case where methods have been adopted as official, protein being determined by the official modified Gunning method.(27) In determining the moisture, the weighed samples were dried in a vacuum drying oven until constant weight was obtained. Acidity was determined by the method adopted by this Station,(28) which, in brief, is titrating a certain aliquot of flour extract obtained by shaking a weighed quantity of flour with pure distilled water for a definite period of time, with a very dilute standard alkali.

The proteins in the form of globulin, albumen, and amids were also determined by the Chamberlain method.[29] The amount of globulin and albumen is the protein soluble in a 5% potassium sulphate solution. The amid nitrogen is that which is precipitated by a solution of phosphotungstic acid from a 5% potassium sulphate solution. Gliadin, which was determined by the method proposed by Hoagland[30], represents the protein soluble in 50% alcohol.

The results of these analyses are shown in Tables I and II which contain the analytical data for the samples of wheat and of flour, respectively.

The baking test, while not an exact quantitative method, is without doubt the best single index of "baking strength", but very great care must be exercised in carrying out these tests. The method adopted by this Station has been standardized in order that as few factors as possible may be involved. The method is as follows: After a portion of a weighed quantity of flour has reached the desired temperature, it is stirred into a

thin sponge with a definite quantity of yeast and water to which a quantity of salt has been added. When this latter has been allowed to ferment for an hour it is mixed in the mixing machine. The dough being well mixed and the sugar thoroughly incorporated into the mass, it is set aside for further fermentation. At the expiration of forty-five minutes the excess carbon dioxide is worked out by kneading the dough with the hand. This kneading process is repeated three times at intervals of fifteen minutes. The dough is then formed into a loaf, panned, again allowed to ferment and rise to its maximum and then baked.

In all the baking tests, the results of which are shown in Table III, 340 grams of flour were used.

In Table IV, the flours were graded according to their ash content, the high grade flours being designated as those containing from .400 to .500% of ash; the second grade representing flours with ash content from .500 to .644% and low grade from .646 to 1.196%.

Determination of Diastatic Activity of Flours.

In this investigation the method which was used for determining the diastatic activity of flours was first worked out in this Laboratory. It has been published in The Journal of the American Chemical Society, Volume XXXVI, No. 4 (April, 1914), and a reprint of the article will be inserted below, as Appendix "A".

In order that the writer could be positive as to the results obtained by experimentation, when the diastatic activity of flours was determined, triplicate and in most cases quadruplicate analyses were made of every sample and in but very few cases were variations between the final titrations of the duplicate determinations much greater than would be allowable as experimental error. The results, which are shown in Table V, are the average of the several duplicate determinations, thus lessening the possibility of experimental error.

In all of the determinations of diastatic activity 16 grams of flour were extracted for one and a half

hours with 200 cubic centimeters of distilled water and 25 cubic centimeters of the extract, or the equivalent of 2 grams of flour were allowed to act upon 2.5 grams of soluble starch for 30 minutes at 40°C.

A rather limited number of samples were used upon which to base conclusions, but as shown in the foregoing tables, volume of the loaf is the best single index of "flour strength."

As summarized in Table VI there appears to be no very marked differences in percentages of maltose as calculated from the diastatic action in the various patent flours. The same may be said concerning the comparative results of patent and first and second clear flours. It is quite evident, as shown in Table VII, that the low grade flours of poor "baking strength" contain as much active diastase as the high grade flours of good "strength." In brief, then, it may be said that the difference in amount of diastatic activity of various grades of flours is not appreciable, and does not play a very important role in influencing "baking

strength."

This conclusion seems to be in opposition to the frequently observed phenomenon that when a water-extract of a "strong" flour is added to a "weak" flour, the"baking strength" of the latter is thereby improved. It may be possible, however, that the physical and chemical constitution of the different flours may accelerate or retard the diastatic activity, and, further, each flour may contain a certain element beneficial to the action of its diastase, or that contained in the yeast used in bread making. We propose to investigate this phase of the problem very carefully but such a study is a different problem than the one taken for this thesis.

The Invertase Content of Flours.

Since the enzyme activity of flours and their relation to "baking strength" has become a subject of scientific interest to investigators, more attention has been paid to the diastase and very little to the proteolytic or invertase enzymes. In the study of invertases of plants, O'Sullivan and Thompson[31] have published more extensively than any other authors. In their researches they have shown that invertase is present in all plants, and as a rule nearly all the different parts of the plant gave the invertase reaction. Koelle[32] has also contributed largely to the present knowledge of invertase.

The writer has been unable to find any publications dealing with the percentage of invertase as affecting "strength" of flour.

In studying the relation of invertase content of flours to "baking strength", the same difficulty pre-

sented itself as in the case of the diastase, in that no satisfactory method of measuring the enzyme activity had beeen previously worked out. Consequently, a similar method for the measurement of the percentage of invertase in flours to that already employed in the determination of diastases was perfected and adopted as the method of study in this problem.

In view of the fact that the method employed in this phase of the investigation has not yet been published, it will appear as appendix to this thesis. (See Appendix "B".)

In order to ascertain if the results obtained were correct, duplicate determinations of each sample were made on two successive days, and upon examining the results it was found that in but very few cases was the error in the duplicates greater than the experimental error, due to pipetting or titration. Consequently, the data which will appear in the following tables are the average of the results of several determinations.

In all of the determinations of invertase activity

16 grams of flour were extracted for one and a half hours with 200 cubic centimeters of distilled water and 50 cubic centimeters of the extract, or the equivalent of 4 grams of flour was allowed to act upon 4 grams of sucrose for 30 minutes at 40°C.

The results shown in the foregoing tables indicate that there is no appreciable difference in the invertase activity of high grade or low grade flours and that flours milled from wheat grown under different conditions do not show any difference in invertase activity.

Further, it is quite conclusive from the results shown in Table X that the flours of poor strength have as much invertase activity as the strong flours and vice versa, and that the percentage of invertase bears no relation to flour "strength."

Again, determinations were made of the invertase activity of different samples of two different kinds of yeast used in home bread-making. The results, some of which are shown in Table XII, make it evident that

the invertase action of yeasts is relatively very great, in some cases four hundred to seven hundred times that of flours. We may then conclude that the invertase in flours becomes negligible upon the addition of yeast in bread-making.

