The Precipitation of Proteins from Cereal Extracts by Sodium Tungstate[1,2]

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A rapid, convenient method is described, based upon the sodium tungstate reagent of Folin and Wu, for the clarification and removal of proteins from those cereal extracts or suspensions which are intended for the determination of reducing sugars by the Munson-Walker method.

The complete removal of the proteins depends upon the proper acidification of the protein-tungstate suspension to a pH of 2 or less.

All filtrations are eliminated, with a great saving of time.

Diastatic activity is shown to be completely inhibited by the procedure described. This result is not accomplished by clarification with lead acetate.

The natural reducing sugars of cereal extracts are conveniently determined by the method described, owing to the complete inhibition of diastatic activity by the sodium tungstate and acid.

EXPERIMENTS conducted on the determination of the activity of the natural diastatic enzymes in wheat flour made it necessary to determine maltose and other reducing sugars produced in flour-water suspensions. In order to determine the reducing sugars in a suspension of flour in water which has undergone autolytic digestion for various periods of time, it is necessary both to inhibit diastatic activity and remove the colloidal materials, the proteins and dextrins, which are in suspension. The longer the digestion continues the greater is the degree of dispersion of these colloidal materials. Considerable difficulty was encountered in obtaining solutions of the sugars resulting from enzymatic action, which were sufficiently clear for quantitative sugar determinations. Lead acetate as a clarifying agent was found to be practically worthless for this purpose, primarily because of the difficulty of filtration. The previous inhibition of enzymatic activity by acid or alkali is not satisfactory because of the necessity of again neutralizing the solution before adding the lead reagent.

The literature supplies numerous alternative methods for investigation. Blish[3] made a study of protein precipitants and reported that "reagents ordinarily used for precipitating proteins, such as alkali, acetic acid, trichloracetic acid, salts of heavy metals, colloidal iron, aluminium hydroxide cream, phosphotungstic acid, and tannic acid, are for various reasons unsatisfactory for removing gliadin from water extracts of flour."

He recommended 0.1 *N* copper sulfate and sodium hydroxide as the most efficient precipitant of protein nitrogen. Swanson and Calvin[4] and Thatcher and Koch[5] used phosphotungstic acid for the clarification of their flour suspension or extracts. This reagent appears to be the most serviceable for rapid work, but its cost is excessive when used in quantity for control and service laboratories. Folin and Wu[6] developed a new protein precipitant, tungstic acid, which was applied to the precipitation of blood proteins. After the addition of one volume of 10 per cent sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) to diluted blood serum, they added, with shaking, one volume of $^2/_3$ *N* sulfuric acid, and the resulting precipitate was in such form that it could be easily centrifuged and filtered. Because of the efficiency and ease of application, combined with greatly reduced expense, it would seem that this reagent deserves a more general use for the clarification of colloidal protein suspensions. A few trials on flour suspension or extracts and malt sirups gave promise of its being better suited to these than any other method yet employed.

Preliminary Trials

For preliminary trials with Folin and Wu's reagent, 10-g. samples of flour were shaken up in 100 cc. of distilled water and allowed to stand 1 hr. with frequent shaking, diluted to 200 cc., and then clarified. It was found that 3 cc. of 15 per cent sodium tungstate solution were sufficient to precipitate the proteins from 10 g. of flour. In these preliminary trials the supernatant liquid after clarification sometimes became clear immediately, the flocculated proteins settling out rapidly, but at other times the cloudiness persisted after a half-hour's centrifuging. The reason for this failure in some instances was obviously dependent upon the final pH of the colloidal suspension. The addition of sodium tungstate to a suspension of normal flour in water produces an alkaline reaction. The complete precipitation of the proteins from a colloidal suspension depends upon their adsorption to or combination with the tungstate ion and the subsequent precipitation of the coagulated aggregate by throwing the hydrogen-ion concentration over sufficiently far to the acid side of the isolectric point. This was accomplished by Folin and Wu through the addition of $^2/_3$ *N* H_2SO_4 in a quantity which neutralized the combined alkalinity of the tungstate and blood serum, with a slight excess. In the case of flour extracts, however, the higher buffer value of the phosphates and proteins present requires a much larger excess of acid to produce the necessary hydrogen-ion concentration. The hydrogen-ion concentrations of these solutions after clarification were determined electrometrically in Bailey[7] hydrogen electrodes, and they were found to have values in terms of pH ranging from 2.12 to 1.34. Those solutions in which the clarifications were unsatisfactory invariably had a pH of above 2.6. These and subsequent results confirmed the fact that the success of the sodium tungstate clarification depends upon proper acidification of the sodium-tungstate-protein suspension to a hydrogen-ion concentration of 1×10^{-2} or more, corresponding to a pH of 2 or less. There is no danger of precipitating the hydrated colloidal tungstic acid, even with a much larger concentration of acid.

