and the crystalline product left behind purified by recrystallization from boiling water. It separated, on cooling, in the form of prismatic crystals which melted at 183–184° to an oil. A mixture of this with *phenyl-alanylglycine-hydantoin* (m. 184°) melted at the same temperature.

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A STUDY OF CERTAIN CONDITIONS WHICH AFFECT THE ACTIVITY OF PROTEOLYTIC ENZYMES IN WHEAT FLOUR.

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Received February 16, 1916.

Introduction.

A study on conditions which affect the activity of amylolytic enzymes in wheat flour was reported in a previous paper.[1] In that study several facts were noticed which deserve further investigation. In planning for such work it was seen that the proteins of the flour were involved, and therefore it was thought best first to make a study of certain conditions which affect the activity of proteolytic enzymes of wheat flour.

In this investigation we used a high-grade patent flour, made by the Department of Milling Industry. Such a flour is desirable because it consists mostly of the pure floury endosperm of the wheat kernel, and has a low ash content.

The proteins of wheat flour have been the object of a large amount of investigation. Osborne and Voorhees[2] separated the nitrogenous substances in wheat flour into five distinct proteins known as gliadin, glutenin, globulin, leucosin, and proteose. Because of the number and complex nature of the proteins of wheat flour it was decided to confine this study to the protein leucosin freely soluble in pure water.

It is recognized that the figures obtained would have been different had we used the whole ground wheat kernel, or flour less free from proteins of the bran coat and germ. The results would probably also have been different had the other proteins of the flour been included. It is planned to continue this experiment under conditions where the other proteins of the flour are included, and also on the whole ground wheat kernel.

Methods of Experimentation.

A large number of preliminary trials were made in order to determine the best methods of procedure. In this report are presented only the results of trials that were found workable and of value in this study. The data obtained in the different trials are not given in the chronological order of performance, but in a manner calculated to make the report clear.

[1] THIS JOURNAL, 35, 1635–1643 (1913).

[2] *Am. Chem. J.*, 15, 392–471 (1893).

All of the determinations were made in quadruplicate, and wherever the agreement was not within reasonable limits, the trial was repeated. The results here given are the average of the determinations made under acceptable conditions.

The general plan of procedure was to digest a water extract of flour under the conditions of the trial and then determine the increase in the content of nitrogen in the amino form. For determining the amount of nitrogen in this form at any stage of the experiment, the formal titration as elaborated by Sörensen[1] was employed. By using the water extract, the study is confined principally to the action of proteolytic enzymes on the water-soluble protein known as leucosin. The acids, alkali, salts, or other substances used in this study were added to the flour extract.

The water extract from sound flour contains nitrogenous compounds having free amino and carboxyl groups. If this extract is neutralized and then formaldehyde added, the amount of titrable material is always a measurable quantity. It is necessary to determine the amount of such titrable material before we can measure the protein cleavage due to the action of proteolytic enzymes. This determination is reported as the blank in the tables that follow.

In this investigation the influence of bacterial action was eliminated by the use of neutral toluene water in making the extractions and digestions. Ordinary distilled water was made neutral by passing through it air freed from carbon dioxide. To such water was added 5 cc. of neutral toluene for 8 liters, or a little more than $^1/_2$ cc. per liter. We believe that this amount of toluene was sufficient to inhibit bacterial action, because the extracts were always found neutral to litmus paper tested according to the method of Henriques and Sörensen,[2] and toluene was seen floating on the surface of the extract. If such an excess of toluene was not noticed more was added. In several preliminary trials four times the above amount of toluene was used, but the results were not essentially different from those obtained with the smaller quantity which was sufficient to provide an excess.

The following is a brief outline of the method we used in determining the amount of nitrogen in the amino form present in flour. For the sake of brevity, the nitrogen in amino form will be called the titrable nitrogen. A definite amount of flour is mixed with ten times its weight of neutral toluene water, and shaken at frequent intervals for one hour. This is then allowed to settle for 30 minutes. If the extraction is to be made at a given temperature it is necessary to heat the water and flour to this temperature before mixing and then keep the container in a thermostat for the re-

[1] H. Jessen-Hanse in "Handbuck der Arbeitsmethoden von Abderhalden, V," 6, 262–277.