SUMMARY.

The results of this investigation show that the diastase and invertase activity are not directly responsible for flour "strength." The experimental data point to the fact that all flours contain a considerable amount of diastase, but there does not appear to be any difference in percentage of diastase in the high and low grades of flour.

All grades of flour, even though they contain a low per cent, show about the same invertase activity. The enzymatic action is so small that during fermentation in the presence of yeast it would appear almost negligible.

The work recorded in this thesis opens a very wide field for further investigation. It affords a new, and accurate method for estimating enzymatic activity. It suggests the possibility of interacting substances other than enzymes exerting an accelerating or retarding ef-

fect upon the rate of diastatic activity in doughing flours for bread-making. It clears the way and provides the material for similar studies of the proteoclastic enzymes. It is the writer's hope to continue these studies, along the lines just suggested.

Table I. The Results of the Chemical
Secured from

Lab. No.	Source.	Grade of Flour.	Moisture.	Amount of Ash.	Acidity.
			Per ct.	Per ct.	Per ct.
30K	Nebraska	Patent	12.95	.452	.107
31K	Nebraska	Straight	12.85	.461	.112
32K	Nebraska	1st Clear	12.97	.576	.147
40K	Kansas	Patent	9.03	.402	.090
41K	Kansas	1st Clear	9.08	.498	.130
42K	Kansas	2d Clear	8.59	.644	.172
50K	No. Dakota	Patent	11.60	.410	.095
51K	No. Dakota	1st Clear	10.55	.646	.172
60K	Illinois	Patent	9.89	.460	.107
70K	Ritzville, Wash.	Patent	10.34	.500	.102
80aK	Montana	Patent	10.50	.496	.097
80K	Montana	Patent	8.75	.440	.112
81K	Montana	1st Clear	8.75	.830	.177
82K	Montana	2d Clear	8.62	1.196	.312
83K	Montana	Straight	8.80	.442	.110
90K	Prosser, Wash.	Patent	11.20	.464	.100
91K	Prosser, Wash.	1st Clear	11.61	.606	.145
100K	Minnesota	Patent	11.19	.454	.102

Analyses of the Different Samples of Flour the Various Sources.

Crude Protein (N x 5.7) Per ct.	Gliadin. (N x 5.7) Per ct.	Total Nitrogen as Gliadin. Per ct.	Globulin and Albumen. Per ct.	Amids. Per ct.
10.74	5.79	54.06	12.66	2.37
10.65	5.92	55.55	13.93	2.54
11.82	6.68	56.67	13.38	2.36
11.00	6.32	57.45	11.60	3.26
11.68	6.86	58.75	12.29	2.73
14.64	8.39	57.30	13.24	3.10
10.34	6.10	59.17	13.15	2.70
11.22	6.08	54.15	16.70	3.34
11.08	6.24	53.43	11.51	2.67
10.43	5.60	53.70	14.31	3.82
10.03	5.98	59.65	15.43	3.27
10.83	5.98	55.26	12.67	2.43
11.79	6.75	57.28	15.21	2.70
11.08	5.34	48.35	23.59	4.90
9.49	5.38	56.93	13.91	2.89
12.22	6.82	55.93	11.97	3.07
13.85	7.63	54.68	12.79	2.88
9.23	5.53	60.06	17.89	3.02

Table II. The Results of the Chemical
Wheat Secured from

Lab. No.	Source.	Moisture. Per ct.	Amount of Ash. Per ct.	Acidity. Per ct.
1 30B	Nebraska	14.12	1.868	.267
2 40B	Kansas	8.53	1.702	.230
3 50B	No. Dakota	10.90	1.788	.215
4 60B	Illinois	7.74	1.578	.187
5 70B	Ritzville, Wash.	10.35	1.502	.257
6 80B	Montana	10.73	1.510	.265
7 90B	Prosser, Wash.	7.95	1.596	.245
8 100B	Minnesota	9.60	1.880	.280

Analyses of the Different Samples of "Turkey Red" the Various Sources.

Crude Protein. (N x 5.7) Per ct.	Gliadin. (N x 5.7) Per ct.	Total Nitrogen as Gliadin. Per ct.	Globulin and Albumen. Per ct.	Amid. Per ct.	
11.94	5.76	48.36	20.56	4.22	1
13.68	6.78	49.58	18.37	4.95	2
11.94	5.58	46.88	23.00	4.82	3
12.14	5.78	47.65	23.33	4.10	4
11.00	5.30	48.75	20.40	5.85	5
11.34	5.76	50.79	20.62	5.48	6
12.59	6.54	51.95	17.40	4.81	7
10.71	4.78	44.60	22.11	4.84	8

Table III. Results of Baking Tests of the Flour Samples Secured from the Different Localities.

Lab. No.	Water Absorbed. Cc.	Loaf Volume. Cc.	Color Score.
30K	203	2490	103
31K	205	2390	100
32K	212	2300	88
40K	236	2440	101
41K	235	2375	96
42K	253	2120	75
50K	220	2240	100
51K	225	1820	88
60K	208	2220	98
70K	223	2120	97
80aK	225	2130	99
80K	248	2195	97
81K	248	1845	80
82K	235	1195	--
83K	248	2160	103
90K	227	2310	98
91K	242	2120	85
100K	212	2210	98

Table IV. Comparative "Strength" of the Different Samples of Flour Secured from the Different Sources as Determined by the Volume.