Sodium Tungstate as Clarifying Agent for Flour Suspensions

The efficiency of the sodium tungstate reagents as a clarifying agent for flour suspensions was determined by nitrogen analyses on aliquots of the clarified solutions. The amount of soluble nitrogen remaining in solution was determined before and after clarification, using different amounts of

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[3] *J. Biol. Chem.*, **33** (1918), 551.

[4] *J. Am. Chem. Soc.*, **35** (1913), 1635.

[5] *Ibid.*, **36** (1914), 759.

[6] *J Biol. Chem.*, **38** (1919), 81.

[7] *J. Am. Chem. Soc.*, **42** (1920), 45.

tungstate and varying acidities. Table I is a summary of these results and shows the amount of nitrogen remaining in solution after clarification under different conditions.

TABLE I

Flour G.	Final Volume of Clarified Solution Cc.	Na_2WO_4 15% Cc.	Acid Added	pH of Resulting Solution	Nitrogen Remaining in 100 Cc. of Solution G.	Remarks on Clarification
5	100	4	5 cc. $^2/_3$ N H_2SO_4	3.88	0.0034	Fair
5	100	2	2.5 cc. N H_2SO_4 10 drops in excess by thymol blue[1]	2.60	0.0021	Excellent
5	100	3	Acid by methyl orange	5.04	0.0129	Poor, very cloudy
5	100	2	10 drops in excess by methyl orange	3.44	0.0027	Good
5	100	2	Acid by thymol blue[1]	2.54	0.0021	Very good
10	200	3	4 cc. N H_2SO_4	2.12	0.0026	Excellent
10	200	3	0.4 cc. conc. H_2SO_4	1.47	0.0021	Excellent after 1 hr. digestion
10	200	3	0.4 cc. conc. H_2SO_4	1.46	0.0021	Excellent after 3 hrs. digestion
10	200	0	.....	5.78	0.0241	No clarification after 1 hr. digestion
10	200	0	.....	5.96	0.0379	No clarification after 3 hrs. digestion

[1] Seven drops thymol blue indicator.

The residual nitrogen appears to reach a fairly constant minimum for the flour samples used under the conditions of clarification as shown in the table. Other materials than flour contain different quantities of soluble amino nitrogen and ammonia nitrogen which are not removed by the tungstate procedure but which have no vitiating effect upon the determination of reducing sugars. As a result of these data the following method has been developed for the removal of protein material from the flour suspensions in which the reducing sugars are to be determined:

The flour suspension or extract, containing in this case 10 g. of material per 100 cc., is transferred to a 200-cc. volumetric flask and diluted, with several drops of thymol blue added as an indicator, to about 175 cc.; 3 cc. of 15 per cent sodium tungstate ($Na_2WO_4.2H_2O$) are added, and the contents of the flask thoroughly shaken up. Then the concentrated sulfuric acid is added drop by drop from a micropipet, with constant shaking until the thymol blue turns pink, with 2 or 3 drops in excess. The resulting solution should correspond to a pH of about 2. The suspension in the flask is diluted to the mark and thoroughly shaken. It is then poured into centrifuge cups and whirled for a few minutes. Aliquots of the clear supernatant liquid may then be used without filtration for the determination of reducing sugars by the Munson-Walker method. The concentrated sulfuric acid appears to be more efficient in precipitating the protein tungstate than the $^2/_3$ N acid of Folin and Wu. The precaution of adding the acid slowly drop by drop with shaking of the solution must be observed, otherwise a local concentration will produce a precipitation of flocks of colloidal tungstic acid and with further danger of decomposing some of the carbohydrates. Thymol blue[8] with its acid range at pH 2 serves as a very convenient indicator for the first few trials with a new sample, since it is necessary to add only 2 or 3 drops of the concentrated acid in excess of the pink color to produce the proper acidity for a complete precipitation. After a few trials uniform results were always obtained by measuring the acid from a 1-cc. micropipet or counting the number of drops required to produce the proper color by thymol blue.

Because of their higher buffer value, malt flour suspensions are found to require a slightly larger amount of acid. Difficulties were encountered in the clarification of suspensions to which considerable quantities of acid had previously been added. The same principle of colloidal protein precipitation applies here as well, and the clarification is satisfactory if the suspension is first neutralized by means of a few drops of strong sodium hydroxide. The thymol blue, alkaline range, blue color (pH 8.0 to 9.6) likewise serves for this point, and although the hydroxyl-ion concentration does not need to be carried so far, it does no harm as the subsequent addition of acid brings it back immediately to a pH of 2 or less. A few minutes' centrifuging of the suspension serves to throw down the precipitated proteins into a compact mass in the bottom of the tube, and the clear supernatant liquid may be poured off or pipetted out and used for a determination of reducing sugars without filtering. If a centrifuge is not available the starch and precipitated proteins settle out clear in about 5 min., and, if desired, the supernatant liquid can be rapidly filtered through a fine quantitative filter paper. This is especially true of the clarified, diluted solutions of malt extracts.

