Summary

1. A rapid and precise method for the quantitative determination of organic amino acids is presented.

2. For the titration of strong acids and bases in acetic acid systems, crystal violet, α-naphtholbenzein, and benzoyl auramine have been found to give results that are in accordance with those obtained potentiometrically with the chloranil electrode.

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[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY OF THE UNIVERSITY OF UPSALA]

The Salt Extractable Proteins of Wheat Flour. Ultracentrifugal Study

BY LAURA KREJCI[1] AND THE SVEDBERG

Gortner, Hoffman, and Sinclair[2] have investigated the peptization of wheat proteins by solutions of different salts. They have found that the amount of nitrogen extracted varies with the ions present in the extracting solution. The potassium halides in particular show a marked lyotropic series: normal potassium iodide extracts almost five times as much as normal potassium fluoride, and almost three times as much as normal potassium chloride. This paper is the report of an ultracentrifugal investigation of the protein matter extracted from wheat flour by solutions of these salts.

Potassium Fluoride, Potassium Chloride, Potassium Bromide and Potassium Iodide Extracts of Wheat Flour

The flour used for this investigation was made from Manitoba wheat specially ground in an experimental mill at the laboratory of Upsala Ångkvarn.

A comparison was made of the protein extracted by half normal solutions of potassium fluoride, potassium chloride, potassium bromide and potassium iodide. All the crude extracts contained much non-centrifugible light-absorbing material. To eliminate this each, with the exception of the fluoride, was precipitated one or more times by saturation with solid ammonium sulfate. The crude iodide extract could not be studied because of the high light absorption of the potassium iodide; the ammonium sulfate precipitates were therefore dissolved in 0.5 *N* potassium chloride. To provide a basis for comparison, half the precipitate from the bromide extract was dissolved in 0.5 *N* potassium bromide, half in 0.5 *N* potassium chloride.

(1) Fellow of the American Scandinavian Foundation.

(2) Gortner, Hoffman, and Sinclair, "Colloid Symposium Monograph," 1928, Vol. V, 179–198.

The results are shown in Table I. The sedimentation constants have been corrected for the density and viscosity of the salts present to a basis of sedimentation in pure water. Estimates are included of the amount of non-centrifugible material present after four hours of centrifuging. The protein is inhomogeneous in every case; the constants therefore represent average values which may be used to characterize the mixture of proteins present in each solution.

In almost every case precipitation with ammonium sulfate causes an increase in sedimentation constant and a decrease in non-centrifugible light absorbing material; the only exception is the fourth precipitation of the chloride extract. The change is more marked for the portion of the bromide extract dissolved in potassium chloride than for that dissolved in potassium bromide. It is also greater for the portion of the iodide extract which dissolved readily in potassium chloride than for the portion which dissolved only on longer contact with the solvent. This indicates that the ammonium sulfate precipitates of the heavier proteins dissolve readily in solutions of low salt content, while those of the lighter proteins require a higher salt content in the solvent, or a longer time of contact with the solvent, or both, to dissolve. The behavior of the four-times precipitated chloride extract can be explained by the fact that the three preceding precipitates had been dissolved in water, and adhering ammonium sulfate was the only source of salt in the solvent. The solutions were cloudy; this cloudiness doubtless consisted of protein matter which was insoluble in the dilute ammonium sulfate solution, but soluble in 0.33 *N* potassium chloride.

The sedimentation constant for the crude fluoride extract is 2.20; for the crude chloride extract, 2.38; and for the crude bromide extract

TABLE I

Centrifugal force 100,000 times gravity; source of light, mercury lamp; light filter, chlorine and bromine; plates, Imperial Process; exposure time, one minute; metol hydroquinone developer, one minute