Lab. No.	Source.	Ash Content. Per ct.	Water Absorbed. Cc.	Loaf Volume. Cc.	Color Score.
		High Grade.			
30K	Nebraska	.452	203	2490	103
40K	Kansas	.402	236	2440	101
31K	Nebraska	.461	205	2390	100
41K	Kansas	.498	235	2375	96
90K	Washington	.464	227	2310	98
50K	No. Dakota	.410	220	2240	100
60K	Illinois	.460	208	2220	98
100K	Minnesota	.454	212	2210	98
80K	Montana	.440	248	2195	97
83K	Montana	.442	248	2160	103
80aK	Montana	.496	225	2130	99
70K	Washington	.500	223	2120	97
		Second Grade.			
32K	Nebraska	.576	212	2300	88
91K	Washington	.606	242	2120	85
42K	Kansas	.644	253	2120	75
		Low Grade.			
81K	Montana	.830	248	1845	80
51K	No. Dakota	.646	225	1820	88
82K	Montana	1.196	235	1195	--

Table V. The Diastatic Activity of the Different Samples of Flour Secured from the Different Sources, Expressed in Terms of Maltose. Results Calculated to 1 Gram Material.

Lab. No.	Copper Reduced After Digestion. Grams.	Copper Reduced by Blank. Grams.	Copper Due to Enzyme. Grams.	Maltose Equivalent. Grams.
30K	.72215	.01628	.70587	0.539
31K	.74176	.01628	.72548	0.555
32K	.73368	.01395	.71973	0.543
40K	.77637	.01977	.75660	0.579
41K	.77060	.02064	.74996	0.575
42K	.76829	.01541	.75288	0.577
50K	.75560	.01337	.74223	0.569
51K	.75545	.01454	.74091	0.567
60K	.74637	.00756	.73881	0.565
70K	.73022	.01395	.71627	0.548
80aK	.76483	.01250	.75233	0.577
80K	.74637	.02849	.72788	0.556
81K	.77406	.02559	.74847	0.574
82K	.76252	.03722	.72530	0.555
83K	.75330	.02791	.72539	0.555
90K	.72100	.01977	.70123	0.536
91K	.70831	.01395	.69436	0.529
100K	.74407	.00814	.73593	0.562

Table VI. The Diastatic Activity of the Patent Flours, Expressed in Terms of Maltose. Results Calculated to 1 Gram Material.

Lab. No.	Copper Reduced After Digestion. Grams.	Copper Reduced by Blank. Grams.	Copper Due to Enzyme. Grams.	Maltose Equivalent. Grams.
30K	.72215	.01628	.70587	0.539
40K	.77637	.01977	.75660	0.579
50K	.75560	.01337	.74223	0.569
60K	.74637	.00756	.73881	0.565
70K	.73022	.01395	.71627	0.548
80aK	.76483	.01250	.75233	0.577
80K	.74637	.02849	.72788	0.556
90K	.72100	.01977	.70123	0.536
100K	.74407	.00814	.73593	0.562

Table VII. The Comparison of the Diastatic Activity of the Patent, First Clear, and Second Clear Flours Milled from the Same Variety of Wheat. Results Calculated to 1 Gram Material.

Lab. No.	Grade of Material.	Copper Reduced After Digestion. Grams.	Copper Reduced by Blank. Grams.	Copper Due to Enzyme. Grams.	Maltose Equivalent. Grams.
30K	Patent	.72215	.01628	.70587	0.539
32K	1st Clear	.73368	.01395	.71973	0.543
40K	Patent	.77637	.01977	.75660	0.579
41K	1st Clear	.77060	.02064	.74996	0.575
42K	2d Clear	.76829	.01541	.75288	0.577
50K	Patent	.75560	.01337	.74223	0.569
51K	1st Clear	.75545	.01454	.74091	0.567
80K	Patent	.74637	.02849	.72788	0.556
81K	1st Clear	.77406	.02559	.74847	0.574
82K	2d Clear	.76252	.03722	.72530	0.555
90K	Patent	.72100	.01977	.70123	0.536
91K	1st Clear	.70831	.01395	.69436	0.529

Table VIII. Comparison of the Diastatic Activity of the Different Grades of Flour Expressed as Maltose with the Baking "Strength" as Determined by the Volume of the Loaf.

Results Calculated to 1 Gram Material.

Lab. No.	Ash Content. Per ct.	Loaf Volume. Cc.	Copper Due to Enzyme. Grams.	Maltose Equivalent. Grams.
		High Grade.		
30K	.452	2490	.70587	0.539
40K	.402	2440	.75660	0.579
31K	.461	2390	.72548	0.555
41K	.498	2375	.74996	0.575
90K	.464	2310	.70123	0.536
50K	.410	2240	.74223	0.569
60K	.460	2220	.73881	0.565
100K	.454	2210	.73593	0.562
80K	.440	2195	.72788	0.556
83K	.442	2160	.72559	0.555
80aK	.496	2130	.75233	0.577
70K	.500	2120	.71627	0.548
		Second Grade.		
32K	.576	2300	.71973	0.543
91K	.606	2120	.69436	0.529
42K	.644	2120	.75288	0.577
		Low Grade.		
81K	.830	1845	.74847	0.574
51K	.646	1820	.74091	0.567
82K	1.196	1195	.72530	0.555

Table IX. The Invertase Activity of the Different Samples of Flour Secured from the Different Sources, Expressed in Terms of Invert Sugar. Results Calculated to 1 Gram Material.

Lab. No.	Copper Reduced After Digestion. Grams.	Copper Reduced by Blank. Grams.	Copper Due to Enzyme. Grams.	Invert Sugar Equivalent. Grams.
30K	.03835	.01628	.02207	.00942
31K	.03922	.01628	.02294	.00988
32K	.03979	.01395	.02584	.01132
40K	.05796	.01977	.03819	.01768
41K	.04614	.02064	.02550	.01115
42K	.04066	.01541	.02525	.01102
50K	.03374	.01337	.02037	.00861
51K	.02797	.01454	.01343	.00527
60K	.03172	.00756	.02416	.01048
70K	.03201	.01395	.01906	.00798
80aK	.02739	.01250	.01489	.00596
80K	.04700	.02849	.01851	.00771
81K	.03316	.02559	.00757	.00252
82K	.04037	.03722	.00315	.00141
83K	.04700	.02791	.01909	.00799
90K	.04297	.01977	.02320	.01001
91K	.02941	.01395	.01546	.00624
100K	.03230	.00814	.02416	.01048

Table X. The Invertase Activity of the Patent Flours, Expressed in Terms of Invert Sugar. Results Calculated to 1 Gram Material.

