2. The method fails in the case of volatile sulfones and compounds from which such sulfones are produced by the action of nitric acid.

3. Reproducible results can be obtained with biological material.

4. The process is adaptable to the estimation of sulfur in materials containing as little as 0.02%.

NEW YORK, N. Y.

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

# THE MOLECULAR WEIGHT OF LEGUMIN

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In recent communications from this Laboratory determinations of the molecular weights and of the *P*H-stability regions of some oil seed globulins, *viz.*, edestin,[1] amandin and excelsin[2] by means of the ultracentrifugal method, have been given. These proteins as well as the three other vegetable proteins, R-Phycocyan, R-Phycoerythrin, C-Phycocyan, so far studied with the ultracentrifuge, were all found to possess a molecular weight of 208,000, which is approximately six times the weight of egg albumin. Their molecules are spherical with a radius of 3.95$\mu\mu$. The stability regions are different for the different proteins but they all show a tendency to dissociate into lower multiples of 34,500 with increasing *P*H. It was thought to be of interest to study also some representative of the proteins of the *Leguminosae* family by means of the ultracentrifuge analysis. Legumin from vetch (*Vicia sativa*) was chosen. According to the investigations of Osborne[3,4] the vetch contains only one globulin, legumin and one albumin, legumelin, while the pea and the horse bean contain legumin, legumelin and one more globulin, the vicilin. Whether the legumins extracted from the various *Leguminosae* seeds are identical is not quite clear. We have not so far made any attempt to answer this question.

**Preparation of Material.**—Two thousand grams of vetch flour was divided in two equal portions and each of them stirred at room temperature during twenty-four hours with 4000 cc. of 10% sodium chloride solution.[5] Enough toluene to form a layer about 1 cm. thick was added. It served the double purpose of dissolving the fat and preventing bacterial growth. The bulk of the insoluble part was removed by means of a cloth filter and the rest by centrifuging. Most of the fat had been taken up by the toluene.

[1] T. Svedberg and A. T. Stamm, THIS JOURNAL, **51**, 2170 (1929).

[2] T. Svedberg and B. Sjögren, *ibid.*, **52**, 279 (1930).

[3] T. B. Osborne and G. F. Campbell, *ibid.*, **18**, 583 (1896); **20**, 406, 410 (1898).

[4] T. B. Osborne, "Vegetable Proteins," Longmans, Green and Co., London, **1916**.

[5] The grinding of the vetch seed was kindly done for us in an experimental mill at the laboratory of Upsala Ångkvarn.

The $P_H$ of this solution was 5.7. Saturated ammonium sulfate solution was added to 85% saturation, the precipitate was centrifuged off, suspended in water and dialyzed for twenty-four hours against water. After that time the precipitate had dissolved leaving only a small residue. The solution was filtered and then dialyzed again until the sulfate reaction was negative. This second dialysis caused a separation of the two proteins; legumin was precipitated while legumelin remained in solution.

The legumin precipitate was centrifuged off, washed repeatedly with ammonium sulfate solution of 65% saturation and dissolved in 5% sodium chloride solution. This legumin solution was precipitated with saturated ammonium sulfate solution to 65% saturation. After standing for twenty-four hours in the ice box the precipitate was centrifuged off and washed with ammonium sulfate of 65% saturation until free from legumelin. The legumin precipitate was dissolved in phosphate buffer of $P_H$ 6.5 (0.14 $M$ in $KH_2PO_4$ and 0.06 $M$ in $Na_2HPO_4$) and dialyzed against the same buffer in the ice box until the sulfate reaction was negative: volume of solution 200 cc., concentration 3.35%.

The legumelin solution remaining after precipitating the legumin by dialysis was saturated with ammonium sulfate. After standing for twenty-four hours in the ice box the legumelin precipitate was centrifuged off, washed with ammonium sulfate, dissolved in water, dialyzed against water at 5° and finally electrodialyzed. During the dialysis and the electrodialysis a considerable quantity of protein was precipitated. The ultracentrifugal study of the solution at high speed (44,000 r. p. m.) showed it to be polydisperse. There was a considerable drift in the apparent diffusion constant indicating the presence of several molecular species. The mean value of the sedimentation constant was very low, *viz.*, $1.20 \times 10^{-13}$ at 20°. The corresponding mean molecular weight would be around 7500. The legumelin is therefore not a simple protein. Most probably it only represents a mixture of decomposition products. The study of it was not pursued any further.

**Specific Volume and Light Absorption.**—The specific volume of legumin was measured pycnometrically at 20.2°. The solution was 0.14 $M$ in $KH_2PO_4$ and 0.06 $M$ in $Na_2HPO_4$, giving a $P_H$ of 6.5. Determinations at two concentrations, 2.45 and 1.03%, agreed within experimental error and gave $V = 0.743$.