[2] *Z. physiol. Chem.*, 64, 133; also "Handbuch der Arbeitsmethoden," *loc. cit.*

quired time. A narrow-mouth glass stoppered bottle is a convenient container. After settling, the supernatant liquid is filtered first through cotton, then through folded filter paper. Measure four 100 cc. portions into Erlenmeyer flasks, marked, say, *a*, *b*, *c*, *d*. Using these proportions of flour and water gives the results as titrable material in the extract from 10 g. of flour. To *c* and *d* add 5 cc. of solution of thymolphthalein, made by dissolving 0.5 g. in 1000 cc. of 93% neutral alcohol, and also 10 cc. of 40% formaldehyde neutral to the thymolphthalein solution, and let stand 15 minutes or more. While waiting, titrate flasks *a* and *b* with 0.05 *N* $Ba(OH)_2$, using 5 cc. of a 0.5% neutral alcoholic solution of phenolphthalein as indicator. The titration of flasks *a* and *b* is called the first stage. When solutions *c* and *d* have stood 15 minutes or more titrate to a faint blue. This end point is quite delicate and there is no difficulty in getting the exact reading. The titration of *c* and *d* is called the second stage. The average reading for *a* and *b* subtracted from the average reading for *c* and *d* gives the figure for calculating the amount of titrable nitrogen. If 0.05 *N* alkali is used the difference times 0.7 gives the milligrams titrable nitrogen in the extract.

As a check on the work, add the formaldehyde to flasks *a* and *b* as soon as the first stage titration is completed, and let stand 15 minutes or more. Titrate to a faint pink, then add the alkali, drop by drop, shaking well, till the rose-red color is obtained. It will be found that the total amount of alkali added to *a* and *b* is less than that added to *c* and *d* if the titration is stopped when the faint pink color is obtained in the second stage, but if the titration is continued to a rose-red the amounts will be practically equal. That it is necessary to titrate to a deep rose-red when phenolphthalein is used as indicator was determined by dissolving several pure amino acids in neutral water and then titrating, similar to what was done by Sörensen. If the titration is carried to a faint pink, only about 95% of the nitrogen in the amino form is obtained.

In making any formal titration, after ascertaining that the solution is neutral, it is necessary to make sure that no ammonia is present. It was found that all flour extracts used in this investigation, unless acids or alkali had been added, were neutral, and that no ammonia was present.

While flour extract is neutral to this litmus paper, it is not neutral to phenolphthalein, as shown by the titrable material obtained in the first stage. The neutralization of the alkali used in the first stage is due to the presence of acid phosphates, organic acids, if such are present, or any substance in the flour that has the power to neutralize an alkali. The substances in the flour extract which neutralize the alkali in the first stage have been discussed by Swanson in a previous paper.[1] This first stage is the usual method of determining acidity in wheat flour.

[1] *J. Ind. Eng. Chem.*, **4**, 274 (1912); also Kans. Agr. Expt. Sta., *Bull.* **202**.

Why is it necessary to titrate portions *a* and *b*? Why not titrate portions *c* and *d* in the two stages? Phenolphthalein is a more sensitive indicator for the first stage, and it is necessary first to measure as far as possible the neutralization due to substances other than the carboxyl groups which are a part of the amino acids, if we are to obtain a true measure of nitrogen in the amino form. That these substances neutralized in the first stage are mostly acid phosphates is probable from the work referred to above. Thymolphthalein is a more sensitive indicator for the second stage with opalescent solutions like these flour extracts. With phenolphthalein it is difficult to determine the amount of alkali to add beyond the faint pink end point to produce the required rose-red color. With clear solutions this difficulty is absent and the two stages of titration can be made on the same portion of extract, using phenolphthalein as indicator. The color end point for each stage is matched against a standard prepared each day, by adding to 100 cc. of neutral water 5 cc. of the phenolphthalein or thymolphthalein solution as the case may be, and exactly 8 drops of 0.05 *N* $Ba(OH)_2$.

Experimental.

The digestions were made at room temperature unless otherwise stated.

Trial I.—In order to determine the best proportion in which to mix flour and water, and the time necessary for complete extraction at 25°, flour and water were mixed in a bottle in the following proportions: 1 : 5, 1 : 10, 1 : 20, 1 : 50. These were shaken at frequent intervals, and at the end of definite periods small amounts were poured on four different folded filters, to get as rapid filtration as possible, and only enough was poured on the filter to give sufficient filtrate to measure portions large enough to represent the extract from 10 g. of flour. The main portion in the bottle was again shaken at frequent intervals, and at the end of the next period the filtration was repeated. The four portions obtained at the end of each period were titrated for the first and second stages, with the result given in cc. of $Ba(OH)_2$ neutralized.