Lab. No.	Copper Reduced After Digestion. Grams.	Copper Reduced by Blank. Grams.	Copper Due to Enzyme. Grams.	Invert Sugar Equivalent. Grams.
30K	.03835	.01628	.02207	.00942
40K	.05796	.01977	.03819	.01768
50K	.03374	.01337	.02037	.00861
60K	.03172	.00756	.02416	.01048
70K	.03201	.01395	.01906	.00798
80aK	.02739	.01250	.01489	.00596
80K	.04700	.02849	.01851	.00771
90K	.04297	.01977	.02320	.01001
100K	.03230	.00814	.02416	.01048

Table XI. Comparison of the Invertase Activity of the Different Grades of Flour as Invert Sugar with the Baking "Strength" Determined by the Volume of the Loaf.

Results Calculated to 1 Gram Material.

Lab. No.	Ash Content. Per ct.	Loaf Volume. Cc.	Copper Due to Enzyme. Grams.	Maltose Equivalent. Grams.
		High Grade.		
30K	.452	2490	.02207	.00942
40K	.402	2440	.03819	.01768
31K	.461	2390	.02294	.00988
41K	.498	2375	.02550	.01115
90K	.464	2310	.02320	.01001
50K	.410	2240	.02037	.00861
60K	.460	2220	.02416	.01048
100K	.454	2210	.02416	.01048
80K	.440	2195	.01851	.00771
83K	.442	2160	.01909	.00799
80aK	.496	2130	.01489	.00596
70K	.500	2120	.01906	.00798
		Second Grade.		
32K	.576	2300	.02584	.01132
91K	.606	2120	.01546	.00624
42K	.644	2120	.02525	.01102
		Low Grade.		
81K	.830	1845	.00757	.00252
51K	.646	1820	.01343	.00527
82K	1.196	1195	.00315	.00141

Table XII. The Invertase Activity of Yeasts as Expressed in Terms of Invert Sugar. Results Calculated to 1 Gram Material.

Lab. No.	Kind of Yeast.	Copper Reduced After Digestion. Grams.	Copper Reduced by Blank. Grams.	Copper Due to Enzyme. Grams.	Invert Sugar Equivalent. Grams.
1	Yeast Foam	1.14998	Negligible	1.14998	4.365
4	Compressed	.67953	Negligible	.67953	2.166

Fig. I. Showing Quality of Loaves of Bread from Flour Tested in These Investigations.

Fig. II. Showing Quality of Loaves of Bread from Flour Tested in These Investigations.

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THE QUANTITATIVE EXTRACTION OF DIASTASES FROM PLANT TISSUES.

By R. W. Thatcher and Geo. P. Koch.

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There are two different opinions as to the nature of enzyme action. One supposes that enzymes are only catalytic in their effect, that is, that they are only accelerators of reactions which actually occur in the absence of the enzyme, but at a very much slower rate. The other holds that enzymes are actual causative agents for their specific reactions, and function by forming definite chemical unions with the initial substance which later break down into a new substance or substances, liberating the enzyme unchanged and in condition to repeat the process an indefinite number of times. Whatever may be the nature of their action, however, it is obvious that they do not participate quantitatively in the reaction which is their specific function. Hence, they cannot be measured quantitatively by the usual methods of determining the *quantity of the end-products of the reaction*. It is necessary, instead, to measure their effect upon the *velocity of the reaction*.

In any comparative study of the enzymes of different samples of vegetable materials, it becomes desirable to obtain comparable measures of the quantity of enzyme present. Since there are no known methods for isolating and determining the exact quantity of an enzyme in any given plant or animal tissue and, further, since in the present state of knowledge concerning enzyme action it is uncertain whether the same actual quantity of the same enzyme from different sources will possess the same accelerating or causative effect upon its substrate, the investigator is necessarily forced to assume that the comparative accelerating effect upon reaction-velocity of the extract from the same quantity of the materials under investigation is a comparable measure of the quantity of enzyme present in each sample. Or he may, as is doubtless a better procedure, use as his basis of comparison the "diastatic strength" or some similar term, instead of the actual quantity of enzyme present.

In a series of investigations of the various factors which may influence what is called the "strength" of wheat flour, we have undertaken a study of the enzymes of the wheat kernel and their distribution to the various products separated from it in milling, and of their effect upon the flour during the fermentation of the dough and baking of the loaf of bread. Quite naturally, we first sought to study the diastatic enzymes, that is, those which cause or accelerate the hydrolysis of starch. We were immediately confronted by the lack of a method of quantitative extraction of the active diastases of different samples, which would yield comparable results. There was, first, the question of a suitable standard of measurement of the acceleration of starch-hydrolysis due to the presence

of the enzyme, and second, the difficulty of extracting an active hydrolyzing enzyme from a material containing large quantities of the hydrolyzable material, without getting continuous activity of the enzyme during the extraction period. This latter difficulty had led a number of investigators who had previously attempted a study of this kind to abandon it as incapable of exact solution, since they found that the activity of the enzyme during the extraction "used up" variable proportions of its accelerating effect, and, consequently, they obtained extracts of variable "diastatic strength" from the same sample if the time or temperature of extraction, the ratio of solvent to material, or the conditions of preservation of the extract until its "diastatic strength" could be measured, varied in even comparatively slight degrees. It occurred to us, however, that this difficulty might easily be overcome by carrying out the extraction at a temperature below that at which the enzymes exert any influence upon reaction velocity, say at 0°, provided the enzymes themselves were sufficiently soluble at that temperature to permit the securing of comparably quantitative extractions. This could, of course, be easily ascertained experimentally.