[8] Clarke, "The Determination of Hydrogen Ions," Williams & Wilkins Co., 1920, p. 65.

Effect of Sodium Tungstate on Reduction of Fehling Solution

Since this method for clarifying was intended to be used in connection with the determination of reducing sugars resulting from diastatic action in wheat flour suspensions, it was necessary to see what effect, if any, the use of sodium tungstate had on the reduction of Fehling solution in the Munson-Walker method. Preliminary trials on flour-water suspensions with and without added dextrose indicated that the addition of the sodium tungstate in excess for clarification did not affect the Fehling reduction. This was confirmed by a series of experiments in which dextrose added to flour samples was recovered, after clarification by varying amounts of the sodium tungstate reagent. Reducing sugars were determined in the clarified solutions by the gravimetric Munson-Walker method (A. O. A. C.).

These experiments, with other data, some of which are recorded in Table II, lead to the following two statements:

1—The use of 15 per cent sodium tungstate in quantities up to 5 cc. and sulfuric acid for clarification of flour suspensions neither interferes with nor affects the determination of reducing sugars in the clarified solution by the Munson-Walker gravimetric method.

2—The use of sodium tungstate clarifying reagent renders the solution clear and protein-free, and when centrifuged to throw down suspended matter it eliminates all necessity for filtration. Further proof that filtration of the clarified centrifugate is entirely unnecessary is evident from data obtained in other experiments at different times and for different purposes but using the same flour sample and method. These data are collected and tabulated in Table II.

TABLE II

Sample	Cu_2O G.
Filtered	
1.....	0.1259
2.....	0.1259
3.....	0.1252
Average.....	0.1256
Unfiltered	
4.....	0.1260
5.....	0.1251
6.....	0.1256
Average.....	0.1255

On the other hand, a very few trials are sufficient to convince one that filtration of the unclarified solution from a flour-water suspension is a most unsatisfactory procedure and should be expected to give inaccurate results for reducing sugars.

Effect of Inhibiting Agents on Diastase of Flour

Several agents to stop enzymatic action have been used by various investigators of diastatic activity. Among these, sodium hydroxide or sodium bicarbonate, sulfuric acid, alcohol, and low temperatures are perhaps the most common. In general, the salts of the heavy metals, antiseptics, and, in fact, most of the so-called catalytic poisons have but little inhibiting effect on the activity of diastase under normal conditions of pH and salt concentration, except as they are able to precipitate the enzyme-carrying protein or to influence the

pH of the medium. The concentration of alkali required for complete inhibition of flour diastase under the conditions here used is considerably higher than that generally recommended, and there seems to be serious danger of destroying some of the sugars present by the addition of sufficient alkali to produce such hydroxyl-ion concentration, as pointed out by Neff.[9] Furthermore, the increased dispersion and solution of proteins by the added NaOH is not desirable. The unsatisfactory results obtained in preliminary experiments whenever NaOH was used to stop diastatic activity or lead acetate for clarification of flour suspensions, in contradistinction to the uniformly satisfactory results by the use of sodium tungstate and sulfuric acid, made it desirable to determine the enzyme-inhibiting action of the tungstate clarification. This was determined in a series of experiments, the data on which are summarized in Table III.

Ten grams of flour were weighed into 300-cc. flasks and 100 cc. of distilled water were added by pipet, with continual shaking to get the flour thoroughly stirred throughout the liquid. The various materials to be used as inhibiting agents were than added and the whole allowed to remain in the water bath at a constant temperature for the lengths of time designated in Column 3 of Table III. The suspensions in the flasks were shaken only at 15-min. intervals. Sample 1 was clarified by the tungstate reagent at the end of the first hour; Sample 2 was clarified at once, then allowed to stand 1 hr. before determining the reducing sugars; Sample 3 was clarified in the same manner as Sample 2, except that it was allowed to stand 2 hrs. before determining the reducing sugars; Sample 4 was a trial with only 3 cc. of sodium tungstate, omitting the precipitation by sulfuric acid until the end of the hour. Samples 5 and 6 showed the effect of two different concentrations of alkali previously recommended in the literature for the inhibition of diastatic activity. Samples 7 and 8 were tried with 2 cc. of basic and 2 cc. of neutral lead acetate, respectively, and allowed to stand 1 hr. before filtration and removal of the lead for sugar determinations. They were then clarified and treated according to the official A. O. A. C. procedure. Sample 7 was cloudy and required 12 hrs. for filtration, while Sample 8 filtered much more rapidly. Samples 9 and 10 were cooled to 0° C. by an ice and salt bath. In Samples 1, 5, 6, 9, and 10, the diastatic activities were stopped by sodium tungstate clarification in the manner described above, while Sample 4 required the addition of 0.4 cc. concentrated sulfuric acid. After clarification the samples were all made up to a volume of 200 cc., centrifuged, and 50-cc. aliquots taken for reducing-sugar determinations. The results are expressed in Table III in terms of cuprous oxide corresponding to the reducing sugars from 50 cc. of the clarified solution (2.5 g. flour).