TABLE I.—BEST PROPORTION IN WHICH TO MIX FLOUR AND WATER AND THE TIME NECESSARY FOR COMPLETE EXTRACTION AT 25°.

1:5.		1:10.		1:20.		1:50.		
1st.	2nd.	1st.	2nd.	1st.	2nd.	1st.	2nd.	Time extracted.
1.20	2.00	0.95	1.55	1.20	1.40	1.50	2.00	10 minutes
1.70	2.20	1.50	2.20	1.40	2.20	1.75	2.25	20 minutes
1.90	2.60	1.90	2.30	2.00	2.20	2.00	3.00	40 minutes
2.10	2.80	2.00	2.40	2.20	2.20	2.50	3.00	60 minutes
2.10	2.90	2.00	2.40	2.20	2.20	2.50	3.00	90 minutes
2.10	2.60	2.00	2.40	2.20	2.40	2.50	3.00	120 minutes

Proportion 1 : 5 was too concentrated and filtering was difficult. Proportion 1 : 50 was too dilute and the error on the end point relatively too large. Proportions 1 : 10 and 1 : 20 gave results that were practically the same.

Trial II.—This was a repetition of Trial I, except that the water was heated to 37° before it was mixed with the flour, and the bottle containing the mixture was kept at this temperature. The results show that more titrable material is extracted at 37° than at 25°, and also that the titrable material in the flour is extracted in an hour's time, and that extraction for another hour gives no important increase.

TABLE II.—BEST PROPORTION IN WHICH TO MIX FLOUR AND WATER AND THE TIME NECESSARY FOR COMPLETE EXTRACTION AT 37°.

1:5.		1:10.		1:20.		1:50.		Time extracted.
1st.	2nd.	1st.	2nd.	1st.	2nd.	1st.	2nd.	
1.80	2.00	2.00	2.20	2.40	1.60	2.00	3.00	10 minutes
2.00	2.40	2.30	2.70	2.40	2.40	2.25	3.75	20 minutes
2.10	2.70	2.55	2.70	2.40	2.40	2.50	4.00	40 minutes
2.10	2.70	2.60	2.70	2.60	2.40	2.50	4.00	60 minutes
2.20	3.00	2.60	2.70	2.60	2.40	2.50	4.00	90 minutes
2.30	3.00	2.60	2.70	2.60	2.60	2.50	4.00	120 minutes

Effect of Inorganic Compounds.

In the following tests it was necessary to have a large amount of flour extract. The extract for each test was prepared as follows: 700 g. of the flour were weighed into an 8-liter bottle. To this was added 7000 cc. of neutral toluene water, and the whole was shaken for one hour. It was then allowed to settle for one hour, and about 6000 cc. siphoned off and filtered, first through cotton and then through a folded filter. From this extract four 100 cc. portions were measured and titrated for the first and second stages. This constituted the blank titration. The rest of the flour extract was placed in a large bottle and used for the tests under the conditions given. Where the digestion was conducted at a temperature higher than that of the laboratory, a thermostat was used. This was provided with a stirrer and thermoregulator, and a constant temperature was easily maintained.

Trial III.—To determine the effect of digesting the flour extract alone. The flour extract was kept at a temperature of very nearly 40° and at the end of each week the amount of titrable material was determined. The amount of titrable material obtained in the first stage shows a slight increase during the first week, after which the results obtained are constant.

TABLE III.—RESULTS FROM DIGESTING THE FLOUR EXTRACT ALONE.

Time.	1st. stage. Cc. 0.05 *N* $Ba(OH)_2$.	2nd stage. Cc. 0.05 *N* $Ba(OH)_2$.	Milligrams N in amino form.
blank	3.3	2.7	1.89
1 week	4.3	3.6	2.52
2 weeks	4.1	4.4	3.08
3 weeks	4.4	4.9	3.43
4 weeks	4.4	6.0	4.20
5 weeks	4.4	6.0	4.20
6 weeks	4.2	6.4	4.48
7 weeks	4.2	6.2	4.34

The amount of titrable nitrogen obtained in the second stage increases slightly to the end of the fourth week, after which there is no definite change The results in cc. are reported only to the first decimal place, as beyond that they have no significance.