There remained the question of a suitable standard of measurement of the comparative accelerating effect of extracts from different samples. The possibility of comparable measurements depends upon whether the velocity of the reaction is uniform throughout its duration, exhibiting the so-called "straight-line" curve of reaction-velocity, or is modified by the law of mass action, having an approximately logarithmic velocity-curve. In the latter event, it would be impossible to make comparisons of velocity-accelerations of extracts from different samples, as the relative mass of hydrolyzable material and of activating enzyme in each different extract would rarely, if ever, be the same. However, Kjeldahl's first assumption[1] that the amount of reducing sugar produced is a true measure of diastatic power, provided that not more than 40% of the original starch is converted into maltose at the expiration of the period of digestion, has been accepted as a working basis by many investigators and has been indirectly confirmed by Brown and Glendinning,[2] who found that equal amounts of starch were hydrolyzed by diastase in equal times during the earlier part of the reaction, but that toward its close, when the concentration of enzyme becomes large as compared with that of the substrate, the reaction obeys the law of mass action. Kjeldahl's "law of proportionality" was, therefore, accepted as a suitable basis of measurement for our work.

The next question was as to the method of measurement of the velocity of the reaction. Several methods for this have been suggested.

[1] See *Dingler's polytechn. J.*, 235, 379–387, 452–460 (1880).
[2] *J. Chem. Soc.*, 81, 388–392 (1902).

Kjeldahl[1] recommended the determination of the amount of reducing sugar produced by the action of a definite amount of the extract in question upon an excess of starch for a definite length of time at a definite temperature.

Roberts[2] proposed the use of the number of cubic centimeters of a standard starch paste which would be converted into non-iodine-coloring material in five minutes at 40° by one cubic centimeter of the extract under examination, as a standard.

Jungk[3] recommended measuring the time required for 10 cc. of extract, acting at 40°, to convert 10 g. of anhydrous starch into maltose.

Lintner[4] modified the Kjeldahl process so as to make it possible to calculate the diastatic strength upon the basis of the production of a constant quantity of maltose from a constant amount of soluble starch, expressing the "diastatic strength" of the extract in terms of percentage of the strength of a standard diastase, of which 0.12 mg., acting upon a definite quantity of soluble starch for 1 hour at 21°, produced enough maltose to completely reduce 5 cc. of Fehling's solution.

Johnson[5] published a new form of the method, in which the amount of diastase necessary to digest a fixed amount of starch to no color with iodine in ten minutes at a temperature of 40° was determined. He found that the same measure of the velocity of reaction was obtained in this way as by a measurement of the quantity of sugar produced.

Various other modifications of the Lintner process have been suggested from time to time and have recently been submitted to critical review by Sherman, Kendall, and Clark,[6] who recommended, as a result of their studies, the general adoption of 30 minutes as the time and 40° as the temperature for all digestions for the determination of diastatic strength. Their investigations included comparisons of reaction-velocities as measured by the disappearance of iodine-coloring material and by the quantity of maltose produced. Later and more extensive studies of these two methods of estimation of diastatic strength by Sherman and Schlesinger[7] emphasize the distinction between the amyloclastic and the saccharogenic activity of diastatic preparations and point out the relationships which exist between these, and some of the advantages and disadvantages of the two processes.

For the purposes of our investigations, it was very clearly the sugar-producing, or saccharogenic, power of the diastases of flour which it would

[1] *Loc. cit.*

[2] *Proc. Roy. Soc.*, 32, 145–161; abs. in *J. Chem. Soc.*, 40, 1051 (1881).

[3] *Pharm. J. Trans.*, [3] 14, 104–108; abs. in *J. Chem. Soc.*, 46, 529–531 (1884).

[4] *J. prakt. Chem.*, [2] 34, 378–394 (1886).

[5] THIS JOURNAL, 30, 798–805 (1908).

[6] *Ibid.*, 32, 1073–1086 (1910).

[7] *Ibid.*, 35, 1784–1790 (1913).

be of value to study, and we, therefore, decided to use the maltose produced by digestion at 40° for 30 minutes as the measure of the reaction velocity or the "diastatic strength" of the extracts from the samples under investigation.

As a rapid and accurate method of measuring the maltose produced, the determination of the residual copper, after boiling a suitable aliquot portion of the solution with a definite volume of standardized Fehling's solution, by titration by the iodine method, as perfected by Peters,[1] seemed promising, and upon trial proved thoroughly satisfactory.

The process of preparing the portion of the extract used as a blank, and the solutions after digestion with soluble starch, for their action upon the standardized Fehling's solution was that used by Swanson and Calvin in their investigation of the conditions affecting the activity of the amylolytic enzymes in wheat flour,[2] and was found to work very satisfactorily.

Experimental Data.

The object of this preliminary work was to ascertain whether the diastases present in wheat-products could be quantitatively extracted at 0°, in order that none of their catalytic power might be "used up" during the process of dissolving them from the material under investigation The plan of the work involved the digestion of weighed quantities of ground wheat, bran, and different grades of flour in water at 0° for varying lengths of time and the determination of the maltose-producing power of the resultant extracts. It was believed that, if the results should show a uniform diastatic power for extracts made at different lengths of time, and a uniform quantity of reducing sugars in the aliquot part of the extract used as a blank, the principle that the diastases are quantitatively extracted, and that no diastatic action occurs during the extraction at that temperature, would be established.

In order to facilitate computations of fractions resulting from taking aliquot parts, we decided to use 16 g. of material for each extraction. Swanson and Calvin[3] found that the maximum amylolitic activity was secured when flour and water were used in proportions varying from 1 : 4 up to 1 : 10. We, therefore, decided to use 200 cc. of water for each extraction, this being a convenient volume for subdivision and not far outside the optimum limits just mentioned.

Ordinary half-pint milk sample bottles were used to hold the materials during the extraction. These bottles were furnished with a tin cap held down by a spring clamp. To insure against leakage, a disk cut from thin rubber was placed under the cap before it was clamped down. In preparing the material for extraction, the charge of dry material was

[1] See THIS JOURNAL, 34, 433–452 (1912).
[2] *Ibid.*, 35, 1636–1637 (1913).
[3] *Loc. cit.*

weighed into the bottle, the cover clamped down and the bottle placed in the ice-water bath until its contents reached the desired temperature. The bottle was then removed from the bath, its stopper loosened, and 200 cc. of redistilled water (previously cooled to 0°) quickly pipetted into it. The cap was again clamped down and the bottle immediately returned to the ice-water bath. The filling of the several bottles of each series was so timed that all the desired periods of extraction would terminate at the same time, in order that all subsequent operations upon the extracts might be carried on simultaneously. Each bottle was shaken vigorously at intervals during the extraction.