The light absorption was measured by means of the Judd–Lewis spectrophotometer. At $P_H$ 6.5 the solvent was 0.14 $M$ in $KH_2PO_4$ and 0.06 $M$ in $Na_2HPO_4$ and the legumin concentration 0.1 and 0.4% with a thickness of layer of 2.0 and 1.0 cm., respectively. At $P_H$ 12.4 the solvent was 0.011 $M$ in $KH_2PO_4$, 0.001 $M$ in $Na_2HPO_4$, 0.059 $M$ in NaOH and 1% in NaCl and the legumin concentration 0.2% with a thickness of layer of 1.0 cm. The position of the maximum and the minimum as well as the values of

the specific extinction coefficient $\epsilon/c = 1/cd \times \log I_0/I$ (where $c$ is the concentration, $d$ the thickness of the solution, $I_0$ the intensity of the light beam after passing through the solvent and $I$ the intensity after passing through the same thickness of solution) was found to be the same at $P_H$ 6.5 and 12.4 although, as shown by the ultracentrifugal analysis, the legumin molecule is broken up into smaller units at $P_H$ 12.4. The absorption maximum was situated at 280$\mu\mu$ with $\epsilon/c$ = 7.5 and the minimum at 255$\mu\mu$ with $\epsilon/c$ = 3.0. In Fig. 1 the absorption curve is given.

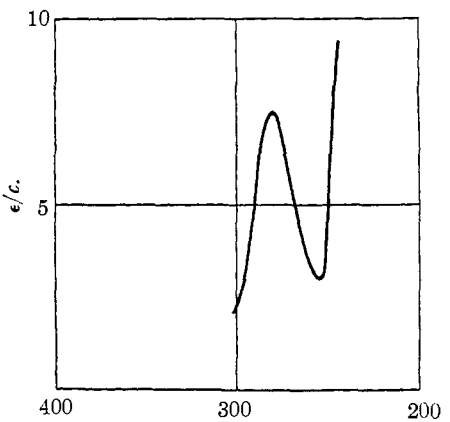

Fig. 1.

**Determination of Molecular Weight.**—Using the sedimentation equilibrium method the molecular weight is given by the relation

$$M = \frac{2RT \ln (c_2/c_1)}{(1 - V\rho)\omega^2(x_2^2 - x_1^2)}$$

where $R$ is the gas constant, $T$ the absolute temperature, $V$ the partial specific volume of the solute, $\rho$ the density of the solvent, $\omega$ the angular velocity and $c_2$ and $c_1$ are the concentrations at the distances $x_2$ and $x_1$ from the center of rotation.

In Table I the result of a typical run is given and in Table II are collected the data of the three equilibrium runs made on legumin.

As mean value we get 208,000 ± 5000 for the molecular weight. The fact borne out by Table I that there is no drift in the molecular weight values with distance from the center of rotation shows that legumin is homogeneous with regard to molecular weight.

TABLE I

LEGUMIN, SEDIMENTATION EQUILIBRIUM RUN

Concn., 0.11%; phosphate buffer, $P_H$ 6.8 (0.1 $M$ in $KH_2PO_4$, 0.1 $M$ in $Na_2HPO_4$); $V$ = 0.743; $\rho$ = 1.017; $T$ = 293.3; length of col. of soln., 0.43 cm.; thickness of col., 0.80 cm.; dist. of outer end of soln. from axis of rotation, 5.95 cm.; speed, 5000 r. p. m. ($\omega$ = 166.7$\pi$); light absorption standard, $M$/200 in $K_2CrO_4$ with a thickness of layer of 0.40 cm.; source of light, mercury lamp; light filters, chlorine and bromine; aperture of objective, F:36; plates, Imperial Process; time of exposure, 30, 60 and 120 seconds; exposures made after 40, 45 and 50 hours of centrifuging.

| Distances, cm. | | Mean concn., % | | Number of | |
|---|---|---|---|---|---|
| $x_2$ | $x_1$ | $c_2$ | $c_1$ | exposures | Mol. wt. |
| 5.87 | 5.82 | 0.084 | 0.073 | 12 | 213,100 |
| 5.82 | 5.77 | .073 | .064 | 12 | 203,100 |
| 5.77 | 5.72 | .064 | .056 | 9 | 215,700 |
| 5.72 | 5.67 | .056 | .049 | 9 | 212,200 |
| 5.67 | 5.62 | .049 | .043 | 9 | 194,000 |
| | | | | Mean | 208,000 |

TABLE II

LEGUMIN, SUMMARY OF SEDIMENTATION EQUILIBRIUM MEASUREMENTS

| Solvent $KH_2PO_4$, $M$ | Solvent $Na_2HPO_4$, $M$ | $P_H$ of soln. | Concn. of protein at start, % | Mean speed, r. p. m. | Mol. wt. |
|---|---|---|---|---|---|
| 0.14 | 0.06 | 6.5 | 0.40 | 5600 | 213,000 |
| .1 | .1 | 6.8 | .11 | 5000 | 208,000 |
| .008 | .192 | 8.0 | .10 | 4800 | 204,000 |

**Determination of Sedimentation Constant.**—The high-speed oil-turbine ultracentrifuge was used for measuring the sedimentation constant