Trial IV.—To determine the effect of adding to the flour extract 50 mg., respectively, of potassium dihydrogen phosphate, potassium hydrogen phosphate, or potassium orthophosphate, for each 10 g. of flour, extracted, and digesting at 40°.

Potassium Dihydrogen Phosphate is acid and the amount of $Ba(OH)_2$ neutralized in the first stage is very much increased. In Trial III the amount of 0.05 *N* $Ba(OH)_2$ neutralized in the blank for the first stage was 3.3 cc. and here it was 12.7 cc. The 9.4 cc. $Ba(OH)_2$ was neutralized by the 50 mg. KH_2PO_4. The amount of titrable nitrogen increases during the first two weeks, but the increase is not materially different from the results obtained when only neutral water was used, showing that this salt has no catalytic action on protein cleavage. If there is any influence it is retarding.

TABLE IV.—EFFECT OF POTASSIUM PHOSPHATES.

Time.	1st stage. Cc. 0.05 *N* $Ba(OH)_2$.	2nd stage. Cc. 0.05 *N* $Ba(OH)_2$.	Milligrams N in amino form.
	Potassium Dihydrogen Phosphate.		
blank	12.7	2.6	1.82
24 hours	12.5	2.9	2.03
1 week	12.4	4.8	3.36
2 weeks	12.7	5.6	3.92
3 weeks	12.7	5.4	3.78
4 weeks	12.1	5.7	3.99
5 weeks	12.5	5.2	3.64
	Potassium Hydrogen Phosphate.		
blank	2.4	3.3	2.31
1 week	2.9	4.3	3.01
2 weeks	4.4	4.0	2.80
3 weeks	4.4	4.2	2.94
4 weeks	4.6	4.1	2.87
5 weeks	5.0	4.4	3.08
6 weeks	5.1	4.4	3.08
	Potassium Orthophosphate.		
blank	0.4[1]	3.2	2.24
1 week	0.0	4.2	2.94
2 weeks	0.6	4.6	3.22
3 weeks	0.7	5.1	3.75
4 weeks	1.2	5.2	3.64
5 weeks	1.5	4.5	3.15
6 weeks	1.8	5.0	3.50
7 weeks	1.7	5.3	3.71
8 weeks	2.0	5.9	4.13

[1] 0.4 HCl added.

Potassium Hydrogen Phosphate was slightly basic as shown by the fact that the blank titration for the first stage is a little less than in Trial III. This basicity decreases slowly but definitely. The amount of titrable nitrogen shows no increase after the first week. This salt has, therefore, in the amount used, an inhibiting effect on protein cleavage.

Potassium Orthophosphate is basic, and the addition of this amount of salt is more than sufficient to neutralize the acidity of the flour extract as shown by the titration of the blank. The acidity of the flour extract is probably due to the presence of salts similar to KH_2PO_4, judging from the amount of bases and phosphorus present. The addition of K_3PO_4 would react with the KH_2PO_4, forming K_2HPO_4, which, as shown, is almost neutral or only slightly basic. The basicity decreases. At the end of the first week the extract was neutral to phenolphthalein, and the acidity gradually increased. The K_3PO_4 has a retarding effect on protein cleavage.

Trial V.—To determine the effect of adding to the flour extract 80 mg. KOH for every 10 g. of flour extracted. This made the solution alkaline.

TABLE V.—EFFECT OF ADDING POTASSIUM HYDROXIDE.

Time.	1st stage. Cc. 0.05 *N* HCl.	2nd stage. Cc. 0.05 *N* $Ba(OH)_2$.	Milligrams N in amino form.
blank	14.6	3.4	2.38
1 week	9.6	3.4	2.39
2 weeks	7.3	3.3	2.31
3 weeks	7.1	4.0	2.82
4 weeks	6.5	3.3	2.31
5 weeks	6.2	3.0	2.10
6 weeks	5.0	2.8	1.96
7 weeks	5.1	2.4	1.68
8 weeks	5.4	2.6	1.82

The alkalinity decreases regularly till the end of the fifth week. The result is very similar to that obtained in Trial IV with K_3PO_4. It may mean that more KH_2PO_4 is formed through hydrolytic action. The amount of titrable nitrogen does not increase, showing that the presence of this amount of KOH stops protein cleavage.

Trial VI.—To determine the effect of adding 100 mg. K_2S for each 10 g. of flour extracted. The effect of this salt was a slight increase in the acidity during the first week, after which the results were constant. The increase in the amount of titrable nitrogen is not greater than when water

TABLE VI.—EFFECT OF ADDING POTASSIUM SULFIDE.