At the expiration of the extraction period, the bottles were removed from the bath and their entire contents quickly poured upon large S and S folded filters. The first few drops which came through were usually cloudy, but by pouring back once or twice a filtrate clear enough for all practical purposes was easily obtained. Filtration was usually rapid enough so that a sufficient volume of extract for the necessary aliquots could be obtained in a few minutes and without serious rise in temperature of the extract. (In working with materials which filter slowly the temperature might be kept down by placing the funnel in a jacket, such as is used for hot filtration, filled with ice-water; but this was not found necessary in any of our work.) As soon as a sufficient volume of extract was secured, it was at once warmed to 40°, the temperature at which its action upon starch was to be ascertained, and two aliquots of 50 cc. each drawn out. To one of these $N/10$ sulfuric acid was added, in such an amount that the resultant mixture would be a 0.02 N solution (this being the strength found by Swanson and Calvin to be the correct one) in order to stop all diastatic action. To the other aliquot, 25 cc. of a 10% solution of soluble starch (previously warmed to 40°) were added, and the mixture placed in an incubator, kept automatically at 40°, for exactly 30 minutes. At the expiration of this time, further action was stopped by bringing the mixture to a 0.02 N sulfuric acid. In both the blank and the digested solution, the soluble proteins were precipitated with salt and phosphotungstic acid, and the maltose in an aliquot of each determined, by the methods mentioned above. The results obtained in the first series of experiments, using ground wheat, ground barley, bran, and three different grades of wheat flour, calculated to the basis of 1 g. of original material in each case, are shown in Table I.

As a further confirmation that the diastases were quantitatively extracted from materials such as those under examination by diffusion into water at 0°, we conducted another series of extractions using in each case, first, the material ground as for ordinary analyses, and, second, the same weight of material after grinding vigorously for ten minutes in a mortar with an equal weight of sharp quartz sand. It would seem that

TABLE I.—SHOWING EFFECT OF DIFFERENT LENGTHS OF TIME OF EXTRACTION AT 0° UPON DIASTATIC STRENGTH OF EXTRACT.

(16 g. material, 200 cc. water, 50 cc. aliquots used.)

		Results calculated to 1 g. material.			
Material.	Time of extraction. Hours.	Copper reduced after digestion. Gram.	Copper reduced by blank. Gram.	Copper due to enzyme. Gram.	Maltose equivalent. Gram.
Wheat	5	0.454	0.023	0.431	0.339
	3	0.422	0.016	0.406	0.319
	1	0.422	0.014	0.408	0.320
Barley	5	0.474	0.038	0.436	0.342
	3	0.465	0.036	0.429	0.337
	1	0.451	0.023	0.428	0.336
Bran	5	0.468	0.008	0.460	0.362
	3	0.434	0.005	0.429	0.337
	1	0.451	0.005	0.446	0.351
Patent flour	5	0.439	0.015	0.424	0.334
	3	0.451	0.013	0.438	0.344
	1	0.477	0.013	0.464	0.365
First clear flour	5	0.445	0.013	0.432	0.339
	3	0.436	0.007	0.429	0.337
	1	0.425	0.005	0.420	0.330
Second clear flour	5	0.477	0.024	0.453	0.356
	3	0.474	0.012	0.462	0.363
	1	0.471	0.007	0.464	0.365

by this vigorous treatment the cell tissues would be so thoroughly ruptured that there could be no possible danger of incomplete solution of the diastases. Two check samples, prepared in this way, of several different materials were treated with water at 0° for $1\frac{1}{2}$ hours and the diastatic strength of the resultant extracts determined as before, with the results shown in Table II.

TABLE II.—SHOWING EFFECT OF GRINDING MATERIAL WITH SAND UPON DIASTATIC STRENGTH OF EXTRACT.

		Results calculated to 1 g. material.			
Material.	Mode of preparation.	Copper reduced after digestion. Grams.	Copper reduced by blank. Gram.	Copper due to enzyme. Grams.	Maltose equivalent. Grams.
Barley	ordinary	0.459	0.020	0.439	0.345
	ground with sand	0.474	0.015	0.459	0.361
Wheat	ordinary	0.498	0.023	0.475	0.373
	ground with sand	0.477	0.023	0.454	0.357
Flour	ordinary	0.449	0.015	0.434	0.341
	ground with sand	0.436	0.015	0.421	0.331
Shorts	ordinary	0.965	0.012	0.953	0.736
	ground with sand	0.907	0.020	0.887	0.682
Malt	ordinary	8.433	0.033	8.400	6.460
	ground with sand	8.433	0.035	8.398	6.459

These data clearly indicate a complete extraction of active diastases at 0°. The diastatic strength of the extracts obtained after grinding with sand was sometimes slightly greater and sometimes slightly less than that from the sample prepared in the ordinary manner, indicating that the differences were due to lack of uniformity in the original samples and not to unequal extraction of the active diastases. In other work on similar samples, we were able to make these differences almost completely disappear by long-continued mixing of the material before weighing out samples for the determinations. The extractions for five hours show sometimes a slightly higher, and sometimes a slightly lower, activity than those for one or three hours, indicating that these differences are due to slight variability in the sample or some similar cause, and not to the length of time of extraction.

In each case, a slightly larger amount of reducing sugar was found in the blanks from the longest period of extraction. This might be due either to slight hydrolysis of starch during the extraction, or to slow solution of the reducing sugars in water at this low temperature. It was later ascertained that the sugar in the blank would vary if the proportion of water to material used were varied, while the diastatic activity per gram of material remained practically constant, even though the dilution were doubled or tripled. This clearly indicates that the variation in reducing sugars in the blank is due to gradual solution of the sugars in the material at the low temperature used, and not to diastatic action during the extraction. Further, it was found that the filtered extract could be kept for some hours at the temperature of ice-water without any increase in its sugar content, indicating either that there is no diastatic action at this temperature or that no soluble carbohydrates which can be hydrolyzed by the enzymes present are extracted from these materials at 0°.