Extracting agent	Description of treatment	Solvent for ammonium sulfate ppt.	Solvent in ultracentrifuge	Non-cent. material, %	$s_{20} \times 10^{13}$
0.5 *N* KF	Crude		0.5 *N* KF	66	2.20
0.5 *N* KCl	Crude		.5 *N* KCl	50	2.37
	Pptd. once	H_2O	.13 *N* KCl	37	2.93
	Pptd. twice	H_2O	.13 *N* KCl	12	3.11
	Pptd. 3 times	H_2O	.17 *N* KCl	12	3.18
	Pptd. 4 times	0.33 *N* KCl	.33 *N* KCl	20	2.63
0.5 *N* KCl	Crude (fresh)		.17 *N* KCl	45	2.38
0.5 *N* KBr	Crude		.5 *N* KBr	43	3.56
	Pptd. once	.5 *N* KCl	.5 *N* KCl	20	4.33
	Pptd. once	.5 *N* KBr	.5 *N* KBr	30	3.53
0.5 *N* KI	Pptd. once (portion readily soluble)	.5 *N* KCl	.5 *N* KCl	39	4.43
	Pptd. once (portion soluble with difficulty)	.5 *N* KCl	.5 *N* KCl	55	3.58
	Pptd. twice	.5 *N* KCl	.5 *N* KCl	22	4.89
	Pptd. 3 times	.5 *N* KCl	.5 *N* KCl	18	6.42

3.56. By analogy with the bromide results one may conclude as a first approximation that the constant for the crude iodide extract should lie somewhere between 3.58 and 4.43; it is almost certainly higher than 3.56, the constant for the bromide extract. These figures are of interest in connection with Gortner's extraction figures because they show that solutions of potassium fluoride, potassium chloride, potassium bromide and potassium iodide extract not only increasing amounts of protein from wheat flour, but mixtures with an increasing content of proteins of high molecular weight as well.

Successive Extraction of Wheat Flour

In an attempt to separate the mixture of proteins into more nearly homogeneous fractions, the flour was extracted successively with 0.5 *N* solutions of potassium fluoride, potassium chloride and potassium bromide. The results are shown in Table II.

The chloride solution contains much less noncentrifugible material than the corresponding extract of the fresh flour, and the sedimentation constant is unexpectedly high—6.09. This is evidence that the fractions of high molecular weight which had been concentrated from previous extracts by ammonium sulfate precipitation had been actually present in the crude extracts and were not aggregates formed by the action of the ammonium sulfate.

The bromide extract has a lower sedimentation constant (3.75) than the chloride extract. It is very near that of the bromide extract of the fresh

TABLE II

Experimental conditions as in Table I (exposure time, 1 min.)

Solution	Solvent	Non-cent. material, %	$s_{20} \times 10^{13}$
KF extract	0.5 *N* KF	66	2.20
KCl extract of gluten residue from KF extraction	.5 *N* KCl	31	6.09
KBr extract of residue from KF and KCl extraction	.5 *N* KBr	36	3.75
Fraction of KCl extract pptd. by half-saturation with $(NH_4)_2SO_4$	.5 *N* KCl	20	5.90
Fraction of KCl extract not pptd. by half-saturation with $(NH_4)_2SO_4$ but pptd. by saturation	.5 *N* KCl	10	9.25

flour. However, this solution contains a less heterogeneous mixture of proteins and less noncentrifugible material.

The chloride extract was divided into two portions by fractional ammonium sulfate precipitation. The portion of the protein precipitated by half-saturation of the solution was partially dissolved in a small volume of water, reprecipitated by half-saturation, dissolved again, and reprecipitated. The final precipitate was suspended in 0.5 *N* potassium chloride and allowed to stand overnight. Solution took place very slowly; this was shown by the considerable increase in light absorption during the interval. The sedimentation constant of this fraction was nearly the same as that of the crude chloride extract from which it had been prepared.

The protein which had not been precipitated by

half-saturation of the chloride extract was precipitated by the addition of further ammonium sulfate to saturation. This was suspended in 0.5 *N* potassium chloride; it dissolved rapidly to an almost clear solution. The sedimentation constant was high and the content of non-centrifugible material was low; and the sharper sedimentation boundary indicated that the fraction was more nearly homogeneous than the solution from which it had been prepared. This shows that the original solution had been separated into a fraction of higher average molecular weight which is precipitated only at high concentrations of ammonium sulfate but when once precipitated dissolves readily in 0.5 *N* potassium chloride, and a fraction of lower average molecular weight which forms on half-saturation with ammonium sulfate a precipitate which dissolves only slowly in 0.5 *N* potassium chloride.