Time.	1st stage. Cc. 0.05 *N* $Ba(OH)_2$.	2nd stage. Cc. 0.05 *N* $Ba(OH)_2$.	Milligrams N in amino form.
blank	4.7	3.1	2.17
1 week	7.5	3.8	2.66
2 weeks	7.9	4.6	3.22
3 weeks	7.4	6.0	4.20
4 weeks	7.2	6.2	4.34

alone was used, showing that K_2S in the amount used, has no influence on the rate of protein cleavage.

Trial VII.—To determine the effect of adding to the flour extract 50 mg. of NH_4Cl for each 10 g. of flour extracted. There was no notable change in the amount of titrable material obtained in the first stage until after the fourth week. The amount of titrable nitrogen obtained in the second stage was large. The great increase in the blank is due to the presence of ammonia.

TABLE VII.—EFFECT OF ADDING AMMONIUM CHLORIDE.

Time.	1st stage. Cc. 0.05 *N* $Ba(OH)_2$.	2nd stage. Cc. 0.05 *N* $Ba(OH)_2$.	Mg. N in amino form, due to presence of NH_4Cl.
blank	4.7	21.0	12.70
1 week	4.7	23.7	14.59
2 weeks	5.1	26.0	16.20
3 weeks	4.7	28.0	17.60
4 weeks	4.9	29.2	18.44
5 weeks	7.0	34.2	21.94

The presence of NH_4Cl increases the amount of protein cleavage more than any of the previous salts used in this series of experiments. The average mg. nitrogen obtained in the blanks of the preceding trials is very nearly 2. The increase beyond this amount is due to the presence of the NH_4Cl.

Trial VIII.—To determine the effect of adding to the flour extract 50 mg. of $CaCl_2$ for each 10 g. of flour extracted. The amount of titrable material obtained in the first stage shows a very slight increase during the first week.

TABLE VIII.—EFFECT OF ADDING CALCIUM CHLORIDE.

Time.	1st stage. Cc. 0.05 *N* $Ba(OH)_2$.	2nd stage. Cc. 0.05 *N* $Ba(OH)_2$.	Milligrams N in amino form.
blank	3.6	2.6	1.82
1 week	4.2	5.2	3.64
2 weeks	4.5	7.2	5.04
3 weeks	5.4	11.0	7.70
4 weeks	5.2	13.5	9.45
5 weeks	4.4	14.7	10.29

The amount of titrable nitrogen obtained in the second stage shows that $CaCl_2$ is practically equal to NH_4Cl in affecting the rate of protein cleavage.

Trial IX.—To determine the effect of adding to the flour extract HCl, in such amounts as to make the concentration of the solution equivalent to 0.01 *N*. As it would take 20 cc. of the 0.05 *N* $Ba(OH)_2$ to neutralize the HCl present in the 100 cc. portion titrated, the difference between 20 and the figures given shows the amount of $Ba(OH)_2$ neutralized by the substances present in the flour extract. These figures are very nearly the same as would be obtained in titrating the flour extract alone as in

Trial III. The amount of titrable nitrogen obtained in the second stage shows that at this concentration HCl inhibited protein cleavage.

TABLE IX.—EFFECT OF ADDING HCl.

Time.	1st stage. Cc. 0.05 *N* $Ba(OH)_2$.	2nd stage. Cc. 0.05 *N* $Ba(OH)_2$.	Milligrams N in amino form.
blank	22.44	2.40	1.68
1 week	23.44	3.45	2.42
2 weeks	23.12	3.10	2.17
3 weeks	23.00	3.80	2.66
4 weeks	23.10	4.10	2.87

Trial X.—To determine the effect of adding to the flour extract HCl in such amounts as to make the concentration equivalent to 0.1 *N*. It would require 200 cc. of the 0.05 *N* $Ba(OH)_2$ solution to neutralize the HCl present in the 100 cc. portions titrated. In addition to this it would take about 2.5 cc. 0.05 *N* $Ba(OH)_2$ to neutralize the substances in the flour extract. The figures show that it required only 196 cc. $Ba(OH)_2$. This means that the equivalent of 6.5 cc. 0.05 *N* HCl was absorbed or neutralized by the substances in the flour extract. The HCl at this concentration completely inhibits protein cleavage.