In order to be certain that the maltose found was wholly due to diastatic activity, and not to hydrolysis by reagents used in the process or to any other cause, another set of extractions for varying lengths of time was made, using the same samples of ground wheat and barley as in the first series. The only variation in the process was that, just before taking the aliquots of the filtered extract for estimation of its diastatic strength, it was boiled for 10 minutes. The results obtained are shown in Table II.

TABLE III.—SHOWING EFFECT OF BOILING EXTRACTS UPON THEIR DIASTATIC STRENGTH.

Material.	Time of extraction. Hours.	Copper reduced by blank. Gram.	Copper reduced by solution from boiled extract digested with 2.5 g. starch. Gram.
Barley.....	2	0.033	0.030
	1	0.030	0.029
Wheat.....	2	0.010	0.011
	1	0.009	0.010

These results prove conclusively that the hydrolysis of starch to maltose, during the digestion for 30 minutes at 40° in the first series of experiments, was due to the presence of diastases in the extracts under investigation.

Satisfactory results having been obtained with barley and wheat products, it was decided to study the applicability of the process to barley malt. Since malt would certainly contain larger proportions of active diastases than the other materials which had been studied, it was obvious that smaller original weights of material or smaller aliquots of the extract, or both, would have to be used in order to keep a sufficient excess of hydrolyzed starch in the mixture during the digestion period. Several preliminary trials resulted in complete hydrolysis of the starch, even when as much as 5 g. of soluble starch were used with comparatively small aliquots of the extracts. Finally, a new set of extracts was made, using 8 g. malt to 200 cc. water, and a series of mixtures containing 2.5 g. of starch and 1 cc., 2 cc., 5 cc., and 10 cc. of the extract, respectively, were digested at 40° for 30 minutes and the resulting solutions tested with iodine. The solution containing 10 cc. of extract gave no color, that with 5 cc. a puplish red, and those with 2 cc. and 1 cc. a clear blue. It appeared from this that the limit of the reaction would not be reached by using 5 cc. of the extract from this particular sample of malt and that 2 cc. of extract would probably bring the conditions within the limits required to insure the operation of "Kjeldahl's law of proportionality."

Accordingly, extractions were made at 0° for varying lengths of time, and in varying volumes of mixture of malt and water, upon two different samples of malt, and digestions of varying aliquots of the resultant extracts with starch carried on as described above. Some typical results of these determinations are presented in Table IV.

In connection with these data, attention should be called to the fact that the very large amount of maltose produced by the action of a very small aliquot of the original extract made it necessary to use a very small fraction of the final solution for the reduction with Fehling's solution. The aliquot of the residual copper solution which was finally titrated with thiosulfate solution corresponded to from 0.01 g. to 0.00625 g. of original malt. Consequently, any slight variation in reading the end point was magnified from 100 to 160 times in calculating the results to the basis of 1 g. of malt.

With this fact in mind, the data in the table clearly show that complete extraction of the enzymes is obtained in a period of one hour or longer. In no case did the three-hour or five-hour extractions give significantly greater "diastatic strength," as shown by the maltose equivalent of 1 g. malt, than was obtained by extracting for only one hour. In some cases, the maltose equivalent was slightly higher in the one-hour extract than in those of longer periods, indicating that the differences

were due to experimental error in making the solutions, or to slight variations in composition of the malt. In those cases where the extraction period was shortened to 30 minutes, one of which is included in the table, somewhat lower diastatic strength was always obtained, than in the longer extractions carried on under otherwise identical conditions. It appears, therefore, that this period of extraction is not quite sufficient for complete solution of the diastatic enzymes from materials ground to the customary fineness for analysis.

TABLE IV.—SHOWING RESULTS OF APPLICATION OF PROPOSED METHOD TO ESTIMATION OF DIASTATIC STRENGTH OF BARLEY MALT.

Sample No.	Weight taken. Grams.	Water used. Cc.	Time of extraction. Hours.	Aliquot of extract. used. Cc.	Results calculated to 1 g. malt. Copper reduced after digestion. Grams.	Copper reduced by blank. Grams.	Copper due to enzyme. Grams.	Maltose equivalent. Grams.
1	8	200	5	5	8.330	0.090	8.240	6.300
	8	200	3	5	8.120	0.085	8.035	6.140
	8	200	1	5	8.120	0.085	8.035	6.140
	8	200	1/2	5	7.670	0.070	7.600	5.820
1	4	100	5	5	7.840	0.090	7.750	5.940
	4	100	3	5	7.950	0.085	7.865	6.010
	4	100	1	5	8.060	0.085	7.975	6.120
1	4	100	5	2	10.640	0.090	10.550	8.170
	4	100	3	2	10.260	0.085	10.175	7.865
	4	100	1	2	10.080	0.085	9.995	7.730
1	4	100	5	2 + 3*	8.625	0.090	8.535	6.570
	4	100	3	2 + 3*	8.510	0.085	8.425	6.485
	4	100	1	2 + 3*	8.510	0.085	8.425	6.485
2	16	200	5	2	2.620	0.150	2.470	1.935
	16	200	3	5	2.042	0.142	1.900	1.485
	16	200	1	5	2.097	0.117	1.980	1.549

It will be noted that the maltose equivalent per gram of sample was considerably higher when only 2 cc. of the extract were used for the digestion than when 5 cc. were used. This is in harmony with the results of Brown and Glendinning and the assumption of Kjeldahl mentioned in an earlier part of this paper. Evidently, 5 cc. of these extracts hydrolyzed the starch used past the limit of 60% of unchanged starch considered by Kjeldahl as necessary to insure the operation of the "law of proportionality." Further confirmation of this fact is found in the series in which the starch was first digested for 30 minutes with 2 cc. of extract, then 3 cc. more of extract added and the digestion continued for another 30 minutes. The maltose obtained in this way exceeded that obtained by using 5 cc. for 30 minutes, but did not nearly approach the equivalent

* 2 cc. digested 30 minutes, then 3 cc. additional added and digested again for 30 minutes.

amount per gram of malt obtained when only 2 cc. of extract acted for 30 minutes on the same amount of starch.