Fractionation by Dialysis

Wheat flour was extracted successively with 0.5 *N* solutions of potassium fluoride and potassium chloride; the extracts were fractionated by dialysis. The solutions were studied in higher centrifugal fields than had been available for the work previously described. In some cases this made it possible to measure the separate sedimentation boundaries of the constituents of the mixtures.

Twenty grams of flour was extracted successively with 75, 75 and 50 cc. of 0.5 *N* potassium fluoride; then with 75, 75, 50, 50 and 50 cc. of 0.5 *N* potassium chloride. The extracts were dialyzed in collodion bags for sixty hours against distilled water. The protein precipitated by dialysis of the three potassium fluoride extracts was combined and dissolved in 55 cc. of 0.5 *N* potassium chloride which was also 0.05 *M* in both Na_2HPO_4 and KH_2PO_4 (KF:1). The protein precipitated by dialysis of the first potassium chloride extract was dissolved in 75 cc. of 0.5 *N* potassium chloride (KCl:1); that precipitated by dialysis of the four succeeding potassium chloride extracts was dissolved in 50 cc. of 0.5 *N* potassium chloride (KCl:2). The dialyzed potassium fluoride extracts were combined and saturated with ammonium sulfate; the protein thus precipitated was collected and dissolved in 40 cc. of 0.5 *N* potassium chloride (KF:2). The combined potassium chloride extracts were similarly treated; the precipitated protein was dissolved in 50 cc. of 0.5 *N* potassium chloride (KCl:3). The results are collected in Table III.

Additional determinations were made with solution KCl:1 at different conditions of *p*H and different dilutions, and with a fresh solution prepared in

TABLE III

Centrifugal force 120,000–170,000 times gravity; thickness of column of solution, 0.8–1.2 cm.; exposure time, 20–120 sec.; other experimental conditions as in Table I

Solution	Solvent KCl 0.5*N* — *M*		*M*		Concentration, %	$s_{20} \times 10^{13}$				
KF:1	Na_2HPO_4	0.050	KH_2PO_4	0.050	100	...	6.8[a] 64%	11.4 36%	..	..
KF:2	.....	..	.....	..	50	1.9	..	..	..	..
KCl:1	.....	..	.....	..	100	...	3.4 40%	11.4 30%	17.2 30%	..
KCl:2	.....	..	.....	..	100	...	2.2 50%	11.6 50%	..	..
KCl:3	.....	..	.....	..	100	1.3 50%	3.1 50%	..	..	..
KCl:1	.....	..	.....	..	100	...	6.6[a] 55%	..	15.8 33%	23.9 12%
KCl:1 *p*H 4.3	NaAc	.024	HAc	.004	75	...	5.9[a] 78%	9.4 22%	..	..
KCl:1 *p*H 7.7	Na_2HPO_4	.023	.....	..	77	...	5.2[b] 50%	10.5 50%	..	..
KCl:1 *p*H 7.7	Na_2HPO_4	.008	.....	..	92	...	...	10.8[c]	..	..
KCl:1 *p*H 8.4	Na_2HPO_4	.004	NaOH	.001	92	...	...	11.5[c]	..	..
KCl:1 (new)	.....	..	.....	..	100	...	...	9.4[c] 55%	..	27.1 45%
KCl:1 *p*H 5.3	Na_2HPO_4	.001	KH_2PO_4	.003	75	...	6.5[a] 78%	10.8 22%	..	..
KCl:1 *p*H 6.8	Na_2HPO_4	.013	KH_2PO_4	.013	75	...	5.7[a] 78%	10.2 22%	..	..
KCl:1 *p*H 11.0	Na_2HPO_4	.013	NaOH	.013	75	...	4.4[a] 80%	9.2 20%	..	..
KCl:1 *p*H 12.6	Na_2HPO_4	.002	NaOH	.016	82	...	...	8.1[d]	..	..
KCl:1 *p*H 12.9	Na_2HPO_4	.001	NaOH	.084	82	...	...	6.0	..	..