TABLE X.—EFFECT OF ADDING HCl EQUIVALENT TO A CONCENTRATION OF 0.1 *N*.

Time.	1st stage. Cc. 0.05 *N* $Ba(OH)_2$.	2nd stage. Cc. 0.05 *N* $Ba(OH)_2$.	Milligrams N in amino form.
blank	196.0	3.3	2.3
1 week	196.0	2.0	1.4
2 weeks	196.0	2.0	1.4
3 weeks	196.0	2.0	1.4

Effect of Adding Organic Substances.

Trial XI.—To determine the effect of adding to the flour extract 1.25 grams of desiccated egg albumen for each 10 g. of flour extracted.

TABLE XI.—EFFECT OF ADDING EGG ALBUMEN.

Time.	1st stage. Cc. 0.05 *N* $Ba(OH)_2$.	2nd stage. Cc. 0.05 *N* $Ba(OH)_2$.	Milligrams N in amino form.
blank	3.0	10.2	7.14
1 week	2.9	10.7	7.49
2 weeks	2.9	11.4	7.98
3 weeks	2.9	11.7	8.19

The egg albumen has no effect on the amount of titrable material obtained in the first stage. The amount of titrable nitrogen obtained in the second stage shows a small increase in protein cleavage, but this is not as great as would be obtained were egg albumen not present. The amount of titrable nitrogen obtained in the blank shows that the protein in the desiccated egg albumen has a large amount of nitrogen in the amino form.

Trial XII.—To determine the effect of digesting desiccated egg albumen in water, adding the egg albumen at the rate of 1.25 g. for every 100 cc. Egg albumen mixed with water has a slightly acid reaction, as shown

TABLE XII.—DIGESTING ALBUMEN IN WATER.

Time.	1st stage. Cc. 0.05 *N* $Ba(OH)_2$.	2nd stage. Cc. 0.05 *N* $Ba(OH)_2$.	Milligrams N in amino form.
blank	1.6	10.6	7.42
1 week	1.5	8.9	6.23
2 weeks	1.7	11.2	7.84
3 weeks	1.9	10.9	7.63
4 weeks	2.1	11.0	7.70

by the figures obtained in the first stage titration. This acidity apparently increases during the progress of digestion. The amount of titrable material obtained in the second stage does not show any regular increase or decrease.

Trial XIII.—To determine the effect of adding to the flour extract 1.25 g. of desiccated egg albumen for each 10 g. of flour extracted, and digesting at 40°. The amount of titrable material obtained in the first stage was greater than when the digestion was conducted at the lower temperature. It shows no marked increase beyond the first week. The amount of titrable nitrogen obtained in the second stage is more than doubled in the five weeks. This trial, in connection with Trial XI, shows that 40° is a much more favorable temperature for protein cleavage than room temperature. If the figures in Table XIII are compared with those in Table III, it will be observed that the protein cleavage goes on at a much greater rate when protein from egg albumen is present in the extract. This may be due in part, at least, to the greater concentration of the protein.

TABLE XIII.—EFFECT OF ADDING EGG ALBUMEN AND DIGESTING AT 40°.

Time.	1st stage. Cc. 0.05 *N* $Ba(OH)_2$.	2nd stage. Cc. 0.05 *N* $Ba(OH)_2$.	Milligrams. N in amino form.
blank	4.9	10.3	7.21
1 week	5.6	14.6	10.21
2 weeks	5.5	16.1	11.27
3 weeks	5.2	19.2	13.44
4 weeks	5.0	21.9	15.33
5 weeks	5.4	23.8	16.66

Trial XIV.—To determine the effect of digesting desiccated egg albumen in water at 40°, adding the egg albumen at the rate of 1.25 g. for each 100 cc. The amount of titrable material obtained in the first stage is less than when the digestion was conducted at room temperature.

TABLE XIV.—DIGESTING EGG ALBUMEN IN WATER AT 40°.

Time.	1st stage. Cc. 0.05 *N* $Ba(OH)_2$.	2nd stage. Cc. 0.05 *N* $Ba(OH)_2$.	Milligrams N in amino form.
blank	1.30	9.40	6.58
1 week	1.30	9.40	6.58
2 weeks	1.50	9.50	6.65
3 weeks	1.30	9.80	6.86

There is no increase as the time of digestion is increased. The amount of titrable nitrogen obtained in the second stage is somewhat less than when the digestion was conducted at room temperature, and there is no increase.