Since the extracts obtained by treating these various materials with water at 0° obey all the known laws of diastatic action, and since the diastatic strength of the extracts, as measured by maltose produced from a large excess of starch, is the same whether the extraction be continued for one, three, or five hours, we conclude that this method of operation insures a quantitative extraction of the diastases present in vegetable tissues ground to the customary degree of fineness for ordinary analyses. We plan to use this method in our further investigations, and suggest it for trial by other workers engaged in similar studies.

Directions for Use of the Proposed Method.

Prepare the sample whose diastatic strength is to be tested in the same way as for any of the common analytical processes. Weigh a suitable charge, preferably 16 grams or some even fraction thereof, into an 8 oz. wide-mouthed bottle having a water-tight stopper, and immerse the bottle and its contents in ice-water until its temperature reaches approximately 0°. Pipet into the bottle 200 cc. of pure distilled water (redistilled if necessary to insure complete freedom from acid or salts) previously cooled to 0°. Stopper the bottle tightly and immerse it in ice-water for one to two hours, shaking vigorously at fifteen-minute intervals. At the expiration of the extraction, pour the contents of the bottle quickly upon a large folded filter, pouring back the first few cubic centimeters of cloudy filtrate until the extract comes through fairly clear (a perfectly clear filtrate at this point is not absolutely necessary). If filtration should be so slow as to make any considerable rise in temperature of the contents of the filter or of the filtrate probable, place the funnel in a water-jacket filled with ice-water and immerse the receiver in ice-water.

The diastatic strength of the extract should preferably be determined at once. If this is not convenient, however, the extract must be kept at a temperature of approximately 0° until the operator is ready to proceed.

In working with materials rich in diastatic enzymes, it is desirable to make a preliminary test of the approximate amount of starch which will be hydrolyzed by various aliquots of the extract when digested under the standard conditions, in order that the final determination of maltose-producing power may be made with such proportions of volume of extract and starch that not more than 40% of the starch taken will be hydrolyzed during the digestion period. This preliminary test is easily made by measuring the same volume of starch solution that is to be used in the final digestion into each of several test tubes, warming these to 40° then adding varying volumes, say 1 cc., 2 cc., 5 cc., and 10 cc. of the extract, and carrying on the digestion for 30 minutes at 40°. At the end of this time, the solutions are tested with iodine and the one which gives just

a faint trace of blue color is regarded as showing the approximate limit of starch-hydrolyzing power of the extract. For the final determination, a volume of extract amounting to approximately 40% of that found as this limit in the preliminary test should be used.

To determine the maltose-producing power of the extract, first warm the starch solution which is to be used[1] to 40°, then pipet the volume of it which corresponds to 2.5 grams of anhydrous starch into a 100 cc. graduated flask. Place this flask in the constant temperature oven kept at 40°. Now warm the extract quickly to 40° and pipet the proper volume of it, as found by preliminary test, or 25 cc. in the case of the common cereals, into the flask containing the starch. Place this flask in the constant temperature oven and immediately pipet another aliquot of the extract into a graduated flask and add standard sulfuric acid to it in such a quantity that when filled to the mark the solution will be 0.02 *N* acid. This solution then constitutes the blank for the determination of reducing sugars already present in the extract. At the expiration of exactly 30 minutes from the addition of the extract to the starch solution, remove the flask from the oven and immediately stop the action by adding sulfuric acid of such strength and amount that the solution when brought to the mark will be 0.02 *N* acid.

To determine the amount of reducing sugars present in the blank and in the digested starch-extract mixture, cool both to room temperature and see that they are made just to the 100 cc. mark; then draw off 50 cc. of each solution and transfer it to another 100 cc. flask; add 25 cc. of a 4% solution of salt and 5 cc. of a 10% solution of phosphotungstic acid, make to the mark and shake vigorously. This precipitates the soluble proteins which would otherwise affect the Fehling's solution. After standing a few minutes the precipitate usually settles, leaving a clear supernatant liquid, an aliquot of which can be drawn off for the estimation of its reducing sugar content; if it does not settle clear, it must be filtered through a quantitative filter before taking aliquots.

The Fehling's reduction may conveniently be carried out in a 200 cc. graduated flask, in order to avoid the necessity of filtering and washing the precipitated cuprous oxide. The small volume occupied by the precipitate does not appreciably affect the volume of liquid in the flask, so that an aliquot may be taken for titration of the residual copper. Measure accurately into the flask 25 cc. of the Fehling's copper solution, add 25 cc. of the alkaline tartrate solution and 50 cc. of water; heat to boiling and then add 25 cc. of the clear filtrate from the protein-precipitation of the solutions under investigation; bring again to boiling and boil for exactly 2 minutes. At the expiration of the 2 minutes, add cold water nearly to

[1] For the method of preparing soluble starch suitable for this purpose, see the article by Sherman and Kendall previously referred to.

the mark and cool the contents of the flask quickly to room temperature and complete the volume to 200 cc. and mix thoroughly. After standing a few minutes the precipitate settles so clearly that 50 cc. of the clear supernatant liquid can usually be pipetted off for titration of the copper which it contains; or the supernatant liquid may be poured through a quantitative filter and 50 cc. of the filtrate pipetted for the titration. The titration should be carried out as recommended by Peters in the article referred to above. At the same time, another 25 cc. of Fehling's solution should be accurately measured into another 200 cc. flask, the volume made up to the mark, and a 50 cc. aliquot pipetted out and titrated for its copper content. The difference between the thiosulfate used for the titration of this 50 cc. and that obtained from the reduction by the solution under investigation gives the thiosulfate equivalent of one-fourth of the copper reduced by the sugar in the aliquot used for the reduction. From these data the maltose content of the digested solution can be computed. From this should be deducted the reducing sugars, calculated as maltose, found in the blank from the same volume of extract as was used for the starch digestion. This gives the maltose produced by the action of the diastases in the volume of extract used. The equivalent amount of maltose reduced by the extract from 1 gram of material can then be calculated, or the data used to calculate the "diastatic strength" of the material to any other basis of comparison which may be selected.

DIVISION OF AGRICUTURAL CHEMISTRY,
AGR. EXPT. STATION, ST. PAUL, MINN.