[a] Mixture of two components (approximately 3 and 7). [b] Mixture of 11 and two lighter components. [c] Mixture of 17, 11, and two lighter components. [d] Mixture of 17, 11, and two lighter components; alkaline decomposition begun.

the same manner. These results are included in Table III. Beneath each sedimentation constant is given the relative concentration of that constituent in the solution, based on the assumption that the extinction coefficients of the different molecules are identical. The estimates are accurate only within about 10%. The degree of dilution, or rather the percentage of saturated stock solution in each, is also included.

The fractions not precipitated by dialysis consist entirely of molecules of low molecular weight. Only one boundary is present in the fluoride extract; two are visible in the chloride extract.

The fractions precipitated by dialysis contain both light and heavy molecules. All these solutions contain a component of sedimentation constant 11. Solution KCl:1 contains in addition molecules of sedimentation constant 17.

Further determinations were made with solution KCl:1 after it had stood in the refrigerator for two weeks in contact with the undissolved portion of the dialysis precipitate; this had given ample opportunity for the proteins to saturate the solution. A duplicate run on the undiluted solution showed the heavy molecules of sedimentation constant 17 and the inhomogeneous material of low molecular weight, and in addition a boundary of sedimentation constant 25. The further determinations at different dilutions showed that the molecules of sedimentation constant 25 and 17 found in the saturated solution dissociate on dilution to molecules of sedimentation constant 11 and still smaller molecules the constant for which could not be accurately determined because of the uncertainty involved in the analysis of mixtures. Although caution must be observed in identifying sedimentation constants with molecular weights because of possible differences in the shape of the molecule, it seems probable that the molecule of sedimentation constant 11 has the same weight as amandin and the other seed globulins, 280,000. It is also possible that the constants 17 and 25 represent molecules $2 \times 280{,}000$ and $4 \times 280{,}000$ respectively. The similarity in shape of the curves for the dilute solutions and the saturated solutions indicates that the lighter molecules may have a weight $^1/_2 \times 280{,}000$.

Discussion

The material not precipitated by dialysis of the salt extracts of wheat flour probably consists of leucosin and proteose; these two proteins therefore consist of small molecules (possibly 34,000 or less) and are inhomogeneous with respect to molecular weight. The material precipitated by dialysis probably consists largely of globulin and gliadin. The globulin in one of its several forms has the same molecular weight, 280,000, as the other seed globulins. The tendency to associate in concentrated solution and dissociate on dilution may explain the correlation between Gortner's extraction figures and the sedimentation constants of the crude halide extracts. Any factor which would tend to increase the concentration of the globulin would favor the formation of the heavier polymers.

Wheat globulin is obtained in greatest yield and with least contamination from the first chloride extract of flour which has previously been extracted with potassium fluoride. The ammonium sulfate fractionation of this solution described previously shows that half-saturation precipitates only a portion of the globulin; most of it is precipitated only by complete saturation. The fact that the product precipitated by half-saturation has a lower sedimentation constant than the product obtained by saturation calls for some comment; the opposite is usually true. The only example of similar behavior is the white of hen's egg; the sedimentation constant for ovoglobulin is approximately 2.5, according to unpublished determinations by H. Palme, while that for ovalbumin is 3.5.

Sinclair and Gortner[3] have shown that solutions of potassium bromide dissolve considerable quantities of gliadin. The sedimentation constant of the predominant gliadin molecule is low, 2.1×10^{-13};[4] that of the heaviest gliadin molecule, according to the most careful estimates, is not greater than 5.5. A solution of gliadin, therefore, sediments far more slowly than even a dilute solution of wheat globulin. The bromide extract of wheat flour which has previously been extracted with potassium fluoride and potassium chloride gives a lower sedimentation constant than the chloride extract which precedes it. The drop is probably due to the increased content of gliadin on the one hand and the lower globulin content on the other.

The wheat globulin is a protein distinct from gliadin, though it occurs as an impurity in the crude alcoholic extract of gluten. This is shown in Table IV, which contains the results of deter-

(3) Sinclair and Gortner, *Cereal Chemistry*, **10**, 171 (1933).
(4) Krejci and Svedberg, THIS JOURNAL, **57**, 946 (1935).

minations made with aqueous solutions of crude gliadin. It is completely absent from purified gliadin. Like gliadin, wheat globulin has a wide stability range. It is stable from *p*H 11 through *p*H 2.23, the lowest *p*H studied.