TABLE III

LEGUMIN, SUMMARY OF SEDIMENTATION VELOCITY MEASUREMENTS

| No. | Solvent $KH_2PO_4$, $M$ | Solvent $Na_2HPO_4$, $M$ | NaOH, $M$ | NaCl, % | $P_H$ of soln. | Concn. of protein, % | $s_{20} \times 10^{13}$ |
|---|---|---|---|---|---|---|---|
| 1[a] | 0.014 | .. | .. | 1 | 3.15 | 0.24 | 8.80[b] |
| 2 | .096 | 0.004 | .. | 1.5 | 5.26 | .22 | 11.42 |
| 3 | .14 | .06 | .. | ... | 6.5 | 1.34 | 11.23 |
| 4 | .14 | .06 | .. | ... | 6.5 | 0.24 | 11.45 |
| 5 | .08 | .12 | .. | ... | 6.89 | .24 | 11.47 |
| 6 | .008 | .192 | .. | ... | 8.00 | .22 | 11.89 |
| 7 | .008 | .032 | 0.018 | ... | 8.94 | .24 | 11.41[c] |
| 8 | .014 | .037 | .033 | ... | 9.38 | .25 | —[c] |
| 9 | .014 | .035 | .035 | ... | 10.78 | .25 | 3.03 |
| 10 | .011 | .001 | .059 | 1 | 12.40 | .20 | 2.14 |

[a] 0.093 $M$ in HAc. [b] Solution decomposed; 25% non-centrifugible products. [c] Mixture of approximately 44% molecules of weight 208,000 and 56% of weight 104,000.

$s = dx/dt \times 1/\omega^2 x$ at different acidities. The temperature of the solution was between 20 and 23° during centrifuging, the time varied from two to $3^1/_2$ hours and the speed from 25,000 to 40,000 r.p.m. The determinations are summarized in Table III and Fig. 2.

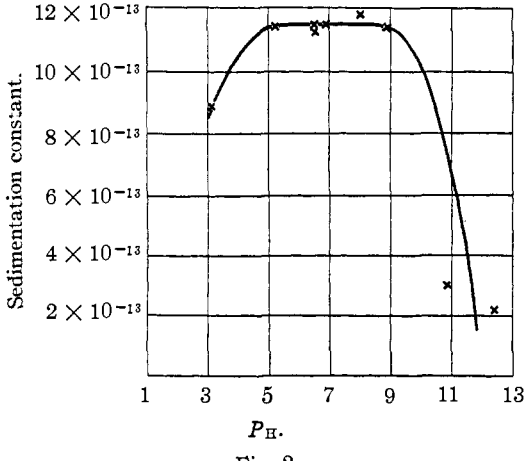


Fig. 2.

The sedimentation is independent of $P_H$ between 5 and 9. The $P_H$ range 5–9, therefore, represents the stability region of legumin. The isoelectric point[6] 5.4 is situated near the acid border of the stability region. In more acid and more alkaline solutions the legumin molecule is broken up into

[6] F. A. Csonka, J. C. Murphy and D. B. Jones, THIS JOURNAL, **48**, 763 (1926); the material used for the determination of the isoelectric point was prepared from pea.

smaller units of unequal weight. At a $P_H$ of about 9.3 molecules of half the normal weight are present. Within the stability region the sedimentation constant is $11.48 \times 10^{-13}$ (mean value from the runs No. 2–7, $P_H$ 5.26–8.94). From the value 208,000 of the molecular weight and this value of the sedimentation constant the value $4.60 \times 10^{16}$ for the molar frictional constant $f = [M(1 - V\rho)]/s$ is obtained. The molar frictional constant for a spherical molecule is $f_0 = 6\pi\eta N(3MV/4\pi N)^{1/3}$, where $\eta$ is the viscosity of the solvent. For a substance of the same molecular weight and specific volume as legumin we get $f_0 = 4.51 \times 10^{16}$. The ratio $f/f_0$ is 1.02 and should be unity if the legumin molecules were actually spherical. The deviation is within the limit of experimental error. The radius of the molecule, derived from the formula $r = (3MV/4\pi N)^{1/3}$, is $3.96\mu\mu$. A comparison of the values of molecular weight, sedimentation constant, frictional constant and radius for legumin with those obtained for the vegetable proteins previously studied by means of the ultracentrifugal methods[2] shows that they are identical within the limits of experimental error.

The expenses connected with these experiments have been defrayed by a grant from the foundation "Therese och Johan Anderssons Minne."

## Summary

1. The molecular weight and $P_H$-stability region of legumin have been determined by means of the ultracentrifugal methods.

2. Legumin is stable from $P_H$ 5 to 9 with a molecular weight of 208,000 ± 5000. At a $P_H$ of about 9.3 molecules of one-half the normal weight are present. At $P_H$-values decidedly lower than 5 and higher than 9 the legumin molecule is broken up into smaller units.

3. Within the stability region the sedimentation constant of legumin is $11.48 \times 10^{-13}$ and the molar frictional constant $4.60 \times 10^{16}$. The molecules are spherical with a radius of $3.96\mu\mu$.

4. The molecular weight, sedimentation constant, molar frictional constant and molecular radius of legumin are within the limits of error identical with the corresponding constants for amandin, edestin, excelsin R-phycoerythrin, R-phycocyan and C-phycocyan as previously determined by means of the ultracentrifugal methods.

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