Trial XV.—To determine the effect of adding to flour extract 1.25 g. of casein for each 10 g. of flour extracted, and digesting at 40°. A weighed amount of buttermilk casein was dissolved in the minimum amount of NaOH solution. This made a neutral solution of sodium caseinate. After filtering it was added to flour in such proportions that each 100 cc. contained 1.25 g. of casein. The titrable material obtained in the first stage is almost the same as when the flour extract was titrated, showing that the sodium caseinate was neutral. The acidity increases slowly but regularly. The amount of titrable nitrogen obtained in the blank for the second stage shows that casein has a larger amount of nitrogen in the free amino group than has the egg albumen. The amount of

TABLE XV.—EFFECT OF ADDING CASEIN AND DIGESTING AT 40°.

Time.	1st stage. Cc. 0.05 *N* $Ba(OH)_2$.	2nd stage. Cc. 0.05 *N* $Ba(OH)_2$.	Milligrams N in amino form.
blank	3.7	13.4	9.38
1 week	5.1	13.7	9.59
2 weeks	5.8	14.9	10.43
3 weeks	6.9	16.6	11.62
4 weeks	7.1	16.9	11.83

increase in the titrable nitrogen as the digestion progresses is no more than was obtained with the flour extract alone in Trial III, showing that the presence of protein from the casein does not cause a more rapid rate of protein cleavage, as was the case when the protein from egg albumen was present.

A fair question is: Are the observed changes due to proteolytic enzymes, or are they simply the result of hydrolytic action of water, or are they due to bacterial action? We believe that we have eliminated bacterial action, because in preliminary trials we compared the use of different amounts of toluene with results essentially the same. We judged the amount to be used by the technique followed by American and European workers. We believe that the changes observed are due to proteolytic enzymes and not to the hydrolytic action of water alone, because the observed changes were according to the generally observed behavior of enzymes in respect to activators, inhibitors, and action on other proteins, such as egg albumen.

Summary.

This paper presents the results of a study on certain conditions which affect the activity of proteolytic enzymes in wheat flour. It describes the use of the formal titration in determining the amount of nitrogen in the amino form present in flour, and also its use as a means of measuring protein cleavage due to proteolytic enzymes present in the flour. Of the

various salts tried, NH_4Cl and $CaCl_2$ had the greatest accelerating effect on the rate of protein cleavage. The proteolytic enzymes present in wheat flour caused a more rapid hydrolysis of the proteins when desiccated egg albumen was present, but not when casein was used.

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ADSORPTION OF INVERTASE.

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Received March 11, 1916.

Hedin and his collaborators, Jahnson-Blohm, and Eriksson,[1] found that the presence of certain substances like serum, egg albumin, saponin, cholestrin, and in some cases the substrate itself, lessened the inhibition of the activity of enzymes like rennet, trypsin and invertase brought about by the presence of solid powders (charcoal), or substances soluble in water as colloids (serum and egg albumin). They considered this inhibition as due to the adsorption and removal of the enzyme from the sphere of action by the charcoal or serum. When, however, certain substances capable of being adsorbed by the inhibitor are present or added to the reaction mixture, they can replace part or all of the enzyme in the enzyme-adsorbent, and the liberated enzyme becoming active again, decreases the amount of inhibition.

It has been shown in a previous paper[2] that, although charcoal does adsorb invertase (and possibly serum and egg albumin do likewise), the apparent inhibiting effect is not due to this, but to a change in the hydrogen ion concentration of the reaction mixture produced by the charcoal, etc. In the light of these results, the explanation given by Hedin for the decrease in inhibition is untenable in the case of invertase at least, since the activity is independent of whether the invertase is adsorbed or not. This is further substantiated by the results indicated below, where the addition of a second substance such as saponin, serum or egg albumin to the enzyme-inhibitor mixture does affect the amount of invertase adsorbed by an inhibitor like charcoal or aluminium hydroxide. In no case is there any noticeable change in the activity as long as the hydrogen ion concentration is kept constant by means of suitable buffers.

Since very little experimental data were given in the previous paper concerning the activity of invertase while adsorbed by charcoal and gelatinous aluminium hydroxide, and since the results have an important bearing on the chemistry of enzyme action in general, as can be seen from the statements based on Hedin's conclusions, occurring in many text-

[1] *Z. Physiol. Chem.*, **72**, 324 (1911); **82**, 175, 178 (1912).

[2] THIS JOURNAL, **38**, 722 (1916).