TABLE IV

AQUEOUS SOLUTIONS OF CRUDE GLIADIN. SEDIMENTATION VELOCITY MEASUREMENTS

Experimental conditions as in Table III

*p*H		$s_{20} \times 10^{13}$ Wheat globulin			Gliadin
2.40	6.0	11.0	15.3	..	2.0
2.23	7.0	10.3	..	..	2.2
2.23	...	..	18.0	26.7	2.3

The expenses connected with this investigation have been defrayed by grants from the Rockefeller Foundation, the Nobel Foundation, and the foundation Therese och Johann Anderssons Minne.

Summary

1. An ultracentrifugal study has been made of the proteins extracted from wheat flour by 0.5 *N* solutions of the potassium halides. These solutions, in the order potassium fluoride, potassium chloride, potassium bromide and potassium iodide, extract mixtures of proteins of increasing average molecular weight.

2. Wheat flour was extracted successively with 0.5 *N* solutions of potassium fluoride and potassium chloride; the extracts were further fractionated by dialysis. The material precipitated by dialysis probably consists largely of gliadin and wheat globulin. The globulin, which ordinarily has a sedimentation constant of 11, polymerizes in concentrated solutions to molecules of sedimentation constant 17 and 25, and dissociates on dilution. The material not precipitated by dialysis, probably a mixture of leucosin and proteose, consists of small molecules and is inhomogeneous with respect to molecular weight.

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Molecular Weight Analysis of Mixtures by Sedimentation Equilibrium in the Svedberg Ultracentrifuge[1]

BY WILLIAM D. LANSING AND ELMER O. KRAEMER

Although Svedberg and his co-workers[2] have found that many of the proteins, when properly purified, are uniform in molecular weight (*i. e.*, are monodisperse), macromolecular substances are generally non-homogeneous in this respect. The typical high polymers, for instance, consist of a mixture of molecules of different sizes, representing different degrees of polymerization. In such cases the usual methods of measuring molecular weight yield average values, but as has been pointed out in a previous paper of this series,[3] different methods may yield different kinds of "average molecular weights" which are not directly comparable. Unfortunately, this fact has not yet been properly considered in previous attempts to compare the results of various investigators, so that a great deal of confusion has resulted.

The authors have already pointed out that at least two "average molecular weights" must be distinguished. The usual physico-chemical methods involve counting of the molecules, and thus yield a "number-average" molecular weight. On the other hand, Staudinger's viscosity method,[4] when applicable, yields a "weight-average" molecular weight. In the present paper a third quantity is introduced, the "*Z*-average" molecular weight, which may be obtained from ultracentrifuge data.

These three different averages are defined by the following equations

$$\text{number-average } M_n = \frac{\Sigma n_i M_i}{\Sigma n_i} = \frac{\Sigma w_i}{\Sigma w_i/M_i} \quad (1)$$

$$\text{weight-average } M_w = \frac{\Sigma n_i M_i^2}{\Sigma n_i M_i} = \frac{\Sigma w_i M_i}{\Sigma w_i} \quad (2)$$

$$\text{and } Z\text{-average } M_Z = \frac{\Sigma n_i M_i^3}{\Sigma n_i M_i^2} = \frac{\Sigma w_i M_i^2}{\Sigma w_i M_i} \quad (3)$$

where n_i is the number of molecules of molecular weight M_i, while w_i is the total weight of that molecular species. For polymeric materials con-

(1) Paper No. III on the Molecular Weight of Linear Macromolecules by Ultracentrifugal Analysis.

(2) Svedberg, *Chem. Rev.*, **14**, 1 (1934); also many papers in THIS JOURNAL, 1926–1934. For complete bibliography see *Naturwissenschaften*, **22**, 225 (1934).

(3) Kraemer and Lansing, *J. Phys. Chem.*, **39**, 153 (1935).

(4) Staudinger, "Die hochmolekularen organischen Verbindungen," J. Springer, Berlin, 1932.