TABLE III

Sample	Inhibiting Agent	Time Hrs.	Temp. ° C.	Weight Cu_2O per 50 Cc. Solution (2.5 G. Flour) G.	pH of Digestion
1	None	1	27	0.0833	.....
2	Na_2WO_4 $+H_2SO_4$	1	27	0.0140	Below 2.0
3	Na_2WO_4 $+H_2SO_4$	2	27	0.0141	Below 2.0
4	Na_2WO_4 only	1	27	0.0491	7.24
5	2 cc. 0.1 *N* NaOH	1	27	0.0316	.....
6	5 cc. 0.1 *N* NaOH	1	27	0.0169	9.13
7	$Pb(CH_3COO)_2$ (Neutral)	1	27	0.1034	.....
8	$Pb(CH_3COO)_2$ (Basic)	1	27	0.0066	.....
9	Ice	1	0	0.0287	5.98 (21° C.)
10	Ice	3	0	0.0388	5.98 (21° C.)

Confirmatory results similar to these and leading to the same conclusions have been obtained on several other flours.

Samples 2 and 3, as examples typical of many similar determinations, show conclusively that the use of sodium tungstate and sulfuric acid with a final pH of 1.5 to 2 effectually stops all diastatic activity. This is accomplished both by a complete precipitation of the enzymic-active protein and by effecting a hydrogen-ion concentration which itself inhibits diastatic activity. The concentration of acid is not sufficient to further hydroliyze the disaccharides or dextrins, even after several hours' standing. This fact had previously been pointed out by Swanson and Calvin,[4] who used a final concentration of 0.02 *N* sulfuric acid which corresponds to pH between 2 and 2.5. This complete inhibition of diastatic action allows an accurate measurement of the reducing sugars by gravimetric Fehling's determination at the operator's convenience.

Samples 5 and 6 indicate that NaOH is not a reliable inhibiting agent to use for diastatic preparations where the quantity of buffer is as high as that found in flours or malts, unless a large concentration is used. Such a high concentration is obviously not desirable.

Lead acetate (Samples 7 and 8, Table III) gives variable results, depending upon the concentration of the reagent, the concentration of the material used to delead the solution, and the time consumed in the operations of filtration.

Samples 9 and 10 clearly indicate that the temperature of an ice bath will not completely stop diastatic activity in solution where the diastase is protected by buffers naturally occurring in the plant medium. Other samples with temperatures around 5° C. showed very nearly the same results.

Samples 2 and 3, which agree with results of nearly a hundred other similar determinations on the same flour sample, serve to show that this "blank" gives very constant and duplicable results and affords a measure of the reducing sugars present in the flour. While this reduction of Fehling solution is due to dextrose, and the total reduction by autolytic digestion of the flour-water suspension is a result of the added maltose produced by diastatic action, the difference in the weights of Cu_2O produced gives a reliable measure of the maltose. This procedure for the determination of the original reducing sugars in the "blank" should be comparable to the values obtained from the official alcohol extraction method. Likewise, it may be possible to substitute the sodium tungstate clarification for the lead acetate procedure in the alcohol extract of sugars from flour and malt products. Preliminary experiments on the application of this new procedure for both reducing and total sugars have indicated its successful application. Collaborative work is now being conducted on this problem and the results will be reported at a later date.

In point of time alone the sodium tungstate procedure requires only about 5 min. with the elimination of all filtration, which is a great advantage in enzyme work. The clarified solution can be allowed to stand for several hours or until all the samples are ready for the Fehling reduction at the operator's convenience.

[9] Armstrong, "The Simple Carbohydrates and the Glucosides," Longmans, Green & Co., 1919, p. 44.

Industrial Research Laboratories

There are more than five hundred industrial laboratories in the United States which are more or less actively engaged in scientific research. The majority of these are rather well equipped to work along certain special lines. Some of them are consulting laboratories which are equipped for general analytical work and for special research. Many of the research laboratories of manufacturing establishments will undertake special investigation for individuals or firms which lack the necessary equipment or personnel.

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